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AÇÃO CONVULSIVANTE DO DISSELENETO DE DIFENILA EM RATOS: ESTUDO DOS MECANISMOS NEUROQUÍMICOS E DA TOXICOCINÉTICA

TESE DE DOUTORADO

Marina Prigol

Santa Maria, RS, Brasil
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por

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de
Doutor em Bioquímica Toxicológica.

Orientador: Prof^{o} Dr^{o} Gilson Zeni
Co-orientadora: Prof^{a} Dr^{a} Cristina Wayne Nogueira

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AÇÃO CONVULSIVANTE DO DISSELENETO DE DIFENILA EM RATOS: ESTUDO DOS MECANISMOS NEUROQUÍMICOS E DA TOXICOCINÉTICA

Elaborada por

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como requisito parcial para a obtenção do grau de **Doutor em Ciências Biológicas: Bioquímica Toxicológica**

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Santa Maria, julho de 2010.

“Morre lentamente
Quem não viaja,
Quem não lê,
Quem não ouve música,
Quem não encontra graça em si mesmo

Morre lentamente
Quem destrói seu amor próprio,
Quem não se deixa ajudar.

Morre lentamente
Quem se transforma em escravo do hábito
Repetindo todos os dias o mesmo trajeto,
Quem não muda de marca,
Não se arrisca a vestir uma nova cor ou
Não conversa com quem não conhece.

Morre lentamente
Quem evita uma paixão e seu redemoinho de emoções,
Justamente as que resgatam o brilho dos
Olhos e os corações aos tropeços.

Morre lentamente
Quem não vira a mesa quando está infeliz
Com o seu trabalho, ou amor,
Quem não arrisca o certo pelo incerto
Para ir atrás de um sonho,
Quem não se permite, pelo menos uma vez na vida,
Fugir dos conselhos sensatos...

Viva hoje !
Arrisque hoje !
Faça hoje !
Não se deixe morrer lentamente !

NÃO SE ESQUEÇA DE SER FELIZ”

Martha Medeiros

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RESUMO

Tese de Doutorado

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica

Universidade Federal de Santa Maria, RS, Brasil

AÇÃO CONVULSIVANTE DO DISSELENETO DE DIFENILA EM RATOS: ESTUDO DOS MECANISMOS NEUROQUÍMICOS E DA TOXICOCINÉTICA

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DATA E LOCAL DA DEFESA: Santa Maria, julho de 2010

Nos últimos anos, têm sido identificadas inúmeras propriedades farmacológicas do composto de selênio disseleneto de difenila [$(PhSe)_2$]. Assim, a pesquisa dos efeitos tóxicos deste composto torna-se importante para a segurança na aplicação farmacológica. Sabe-se que os bebês, em particular, apresentam muitas mudanças fisiológicas e bioquímicas relacionadas ao desenvolvimento, que aumentam a suscetibilidade aos efeitos tóxicos de drogas. Desta forma, **no artigo 1** investigou-se o aparecimento de convulsões induzida pelo $(PhSe)_2$, quando administrado pela via oral (p.o), nas doses de 5 à 500 mg/kg em ratos bebês (12-14 dias de vida) bem como os possíveis mecanismos glutamatérgicos (**Artigo 2**) e GABAérgicos (**Artigo 3**) envolvidos em tal processo. Vários estudos, utilizando diferentes modelos experimentais demonstraram as mais diferentes propriedades farmacológicas e toxicodinâmicas do $(PhSe)_2$ no entanto, pouco se conhece sobre a toxicodinâmica deste composto. Por isso, o objetivo do **artigo 4** foi determinar e quantificar os níveis plasmáticos de $(PhSe)_2$ em ratos e camundongos adultos após a administração p.o. de $(PhSe)_2$ na dose de 500 mg/kg; bem como verificar o envolvimento de diferentes vias de administração, veículos e espécie animal nos níveis plasmáticos do composto e no aparecimento de convulsões induzidas pelo mesmo. No **artigo 5**, determinou-se e quantificou-se os níveis de $(PhSe)_2$ no plasma, fígado e cérebro de ratos bebês e correlacionou-se estes níveis à latência para o aparecimento de convulsões. Devido a necessidade de obter mais informações sobre o composto, que viessem a complementar os dados obtidos, realizou-se modelos cinéticos *in vitro*. O **manuscrito 1** investigou parâmetros relacionados a estabilidade, solubilidade, absorção e ligação às proteínas plasmáticas do $(PhSe)_2$ *in vitro*. No **manuscrito 2** realizou-se um estudo *in vitro* para identificar as vias metabólicas responsáveis pela biotransformação do $(PhSe)_2$ no organismo. Os resultados do **artigo 1** demonstraram que a administração de $(PhSe)_2$

causou toxicidade em ratos bebês, evidenciada pelo aparecimento de convulsões. Estas são dependentes da dose utilizada e estão, pelo menos em parte, relacionadas ao estresse oxidativo. Dentre os mecanismos neuroquímicos envolvidos no efeito convulsivante do $(PhSe)_2$ estão a interação com o sistema glutamatérgico, por estimular os receptores glutamatérgicos ionotrópicos do tipo NMDA e por inibir a captação de glutamato (**Artigo 2**); e com o sistema GABAérgico, por antagonizar os receptores GABAégicos do tipo $GABA_A$, estimulando a enzima GABA transaminase e estimulando a captação de GABA (**Artigo 3**). O **artigo 4** demonstrou que concentração máxima de $(PhSe)_2$ no plasma de ratos e camundongos adultos ocorreu 30 minutos após a administração pela via oral do composto e permaneceu detectável até 8 horas após sua administração. O uso de diferentes vias de administração (intraperitonial (i.p); p.o; subcutânea (s.c)) e veículo (óleo de canola ou dimetil sulfóxido (DMSO)) em ratos e camundongos indicou que o aparecimento de convulsões e os níveis plasmáticos de $(PhSe)_2$ são dependentes da via de administração (i.p > p.o > s.c), do veículo (DMSO > óleo de canola) e da espécie animal (camundongo > rato). No **artigo 5** observou-se ainda que os ratos bebês convulsionaram mesmo apresentando níveis plasmáticos menores de composto que os adultos, o que nos leva a crer que estes são mais sensíveis aos feitos tóxicos do $(PhSe)_2$. Os níveis de $(PhSe)_2$ no fígado e no cérebro de ratos bebês no momento do episódio convulsivo apresentaram uma correlação negativa com a latência para o primeiro episódio convulsivo. O **manuscrito 1** revelou que o $(PhSe)_2$ apresenta estabilidade química e biológica. No entanto, o composto apresenta uma baixa solubilidade em água, um alto coeficiente de partição octanol-água e uma extensa ligação às proteínas plasmáticas. O **manuscrito 2** indicou que o $(PhSe)_2$ não é biotransformado por reações de fase I catalizadas pelo citocromo P450. O composto reage quimicamente com a glutationa reduzida (GSH) e a N-acetilciateína (NAC), formando adutos ou ainda reage com grupos SH de proteínas. A presença de GSH ou NAC no meio de incubação diminuiu a ligação do $(PhSe)_2$ às proteínas. Por fim, foi observado que o $(PhSe)_2$ reduziu a atividade das enzimas do citocromo P450. Em conjunto, os resultados desta tese demonstraram que a intensidade dos efeitos tóxicos causados pelo $(PhSe)_2$ estão diretamente relacionados a sua toxicocinética.

Palavras-chave: Disseleneto de difenila, selênio, convulsão, ratos bebê, neuroquímica, toxicocinética, metabolismo.

ABSTRACT

Thesis of Doctor's Degree

Federal University of Santa Maria, RS, Brazil

CONVULSIVE ACTION OF DIPHENYL DISELENIDE IN RATS: STUDY OF THE NEUROCHEMISTRY MECHANISMS AND TOXICOKINETIC

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ADVISOR: Gilson Zeni

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DATE AND PLACE OF THE DEFENSE: Santa Maria, july, 2010

In recent years have been identifical numerous pharmacological properties of a selenium compound, diphenyl diselenide [$(PhSe)_2$]. Consequently, it is important the investigation of its toxic effect for a safe application in pharmacological studies. It is known that babies, in particular, have many physiological and biochemical changes related to development, which increase the susceptibility to toxic effects of drugs. Thus, the **article 1** investigated the appearance of seizure episodes induced by $(PhSe)_2$ when administered orally at doses of 5 to 500 mg/kg in rat pups (pos natal day 12-14) and the possible glutamatergic (**article 2)** and GABAergic (**article 3)** mechanisms involved in this process. Some studies using different experimental models have demonstrated the most different pharmacological and toxicodynamics properties of $(PhSe)_2$. However, little is known about the toxicokinetic disposition of this compound. Therefore, the aim of **article 4** was to determine and quantify the plasma levels of $(PhSe)_2$ in adult mice and rats after oral (p.o.) administration of 500 mg/kg $(PhSe)_2$; to verify the involvement of different routes of administration, vehicle and animal species in plasma levels of $(PhSe)_2$ and in the onset of the first seizure episode induced by it. In **article 5**, it was determined and quantified the levels of $(PhSe)_2$ in plasma, liver and brain of rat pups and these levels were correlated to the latency for the onset of the first seizure episode. To obtain more information about the compound, which were to supplement the data obtained, we carried out *in vitro* kinetic models. The **manuscript 1** investigated the drug-like properties of $(PhSe)_2$ in regards to stability, solubility, absorption and plasma protein binding (PPB) *in vitro*. In **manuscript 2**, it was conducted an *in vitro* study in order to identify possible metabolic pathways responsible for the biotransformation of $(PhSe)_2$ in the body. Results of **article 1** showed that administration of $(PhSe)_2$ caused toxicity in rat pups, evidenced by the appearance of seizures. These were dose dependent and were, at least in part, related to oxidative stress. Among the mechanisms involved in the convulsive effect of $(PhSe)_2$ were the interaction with: glutamatergic system by stimulating the inotropic glutamatergic receptors NMDA and by inhibiting the uptake of glutamate (**Article 2**); GABAergic system by antagonize the $GABA_A$ receptor, stimulating GABA transaminase enzyme and increasing GABA

uptake (**Article 3**). The **article 4** revealed that the maximum concentration of $(PhSe)_2$ in the plasma of adult rats and mice occurred 30 minutes after p.o administration of the compound and remained detectable up to 8 hours after administration. The use of different routes of administration (intraperitoneal (i.p.), p.o., subcutaneous (s.c.)) or vehicle (canola oil or dimethyl sulfoxide (DMSO)) in rats and mice indicated that the onset of the first seizure episode and plasma levels are dependent on the route of administration (i.p. > p.o. > s.c.), vehicle (DMSO > canola oil) and animal species (mouse > rat). In **article 5**, it was observed that rat pups showed seizures even presenting lower plasma values of $(PhSe)_2$ as compared to adults. This result demonstrated that rat pups are more sensitive to the toxic effects of $(PhSe)_2$ than adult rats. Levels of $(PhSe)_2$ in the liver and brain of rat pups showed a negative correlation with the latency to the first seizure episode. The **manuscript 1** showed that $(PhSe)_2$ has chemical and biological stability. However, the compound has a low solubility in water, a high partition coefficient octanol-water and an extensive plasma protein binding. **Manuscript 2** indicated that $(PhSe)_2$ is not biotranformed by Phase I reactions, catalyzed by cytochrome P450. It reacted chemically with reduced glutathione (GSH) and N-acetylcysteine (NAC) to form adducts or reacts with protein SH groups. The presence of GSH or NAC in the incubation medium decreased the binding of $(PhSe)_2$ protein. Finally, it was observed that $(PhSe)_2$ reduced the activity of cytochrome P450.
Together, the data presented showed that the intensity of toxic effects caused by $(PhSe)_2$ are directly related to its toxicokinetic.

Key words: Diphenyl diselenide, selenium, seizures, rat pups, neurochemistry, toxicokinetic, metabolism.

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LISTA DE ABREVIATURAS

$(PhSe)_2$ - disseleneto de difenila
δ-ALA-D - delta aminolevulinato desidratase ou porfobilinogênio sintase
δ-ALA - ácido 5'-aminolevulínico ou ácido delta-aminolevulínico
CAT- catalase
SOD - superóxido desmutase
ANOVA - análise de variância
SNC - Sistema Nervoso Central
EROs - espécies reativas de oxigênio
TBARS - Substâncias reativas ao ácido tiobarbitúrico
DNA - ácido desoxirribonucléico
RNA - ácido ribonucléico
GABA - ácido γ-aminobutírico
NMDA - N-metil-D-aspartato
AMPA - α-amino-3-hidroxi-5-metil-4-isoxazolpropionato
PTZ - pentilenotetrazol
ATP - adenosina trifosfato
R-SeH - selenol
R-SH – tiol
GSH- glutationa reduzida
NAC- N-acetilcisteína
MK-801- [(+)-5-metil-10,11-diidro-5H-dibenzo[a,d] ciclohepteno-5,10-imina maleato)]
DNQX- (6,7-Dinitroquinoxalina-2,3-diona)
KA- cainato
LPO- Lipoperoxidação
AOAA- acido hemihidrocloreto amino oxalacético
DABA- acido DL-2,4-diamino-n-butírico
DMSO- Dimetil sulfóxido
PBS- Solução tampão fosfato de sódio
Cmáx-Concentração máxima
MeSeCys- metilselenocisteína
MSA- ácido metil selenínico
DMSe- dimetilseleneto
MMSe- metilselenol
SeCys- selenocisteína
SeMet- selenometionina
TMSe- trimetilselenônio

SUMÁRIO

APRESENTAÇÃO

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos, os quais se encontram no item **ARTIGOS CIENTÍFICOS E MANUSCRITOS**. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos científicos e manuscritos e representam na íntegra o presente estudo.

Os itens **DISCUSSÃO E CONCLUSÕES,** encontrados no final desta tese, apresentam interpretações e comentários gerais sobre todos os artigos científicos e manuscritos contidos neste trabalho.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO, REVISÃO BIBLIOGRÁFICA** e **DISCUSSÃO** desta tese.

1. INTRODUÇÃO

A epilepsia é uma desordem neurológica caracterizada pelo aparecimento recorrente e imprevisível de convulsões espontâneas. Embora a epilepsia possa manifestar-se por várias vias diferentes, cada tipo de epilepsia tem em comum a importante característica de aumentar a excitabilidade neuronal, a qual se manifesta através da geração de convulsões (Mcnamara, 1999). Os episódios convulsivos afetam cerca de 1 - 2% da população mundial e podem ocorrer em todas as idades, sendo mais comuns em crianças que em adultos. As convulsões pediátricas são únicas em relação a vários aspectos quando comparadas com a convulsão em adultos (Cowan, 2002). A incidência de convulsões é muito alta no primeiro ano de vida, diminuindo na infância e adolescência (Grunewald, 2002).

O comportamento convulsivo refere-se a uma breve alteração causada pela ativação desordenada, sincrônica e rítmica de grupos de neurônios cerebrais. Um desequilíbrio ocasionado por um aumento da transmissão excitatória glutamatérgica e/ou diminuição da resposta inibitória GABAérgica são importantes mecanismos envolvidos com a gênese da convulsão (Ribeiro et al., 2005).

O aminoácido glutamato é o mais abundante neurotransmissor excitatório no sistema nervoso central (SNC), estando envolvido na epileptogênese, na iniciação, na difusão de convulsões e no dano neuronal relacionado à convulsão. Os efeitos do glutamato são mediados pelos receptores ionotrópicos e metabotrópicos (Chapman, 2000) e a ativação de receptores de aminoácidos excitatórios pode ser um fator desencadeante à formação de espécies reativas de oxigênio (EROs) (Engelborghs et al., 2000).

O ácido γ-aminobutírico (GABA) é o mais importante neurotransmissor inibitório, sendo o receptor $GABA_A$ o mais extensamente envolvido na epilepsia (Gale, 1992). A diminuição na neurotransmissão GABAérgica no SNC contribui para a hiperexcitabilidade, que é responsável pelo estatus epilético (Olsen et al., 1999).

Um significante número de estudos sugere que as EROs estão associadas com os episódios convulsivos (Erakovic, 2001; Liang et al., 2000). O estresse oxidativo ocorre em conseqüência a episódios convulsivos prolongados, sugerindo que isso possa desempenhar um papel importante no dano cerebral induzido pela convulsão (Kaneko et al., 2002). O cérebro é o alvo preferencial do processo peroxidativo, pois contém uma alta concentração

de ácidos graxos poliinsaturados, sendo que os lipídios de membrana, além das proteínas celulares, DNA e RNA são alvos suscetíveis ao dano oxidativo (Halliwell and Gutteridge, 1989; Liang et al., 2000).

O selênio é um elemento traço essencial para o crescimento e desenvolvimento do organismo. Dentre os compostos de selênio que possuem ação farmacológica está o disseleneto de difenila $(PhSe)_2$ (Nogueira et al., 2004; Rosa et al., 2007). O $(PhSe)_2$ é um composto organocalcogênio que possui atividade anti-úlcera (Savegnago et al., 2006), antioxidante (Rossato et al., 2002; Meotti et al., 2004), neuroprotetora (Ghisleni et al., 2003), hepatoprotetora (Borges et al., 2005), anti-inflamatória e antinociceptiva (Savegnago et al., 2007a,b) e anti-hiperglicêmica (Barbosa et al., 2006).

Por outro lado, o $(PhSe)_2$ causa efeitos tóxicos quando administrado em altas doses (Nogueira et al., 2003a). O cérebro é um dos órgãos alvo deste composto, pois, devido a sua lipofilicidade, tem a capacidade de ultrapassar a barreira cérebro-sangue afetando um grande número de processos neuronais (Jacques-Silva., 2001; Maciel et al., 2003). Estudos prévios demonstraram que a exposição ao $(PhSe)_2$ causa modificações na funcionalidade do sistema glutamatérgico *in vivo* e *in vitro* (Nogueira et al., 2001), alterações enzimáticas como na δ-aminolevulinato desidratase (δ-ALA-D) (Meotti et al., 2003) e na Na^+, K^+-ATPase (Borges et al., 2005), também pode ocasionar convulsões (Nogueira et al., 2003a).

A toxicidade induzida pelo $(PhSe)_2$ depende da via de administração (intraperitoneal, intracerebroventricular, subcutânea ou oral) e da espécie considerada (ratos ou camundongos) (Nogueira et al., 2003a). O $(PhSe)_2$ não provoca efeitos neurotóxicos quando administrado pela via subcutânea e intracerebroventricular (Maciel et al., 2003; Jacques-Silva et al., 2001). Porém, quando administrado pela via intraperitoneal, este composto causa convulsão em camundongos, mas não em ratos adultos, demonstrando ser mais neurotóxico em camundongos que em ratos (Nogueira et al., 2003a). Segundo Caldwell (1982) e Blake et al. (2005), a diferença de toxicidade entre espécies ou entre idades pode ser relacionada a diferenças no metabolismo e disposição de um composto.

A absorção de compostos orgânicos no organismo (naturais ou xenobióticos) é seguida pelas reações de biotransformação, as quais podem levar a uma inativação (detoxificação), ativação (bioativação) ou pode levar a formação de compostos mais tóxicos (biotoxificação). A biotransformação de drogas ocorre em duas fases. Durante a primeira fase (Fase I) os xenobióticos são funcionalizados por oxidação, hidrólise ou redução. Na segunda parte (Fase II), os metabólitos primários passam por reações de

conjugação com agentes endógenos, formando assim metabólitos secundários, posteriormente excretados na bile e urina (Levsen et al., 2005; Nassar et al., 2009).

Muitos estudos demonstram que as diversas formas de selênio, inorgânicas ou selenoaminoácidos, utilizadas nutricionalmente, assumem um intermediário comum (selenito), que é utilizado para a síntese de selenoproteínas e o excedente é transformado no fígado em selenoaçúcar A e B (1β-metilseleno-N-acetil-D-galactosamina), para ser excretado na urina (Kobayashi et al., 2002; Suzuki et al., 2005a, Suzuki et al., 2005b, Suzuki et al., 2006). Estudos com o composto orgânico de selênio Ebselen, mostram que ele é reduzido por tióis a 2-selenilbenzenanilida (Ziegler et al., 1992) ou reage com grupos de tióis endógenos de proteínas para formar selenosulfetos em proteínas (Dimmeler et al., 1991). Após a transformação a 2-selenilbenzenanilida, o ebselen sofre glucuronidação ou metilação, formando metabólitos mais polares, para serem excretados pela urina (Muller et al., 1988). Porém, não existem estudos demonstrando a biotransformação do $(PhSe)_2$.

Considerando que o $(PhSe)_2$ apresenta inúmeras propriedades farmacológicas que o tornam potencial candidato a fármaco, os objetivos deste estudo foram investigar a neurotoxicidade aguda induzida por $(PhSe)_2$ em ratos bebês, avaliando o aparecimento de convulsões e os possíveis mecanismos de ação envolvidos na atividade convulsiva induzida pelo $(PhSe)_2$; estudar a toxicocinética do $(PhSe)_2$ e correlacioná-los aos efeitos causados causados pelo composto nos modelos experimentais estudados.

2. REVISÃO BIBLIOGRÁFICA

CAPÍTULO I

2.1 Epilepsia e Convulsão

A epilepsia é uma das muitas desordens cerebrais, que afeta cerca de 50 milhões de pessoas no mundo todo (Scheuer and Pedley, 1990). Referem-se a um grupo diverso de transtornos neurológicos crônicos, etiológica e clinicamente marcados por crises convulsivas recorrentes, as quais resultam da atividade neuronal excessiva, anormal e hipersincrônica (Treiman, 1995; Engelborghs, 2000).

As convulsões se caracterizam por descargas anormais em um grupo de neurônios cerebrais, levando a uma alteração da atividade cerebral caracterizadas por manifestações motoras, sensitivas, sensoriais, psíquicas ou neurodegenerativas. Fundamentalmente se dividem em dois grandes grupos: parciais e generalizadas (Commission, 1981; Loscher, 1997; Holmes, 2005).

As crises parciais são descargas elétricas anormais, provenientes de um foco epileptogênico, limitadas a uma região mais ou menos circunscrita do córtex cerebral, podendo ser: parciais simples: não provocam alteração da consciência e manifestam-se como eventos visuais, motores, autonômicos ou sensoriais e podem se confundir com outros fenômenos transitórios; parciais complexas: caracterizam-se por uma mudança de consciência, definida como incapacidade de responder normalmente a estímulos externos, podendo ocorrer em graus variáveis e associar-se a diversos eventos, como quedas abruptas e movimentos inconscientes e involuntários (automatismos) (Commission, 1989; Loscher, 1997).

Nas crises generalizadas, as descargas neuronais são bilaterais e envolvem simultaneamente amplas áreas de ambos os hemisférios cerebrais. A consciência é quase sempre comprometida, e as manifestações motoras afetam os dois lados do corpo. As crises podem ser convulsivas (com fenômenos motores) ou não. No primeiro caso, são classificadas como: tônicas, quando o corpo fica rígido; clônicas, quando há contrações ritmadas seguidas de relaxamento em rápida sucessão; tônico-clônicas, se os dois sintomas

estiverem presentes e mioclônicas, caso haja contrações não ritmadas e erráticas de apenas um ou alguns grupos de músculos definidos. Caso não haja fenômenos motores, como os anteriormente descritos, as crises são denominadas atônicas (perda do tônus muscular, sem rigidez do corpo) ou de ausência (perda do contato com o meio) (Commission, 1989; Loscher, 1997).

A epilepsia é uma desordem de causa idiopática. Entre as possíveis causas da epilepsia estão lesões cerebrais decorrentes de traumatismos na cabeça, tumores, distúrbios cerebrais degenerativos, infecções (meningite, por exemplo), abuso de bebidas alcoólicas ou de drogas e complicações durante o parto, porém, a maior parte dos casos não tem uma origem clara, ou seja, não são determinados por uma lesão, mas sim por fatores genéticos (Loscher, 1997; Holmes, 2005). Na ausência de um fator etiológico específico, na maioria dos casos a terapia se faz necessária para controlar os sintomas.

2.1.1 Convulsões e o Cérebro em Desenvolvimento

As crises epilépticas são freqüentemente observadas na clínica de neurologia infantil. Em torno de 4% de todas as crianças poderão apresentar um episódio nos primeiros 15 anos de vida, e 90% dos epilépticos têm a primeira crise antes dos 20 anos de idade, o que sinaliza o início precoce da maioria das epilepsias (Fernandes and Dander, 1998).

Lenox (1981) observou que 30% de todas as formas de epilepsia iniciam no primeiro ano de vida, e outros 30,5% no segundo, terceiro e quarto ano. Entre os recém nascidos, a maior freqüência se verifica entre os nascidos a termo, já que a maturação do SNC é importante para o desenvolvimento de crises, tornando as manifestações neurológicas mais importantes deste período (Rotta, 1991). Além disso, durante o nascimento, os bebês podem sofrer insultos como trauma, hipóxia-isquemia, infecções perinatais, hemorragias intracranial, distúrbios metabólicos e febre, o que pode resultar em convulsão (Holmes, 2005).

O tipo de crise epiléptica apresentado pela criança depende do estado de desenvolvimento e organização funcional do SNC. Assim, os tipos de epilepsia podem ser altamente variáveis, desde simples crises de ausência até convulsões tônico-clônicas e crises parciais complexas. Múltiplos tipos de crises, evolução de um tipo a outro e remissão espontânea das crises são características marcantes das epilepsias em crianças

(Cowan, 2002). Cavazzuti (1984) registrou predomínio de formas parciais na faixa de 5 a 14 anos (66%), enquanto Dosse & Sitepu (1993) encontraram predomínio de crises generalizadas na faixa de 0 a 9 anos (62,6%).

Pacientes com epilepsia apresentam um grande risco de desenvolverem alterações cognitivas e anormalidades comportamentais. Existem muitas evidências que demonstram que as convulsões podem causar danos cerebrais que podem ser responsáveis por um declínio cognitivo (Elger et al., 2004). Este fato motiva muitos estudos experimentais utilizando modelos animais, os quais demonstram que o cérebro imaturo é mais vulnerável ao desenvolvimento de convulsões quando comparado ao cérebro adulto.

Em termos de maturação cerebral, tem-se que ratos de 8 a 14 dias de idade correspondem a crianças recém nascidas, enquanto ratos recém nascidos correspondem a fetos humanos com 6 meses de gestação (Morgane et al., 2002). A sinaptogênese no cérebro do rato ocorre principalmente entre o 7º e 21º dia de vida pós-natal, podendo diferir de região a região. O aumento nos contatos sinápticos e a diferenciação destas conexões representam o começo do desenvolvimento químico e funcional do SNC **(Figura 1)**. Desse modo, o cérebro é sensível a alterações no seu desenvolvimento, provocadas por fatores externos durante os períodos de rápido crescimento cerebral, após o nascimento (Rocinholi et al., 1997; Rice, 1999).

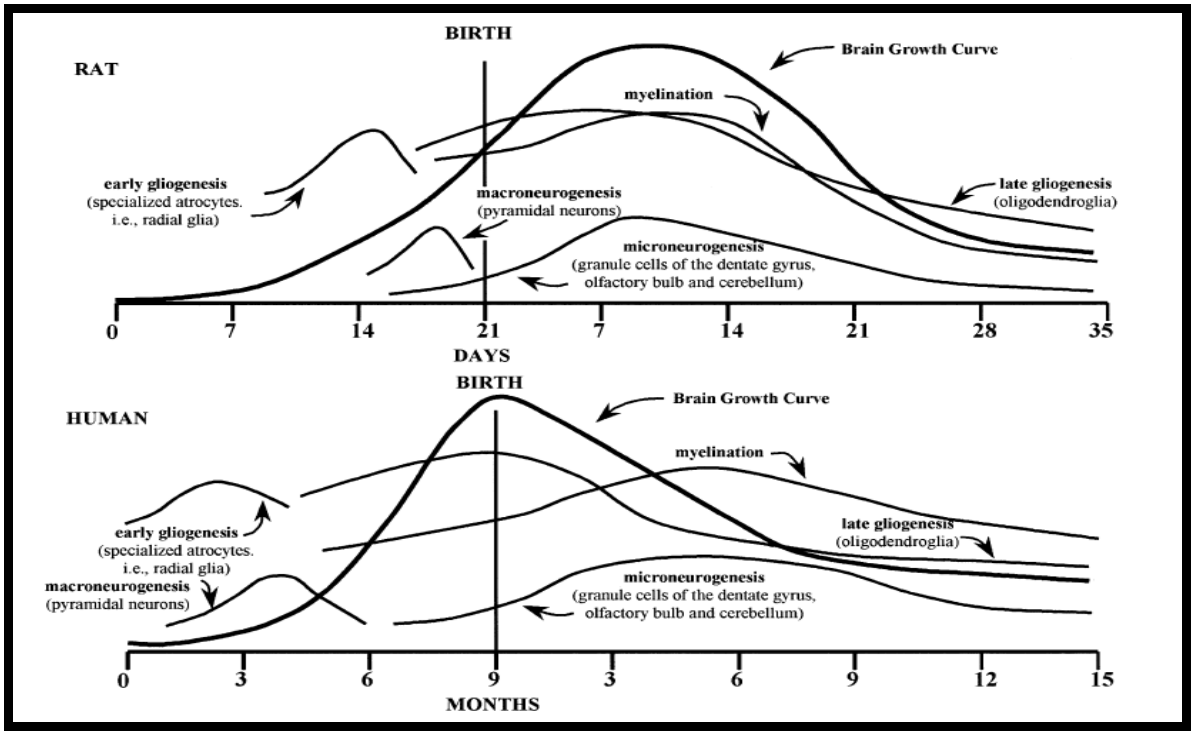

Figura 1 – Curva de Velocidade, comparando os índices relativos, duração e tempo do processo desenvolvimental específico em cérebro de ratos e humanos. As curvas de rápido crescimento cerebral (índices de mudança no peso cerebral) são sobrepostas em relação aos eventos desenvolvimentais no cérebro. Note que a gênese precoce de astroglia e células piramidais em humanos, resultando na aquisição de aproximadamente 27% do peso cerebral adulto no tempo do nascimento, comparado à aproximadamente 12% do peso cerebral adulto visto em ratos ao nascimento. A curva de rápido crescimento cerebral em ratos é alterada para a direita, comparado aos humanos.

Fonte: Adaptado de Morgane et al., 2002.

Por fim, sabe-se que neurônios imaturos tendem a gerar periódicas descargas e essas facilitam a geração de oscilações patológicas, além disto, o sistema GABAérgico exerce uma ação paradoxalmente excitatória em grande parte das estruturas cerebrais neste período (Khazipov et al., 2004). Este fato seria um agravante no desenvolvimento de convulsões neste período, uma vez que vários trabalhos sugerem que os principais mecanismos envolvidos na gênese da convulsão estão relacionados a um desequilíbrio ocasionado por um aumento da transmissão excitatória glutamatérgica e/ou diminuição da resposta inibitória GABAérgica (Ribeiro et al., 2005).

2.2 Os Sistemas Glutamatérgico e GABAérgico

Representando os principais neurotransmissores inibitórios e excitatórios, respectivamente, o ácido γ-aminobutírico (GABA) e o glutamato ocupam um papel central em processos neuropatológicos, tais como epilepsia, déficits de função cognitiva e doenças neurodegenerativas (Meldrum, 1995; Ribeiro et al., 2005). Particularmente, um desequilíbrio em qualquer um dos sistemas de neurotransmissão excitatória ou inibitória no SNC pode estar implicado no desenvolvimento de processos convulsivos (Meldrum, 1995). Além disso, a intensificação do desequilíbrio neuronal que conduz aos episódios convulsivos, assim como o seu abrandamento, pode ocorrer a partir da interação de mecanismos inibitórios GABAérgicos e excitatórios glutamatérgicos (Meldrum, 1995; Ribeiro et al., 2005).

2.2.1 O Sistema Glutamatérgico

O glutamato é o aminoácido encontrado em maior concentração no SNC e medeia a maior parte da transmissão sináptica excitatória no cérebro. É um mediador chave de uma variedade de processos fisiológicos, tais como comunicação intracelular, crescimento e diferenciação, aprendizado e memória (Ozawa et al., 1998) bem como na formação de redes neurais durante o desenvolvimento (Izquierdo, 2006), desempenhando um papel importante na realização de conexões sinápticas normais no cérebro. Conseqüentemente, um desequilíbrio na via glutamatérgica é um fator importante na gênese de muitas desordens neurológicas.

Evidências têm demonstrado que um aumento na neurotransmissão glutamatérgica está envolvido nos mecanismos excitotóxicos das convulsões, além da neurodegeneração (Raol et al., 2001). Efetivamente, as principais vias excitatórias do SNC utilizam o glutamato como neurotransmissor (Meldrum et al., 1999). Sua síntese ocorre nos terminais pré-sinápticos, predominantemente a partir da glutamina, a qual é sintetizada nas células gliais e transportada para os terminais nervosos, onde então é convertida em glutamato devido à ação da enzima glutaminase. Entretanto, ele ainda pode provir do α-cetoglutarato, nas reações de transaminação e na aminação redutora pela glutamato desidrogenase (Kvamme, 1998).

Além disso, quando liberado na fenda sináptica, as respostas fisiológicas ao glutamato ocorrem via ativação de receptores ionotrópicos e metabotrópicos farmacologicamente e funcionalmente distintos, localizados nas membranas pré e pós-sinápticas, bem como na membrana das células gliais (Meldrum et al., 1999).

Como mediador de sinapses excitatórias, o glutamato tem um papel importante em funções essenciais do SNC; entretanto, uma ativação excessiva do sistema glutamatérgico pode provocar dano ou até mesmo morte neuronal (Meldrum, 2002). Esta morte neuronal provocada pela estimulação excessiva dos receptores glutamatérgicos, devido a uma administração de altas doses de glutamato, ou de agonistas de receptores glutamatérgicos foi inicialmente descrita pelo termo "excitotoxicidade". Após a descoberta de que antagonistas glutamatérgicos podem ter efeitos benéficos em modelos de desordens neurológicas, tais como isquemia cerebral e epilepsia, o conceito de excitotoxicidade foi modificado. Isto é, passou-se a admitir que o glutamato endógeno possa também ser um mediador de neurotoxicidade, quando se acumula no espaço extracelular (Obrenovitch et al., 2000).

Apesar do grande interesse em torno deste tema, os mecanismos intracelulares responsáveis pela excitotoxicidade ainda não foram completamente elucidados. Entretanto, o aumento de Ca^{2+} intracelular, seguido da ativação de receptores ionotrópicos de glutamato, inchaço osmótico celular, produção de radicais livres e peroxidação lipídica nas membranas celulares, além do aumento na expressão de proteínas apoptóticas, demonstram ter um papel importante (Porciúncula et al., 2001; Rossato et al., 2002; Centurião et al., 2005).

2.2.1.1 Os Receptores de Glutamato

Os receptores glutamatérgicos podem ser classificados, de acordo com estudos farmacológicos e moleculares, em dois grandes grupos: receptores ionotrópicos e metabotrópicos (**Figura 2**) (Ozawa et al.,1998).

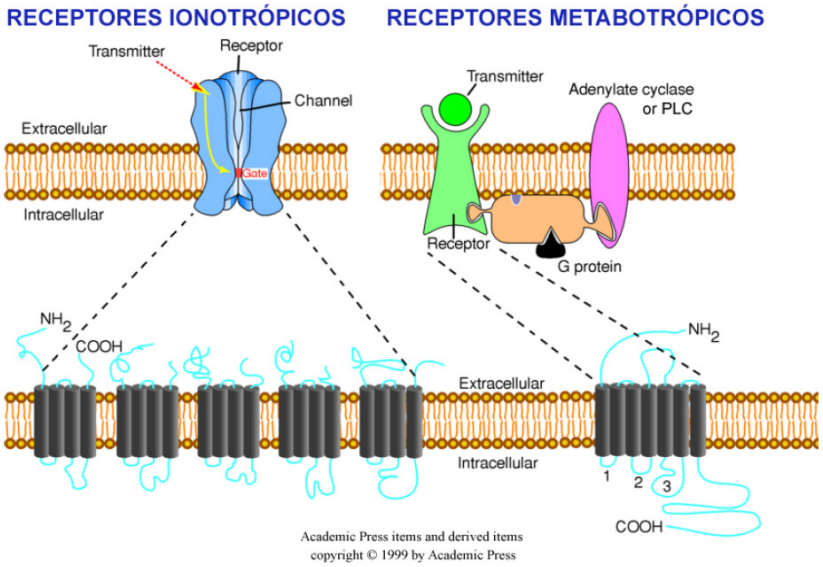

Figura 2. Receptores glutamatérgicos. Os receptores glutamatérgicos ionotrópicos (iGluRs) contêm um canal iônico cátion-específico e são subdivididos em três subtipos: a-amino-3-hidroxi-5-metil-4-isoxazolepropionato (AMPA), cainato e N-metil-D-aspartato (NMDA). Os receptores glutamatérgicos metabotrópicos (mGluRs) são acoplados ao sistema de transdução de sinal via adenilato ciclase ou fosfolipase C (PLC) e estão subdivididos em 8 subtipos (mGluR1-8).

Fonte: Adaptado de Zigmond, Fundamental Neuroscience, Academic Press, 2003.

Os receptores ionotrópicos são canais iônicos que permeiam cátions através da membrana neuronal. Portanto, sua ativação provoca a despolarização da membrana sináptica e desencadeia uma resposta excitatória. Estes receptores são subdivididos em N-metil-D-aspartato (NMDA), a-amino-3-hidroxi-5-metil-4-isoxazol-7 ácido propiônico (AMPA) e cainato (KA), com base na sua sensibilidade a agonistas específicos (Cotmann et al., 1995).

Os receptores AMPA medeiam a neurotransmissão excitatória rápida e são canais com grande permeabilidade a cátions monovalentes (Na^+ e K^+) e com baixa permeabilidade ao Ca^{2+} (Dichter & Wilcox, 1997). Estes receptores possuem, ao menos, três sítios para ligantes: o sítio de união de glutamato (ou AMPA), um sítio de união que modula a desensibilização do receptor e outro que bloqueia o influxo de íons e que está localizado no interior do canal (Ozawa et al.,1998).

Os receptores de KA diferem dos receptores AMPA por serem, além de permeáveis a íons Na^+ e K^+, relativamente permeáveis a íons Ca^{2+} (Ozawa et al., 1998). Esses receptores são encontrados em poucas áreas cerebrais ao contrário dos receptores AMPA,

que apresentam ampla distribuição no SNC. Além disso, a administração intracerebral ou parenteral de KA em ratos possui efeito convulsivo, e resulta num modelo de dano cerebral que se assemelha ao de pacientes com epilepsia lobo-temporal (Kleinrok et al., 1995).

Por sua vez, os receptores NMDA medeiam a transmissão sináptica excitatória lenta, são canais com grande permeabilidade ao Ca^{2+} e baixa permeabilidade ao Na^{+} e K^{+} (**Figura 3**) (Lipton and Rosemberg, 1994; Ozawa et al., 1998). O complexo do receptor NMDA apresenta diversos sítios para ligantes que regulam a abertura do canal iônico: um sítio para glutamato (ou NMDA), um para o coagonista endógeno glicina, um sítio no interior do canal para a união de bloqueadores (MK-801 e PCP (fenciclidina), e sítios modulatórios tais como: um sítio para o Zn^{2+} (antagonista não competitivo do receptor), outro para poliaminas, um sensível a modulação redox (modulado tanto por agentes oxidantes quanto por agentes redutores) e um sítio sensível a H^{+} (Gozlan and Bem-Ari, 1995; Martin et al., 1995; Ozawa et al., 1998)

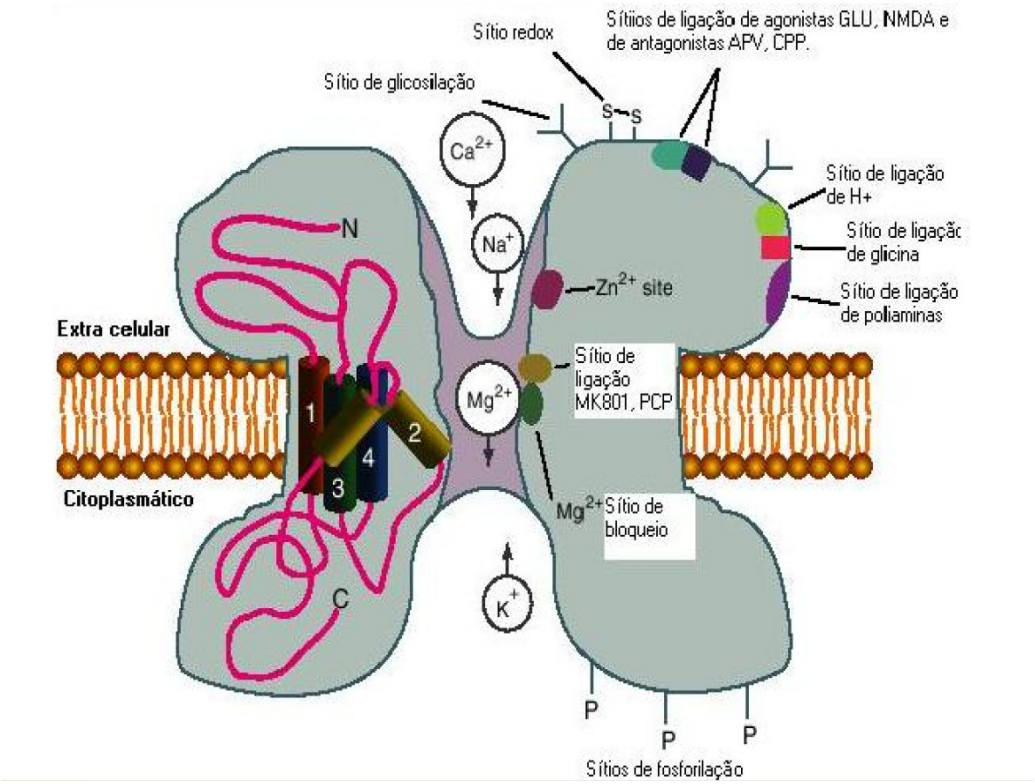

Figura 3. Representação esquemática do receptor NMDA.

Fonte: Zigmond et al. (2003).

Além disso, o canal do receptor NMDA é bloqueado por Mg^{2+} de uma maneira dependente de voltagem, ou seja, nos neurônios em potencial de repouso, a ativação do receptor só ocorre se a membrana neuronal for despolarizada, o que permite a saída de Mg^{2+} do interior do canal. O bloqueio dependente de voltagem do canal NMDA por Mg^{2+} pode ser visto como um mecanismo protetor intrínseco contra a entrada excessiva de Ca^{2+} na célula e a conseqüente toxicidade neuronal (Edmonds et al., 1995).

A ativação do receptor NMDA está envolvida no processo de plasticidade neuronal, entretanto, sua estimulação excessiva acarreta aumento do Ca^{2+} intracelular com conseqüente toxicidade celular (Mody and Macdonald, 1995). Existem evidências de que a excitotoxicidade esteja envolvida na morte neuronal observada em algumas doenças neurodegenerativas, tais como Alzheimer, síndrome de Huntington, esclerose lateral amiotrófica, neurodegeneração associada a infecções por HIV; bem como em patologias agudas, como o dano neuronal isquêmico e hipoglicêmico (Meldrum, 1991, 1995; Price, 1999).

Diferentemente dos receptores ionotrópicos, os receptores glutamatérgicos metabotrópicos estão associados a sistemas de segundos mensageiros intracelulares. Estes receptores são acoplados a proteínas G (proteínas ligantes de nucleotídeos da guanina), as quais modulam a atividade de efetores intracelulares, tais como a adenilato ciclase e a fosfolipase C (Cotmann et al., 1995).

Os receptores glutamatérgicos metabotrópicos, assim como o ionotrópico NMDA, estão envolvidos no processo de indução da plasticidade neuronal. Localizados nos terminais pré e pós-sinápticos e nas células gliais, estes receptores também possuem papel importante na indução de convulsões e morte neuronal (Nicoletti et al., 1996).

Particularmente, a ativação de receptores de glutamato metabotrópicos présinápticos pode promover efeitos excitatórios ou inibitórios (Ozawa et al., 1998).

2.2.1.2 A Captação de Glutamato

A captação de glutamato é o principal mecanismo responsável pela manutenção dos níveis extracelulares de glutamato abaixo dos níveis tóxicos, sendo realizados por transportadores de glutamato presentes na membrana plasmática de neurônios e células gliais, principalmente em astrócitos (Danbolt, 2001).

Esta captação do glutamato envolve dois sistemas de transporte: um carreador com alta afinidade e dependente de Na^+, localizado nas membranas pré-sinápticas e gliais, e outro com baixa afinidade e independente de Na^+, localizado nas membranas das vesículas sinápticas. Devido à ação coordenada destes transportadores o glutamato é armazenado nas vesículas, diminuindo sua concentração na fenda sináptica (Robinson and Dowd, 1997).

Claramente, a captação de moléculas de glutamato apresenta uma função vital na manutenção dos níveis de glutamato, a fim de evitar excessiva ativação dos receptores glutamatérgicos e conseqüente excitotoxicidade. Resultados indicaram que alterações nos transportadores de glutamato podem desencadear um importante papel no processo epileptogênico (Meldrum et al., 1999). Além disso, a captação de glutamato tem vital importância de prover glutamato para síntese de GABA, glutationa, para a liberação de energia desempenha papel importante como acoplador da atividade neuronal com o metabolismo da glicose em astrócitos (Danbolt, 2001).

2.2.2. O Sistema GABAérgico

O ácido γ-amino-butírico (GABA), principal neurotransmissor inibitório do SNC de adultos, é sintetizado em 20 a 30 % dos neurônios, pelos chamados neurônios GABAérgicos e é indispensável para o controle de funções do SNC como a atividade locomotora, o aprendizado e o ritmo circadiano (Chaudhry et al., 1998; Varju et al., 2001). No SNC de vertebrados o GABA é o principal neurotransmissor inibitório. Está localizado em interneurônios de axônios curtos que fazem sinapse com o corpo celular e axônios proximais, e mantém o tônus inibitório que contrabalança a excitação neuronal. Quando este balanço é perturbado, pode ocorrer a convulsão (Treiman, 2001). O GABA é formado dentro dos terminais dos axônios GABAérgicos por meio da transaminação do α-cetoglutarato a ácido glutâmico (Treiman, 2001), que é descarboxilado a GABA pelas duas isoformas da ácido glutâmico descarboxilase GAD_{65} e GAD_{67}. Ambas as isoformas estão presentes em "pools" de GAD solúvel e associadas à membrana (Gadea and Colone, 2001). A distribuição preferencial da GAD_{65} em terminais nervosos e sua associação com vesículas sinápticas sugere que possa estar envolvida na síntese de GABA vesicular que medeia a comunicação sináptica rápida (Gadea and Colone et al., 2001).

Antes da liberação na fenda sináptica, o GABA é transferido para vesículas sinápticas por ação de proteínas integrais de membrana, denominadas transportadores

vesiculares de GABA (VGAT), as quais são cruciais para a função GABAérgica e cuja atividade depende do pH e de gradientes elétricos (Devaud, 2001).

A liberação pré-sináptica de GABA pode ser vesicular, cálcio dependente e estimulada por altas concentrações de potássio e não vesicular, cálcio independente e secundária à despolarização da membrana celular e influxo de sódio. A liberação não vesicular de GABA depende do transporte reverso de GABA (Treiman, 2001). Depois do GABA ser liberado dos terminais dos axônios e de agir sobre os receptores ionotrópicos e metabotrópico, esta ação é terminada pela sua rápida recaptação via transportadores Na^+/Cl^- dependentes, ligados a membranas pré e pós- sinápticas de neurônios e também em células gliais. O GABA é então catabolizado pela GABA transaminase (GABA-T) a semialdeído succínico, que é convertido a ácido succínico pela semialdeído ácido succínico desidrogenase, entrando, então, no ciclo de Krebs (Treiman, 2001). O aumento da atividade da enzima GABA-T no cérebro, diminui as concentrações de GABA e pode levar a convulsões, coma e óbito. A inibição desta enzima aumenta consideravelmente as concentrações de GABA (Devaud, 2001).

2.2.2.1 Os Receptores de GABA

Os receptores $GABA_A$ são ionotrópicos, constituindo-se de um complexo de estrutura hetero-oligômera, cujas subunidades formam um canal de cloreto, o qual, ao aumentar o influxo, hiperpolariza o neurônio. Estes receptores medeiam a resposta inibitória rápida em cérebro de mamíferos (Korpi et al., 2002). A inibição é um processo fundamental na atividade cerebral, e, portanto, a maior parte das células neuronais expressam estes receptores nas suas membranas celulares (Korpi et al., 2002). Os receptores $GABA_A$ apresentam sítios de ligação para GABA, benzodiazepínicos, barbitúricos, neuroesteróides, picrotoxina e etanol (**Figura 4**). Foram isoladas várias subunidades do complexo $GABA_A$ (α1-6, β1-4, γ1-4, δ, π, ε, segundo Bormann, 2000.

Os subtipos de receptores $GABA_A$ são formados por diferentes combinações de subunidades, sendo que há uma variação na sua expressão e suas propriedades farmacológicas em diferentes células e regiões do cérebro (Korpi et al., 2002). Sperk et al. (1997) sugerindo que a distribuição das subunidades de receptores $GABA_A$ difere entre células e mesmo entre regiões do mesmo neurônio. Mudanças na expressão das subunidades ocorrem em resposta a tratamento com drogas, como, por exemplo, o etanol,

barbitúricos, neuroesteróides e benzodiazepínicos. Portanto, a densidade relativa de subtipos de receptores e sua eficácia funcional no cérebro adaptam-se a um ambiente interno alterado (Korpi et al., 2002).

Um segundo tipo de receptores ionotrópicos foi descrito em retina de vertebrados. Compostos de sub-unidades ρ, estes receptores de GABA, insensíveis à bicuculina e baclofen, são frequentemente denominados de receptores $GABA_C$ (Bormann, 2000; Alakuijala et al., 2005).

Já os receptores $GABA_B$ ligados a proteínas G, hiperpolarizam o neurônio aumentando a condutância do potássio e diminuindo o influxo de cálcio, tendo efeito inibitório lento. Estão presentes em terminais de axônios excitatórios e inibitórios e são ativados por baclofen e resistentes a drogas que modulam os receptores $GABA_A$ (Marshal et al., 1999).

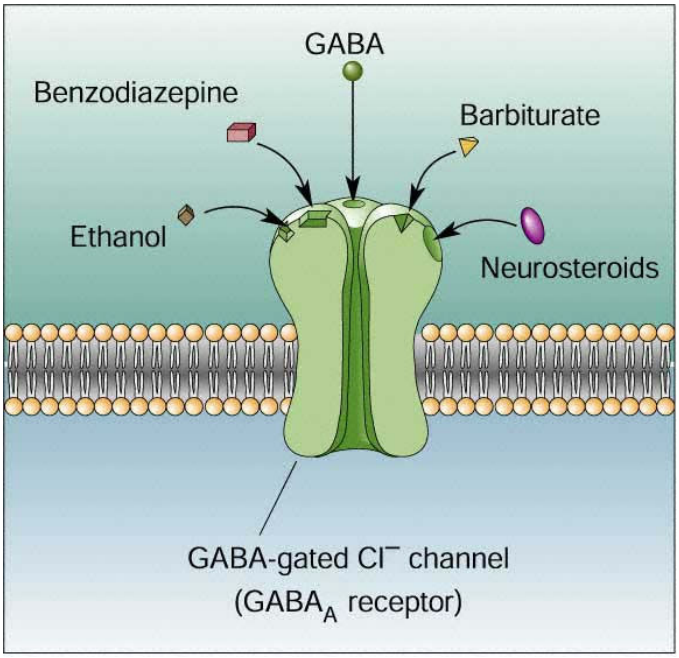

Figura 4 – Representação esquemática do receptor GABAérgico ionotrópico $GABA_A$.
Fonte: http://img.medscape.com/pi/emed/ckb/neurology/1134815.png

2.2.2.2 A Captação de GABA

Além da biossíntese, liberação e interação com seu receptor, a inativação de neurotransmissores é condição para garantir adequadas funções cerebrais. Na finalização

da atividade GABAérgica são responsáveis transportadores de alta afinidade, localizados no terminal pré-sináptico de interneurônios, em processos gliais adjacentes (Cherubini and Conti, 2001; Gadea and Lopez-Colome, 2001) e, possivelmente, também em membranas celulares pós-sinápticas (Frahm and Draguhn, 2001).

Enquanto na neurotransmissão glutamatérgica, os transportadores astrocitários são os mais proeminentes, na neurotransmissão GABAérgica a captação em terminais nervosos pré-sinápticos prevalece sobre a captação pelos astrócitos (Schousboe, 2003), o que dá suporte à noção de que a neurotransmissão GABAérgica está preferencialmente baseada na reciclagem de GABA (Schousboe et al., 2004). Isto não implica o fato de que o transporte glial não tenha importância funcional. Pelo contrário, inibidores do transporte astroglial de GABA são capazes de aumentar a disponibilidade de GABA no "pool" dos neurotransmissores (Schousboe, 2003), sugerindo que a captação glial de GABA constitui importante fator regulador da disponibilidade de GABA na sinapse (Schousboe et al., 2004).

Levando em consideração que o tônus GABAérgico diminuído relativamente ao da neurotransmissão excitatória, pode resultar em atividade convulsivante (Meldrum, 2002), uma série de drogas anticonvulsivantes, atuando na neurotransmissão GABAégica, foram desenvolvidas (Löscher, 1998). Enquanto o GABA captado pelos neurônios GABAérgicos pode ser reutilizado como neurotransmissor, o captado em células gliais circunvizinhas é consumido no metabolismo via GABA transaminase e ciclo do ácido tricarboxílico (Schousboe et al., 2003). Partindo deste princípio e baseados no fato de que existe uma correlação estabelecida entre atividade anticonvulsivante de inibidores de transporte astroglial de GABA, enquanto que esta correlação parece não existir para a inibição neuronal de captação de GABA (White et al., 2002), o desenvolvimento de drogas antiepilépticas, cuja a ação é baseada na inibição seletiva do transporte astrocitário, é de grande importância farmacológica.

2.3 Excitotoxicidade, Formação de Espécies Reativas e Convulsões

Apesar da transmissão GABAérgica e glutamatérgica serem fundamentais para os processos fisiológicos, sabe-se que um desequilíbrio nos neurotransmissores inibitório e excitatório, pode favorecer uma ativação supra-fisiológica dos receptores glutamatérgicos causando morte neuronal e um aumento na produção de espécies reativas. Esse processo é

chamado de excitotoxicidade (Patel, 2004). O termo excitotoxicidade foi inicialmente usado para descrever um aumento na ativação de receptores glutamatérgicos via administração de altas doses de glutamato exógeno. Após a descoberta de que antagonistas glutamatérgicos poderiam proteger contra a perda neuronal induzida por diversos insultos neurológicos, passou-se a admitir que o glutamato endógeno pudesse ser mediador de processos excitotóxicos quando em altas concentrações na fenda sináptica (Obrenovitch, 1999; Obrenovitch et al, 2000).

A neurotoxicidade induzida pelo glutamato pode ter como causa: i) uma redução na transmissão GABAérgica; ii) um aumento na concentração de glutamato no meio extracelular (aumento na liberação ou redução da captação); iii) uma hipersensibilidade dos receptores ao glutamato devido a uma perda na seletividade iônica, um aumento na densidade ou anormalidades na modulação; e/ou iv) uma deficiência no metabolismo energético e nas defesas antioxidantes (Halliwell and Gutteridge, 1989; Galé, 1992; Cotman et al., 1995; Chapman, 2000).

Uma estimulação excessiva de receptores glutamatérgicos, induz a perda do equilíbrio do Ca^{2+} intracelular. O aumento da concentração de Ca^{2+} intracelular provoca um aumento na produção de EROs, disfunção mitocondrial, e conseqüente diminuição na produção de energia (Chapman, 2000). Nesse contexto, a excitotoxicidade também tem sido relacionada a um aumento na produção de espécies reativas (Patel, 2004).

Estudos mostram que a toxicidade induzida por disseleneto de difenila $(PhSe)_2$ está associada à produção de EROs e a inibição de várias enzimas importantes para o metabolismo energético, como a Na^+, K^+- ATPase (Borges et al., 2005). A depleção energética aumenta a liberação de glutamato não só por mecanismos dependentes de cálcio (por despolarização da membrana), mas também pela inibição e/ou reversão de mecanismos de recaptação deste aminoácido excitatório na pré-sinapse e nas células gliais, levando a um aumento de glutamato na fenda sináptica (Madl and Burgesser, 1993) e, a ativação secundária dos receptores glutamatérgicos subtipo NMDA e AMPA/cainato (Zeevalk et al., 1998).

O GABA também pode ter um papel crucial na despolarização neuronal e na formação de EROs. O bloqueio do receptor $GABA_A$ resulta em uma redução do influxo de íons Cl^- e facilita a despolarização da membrana e, assim, o influxo de Ca^{2+} através de canais associados ao receptor NMDA (Sankar et al., 1997). A despolarização da membrana pós-sináptica leva à saída do íon Mg^{2+}, que bloqueia o canal do receptor NMDA de maneira dependente de voltagem, e permite a entrada de íons Na^+ e Ca^{2+} para o meio

intracelular (McDonald & Schoepp, 1993). O dano excitotóxico primário é desencadeado pela entrada de Na^+ através de receptores ionotrópicos do subtipo AMPA/cainato (Chan et al, 1979). O aumento de Ca^{2+}, via ativação de receptores do subtipo NMDA, leva à ativação de enzimas dependentes deste íon, e que estão envolvidas na produção de radicais livres como fosfolipase C (Umemura et al., 1992) e óxido nítrico sintetase, que produz óxido nítrico (NO•) (Snyder and Bredt, 1992). O NO• pode interagir com radicais superóxido (O2•) e formar peroxinitrito (ONOO-). Este radical pode difundir-se para o meio intra, ou extracelular e então promover a oxidação de lipídios, proteínas e DNA (Almeida et al., 1998), que estão associados ao aparecimento de convulsões e morte celular.

Estudos relacionam o envolvimento do estresse oxidativo ao processo convulsivo. Recentes trabalhos demonstraram um aumento em diversos marcadores de formação de espécies reativas, como substâncias reativas ao ácido tiobarbitúrico (TBARS) (Patsoukis et al., 2004) e proteína carbonila (Oliveira et al 2004), assim como uma redução na atividade da enzima Na^+, K^+-ATPase (Oliveira et al., 2004) em modelos de convulsão induzidas por pentilenotetrazol (PTZ), o que sugere que a geração de espécies reativas de oxigênio e nitrogênio estariam relacionadas com os efeitos convulsivantes e neurotóxicos do PTZ (Bashkatova et al., 2003). Do mesmo modo, o tratamento com antioxidantes foi capaz de atenuar as convulsões induzidas por PTZ e/ou o dano induzido pelas espécies reativas (Bashkatova et al., 2003).

2.3.1 Espécies Reativas e Estresse Oxidativo

Do ponto de vista químico, um radical livre (RL) é definido como qualquer átomo, grupo de átomos ou moléculas capazes de existir sob a forma independente e que contém um ou mais elétrons desemparelhados (Halliwell and Gutteridge, 2007). Portanto, os radicais livres podem ser formados pela adição ou pela perda de um elétron de uma substância não-radical. Entretanto, existem compostos igualmente reativos quanto aos radicais livres que não possuem elétron não-pareado na última camada e, portanto não podem ser classificados como radicais livres (Dröge, 2002). Essas substâncias são classificadas de maneira mais ampla como espécies reativas de oxigênio (EROs) ou espécies reativas de nitrogênio.

As (EROs) são capazes de gerar estresse oxidativo em conseqüência de suas propriedades oxidantes e reação com os constituintes celulares (Timbrell, 2000). Estas são geradas por uma variedade de processos, podendo atacar uma diversidade de biomoléculas alvo, tais como, DNA, lipídeos e proteínas.

As membranas biológicas apresentam uma estrutura geral comum. Estas são constituídas de uma bicamada lipídica as quais estão associadas a proteínas. As proteínas presentes na membrana celular são responsáveis pelo transporte de moléculas específicas através da bicamada lipídica. Além disso, essas proteínas podem agir como catalisadoras de reações associadas às membranas, como a síntese de ATP (Alberts et al., 1994).

As membranas biológicas são constituídas principalmente por fosfolipídeos, os quais possuem uma cabeça polar e duas caudas hidrofóbicas. Geralmente, as caudas hidrofóbicas são compostas por ácidos graxos, que podem diferir no comprimento e na configuração em que se apresentam, podendo uma das caudas apresentar uma ou mais ligações duplas (insaturações) (Alberts et al., 1994; Halliwell and Gutteridge, 2007). Quando os RL reagem com esses ácidos graxos insaturados modificam os lipídeos e a membrana perde suas características arquitetônicas, tornando-se menos firme e menos flexível, criando-se verdadeiras fendas iônicas que alteram sua semipermeabilidade, o que favorece a entrada e saída indiscriminada de metabólitos e detritos da célula, provocando sua ruptura e lise com necrose (Timbrell, 2000).

As principais EROs vinculadas ao estresse oxidativo são: o radical ânion superóxido ($O^{-}_{2}{}^{\cdot}$), radical hidroxil ($^{\cdot}OH$), peróxido de hidrogênio (H_2O_2), óxido nítrico (NO) e peroxinitrito ($ONOO^{-}$). Estes por sua vez são neutralizados por um elaborado sistema de defesa antioxidante. Neste contexto o estado de estresse oxidativo pode resultar tanto de um aumento na produção de EROs quanto da redução da capacidade antioxidante celular total (Dawson and Dawson, 1996).

Estudos bioquímicos sugerem que reações de oxidação podem ser importantes em patologias cerebrais, e estão associadas com um desequilíbrio da regulação redox no SNC (Halliwell and Gutteridge, 2007).

2.3.2 Espécies Reativas e Sistema Nervoso Central

Todos os tecidos dos organismos aeróbicos podem sofrer dano oxidativo, porém o tecido nervoso é o mais suscetível em comparação aos demais tecidos. Uma das razões é

seu alto consumo de O_2, já que é responsável por aproximadamente 20% do consumo basal de O_2 corporal (Halliwell and Gutteridge, 2007). Assim, o alto consumo de O_2 pode resultar em um aumento da formação de $O_2^{\bullet}$. Além disso, outros fatores também contribuem para tornar o cérebro vulnerável às reações de oxidação. Dentre eles estão a baixa quantidade de defesas antioxidantes; as altas concentrações de lipídios poliinsaturados, que servem como substrato para peroxidação lipídica e a presença do ferro encontra-se presente em altas concentrações em algumas regiões cerebrais, favorecendo a lipoperoxidação e a autooxidação de neurotransmissores devido ao $OH^{\bullet}$ formado na reação de Fenton (Sousa et al., 2003; Wajner et al., 2004).

2.3.3 Sistema de Defesas Antioxidantes Contra as Espécies Reativas de Oxigênio e Nitrogênio

Os seres vivos dispõem de mecanismos protetores para evitar o acúmulo de espécies ativas de oxigênio que incluem mecanismos enzimáticos e não enzimáticos (**Figura 5**). As principais enzimas antioxidantes são a superóxido dismutase, a catalase e a glutationa peroxidase. Essas enzimas evitam o acúmulo de O_2• e de H_2O_2, e a conseqüente produção de •OH, contra o qual não existe nenhum sistema enzimático de defesa. Existem substâncias que neutralizam ação do •OH, levando à formação de produtos menos tóxicos. As substâncias que neutralizam os radicais livres na fase de iniciação ou de propagação da lipoperoxidação, levando à formação de produtos menos tóxicos, são os denominados *scavengers*, enquanto aquelas que atuam absorvendo a energia de excitação dos RLs, neutralizando-os, são chamadas de *quenchers*. As defesas não-enzimáticas incluem os antioxidantes lipofílicos (tocoferóis, carotenóides e bioflavonóides) e hidrofílicos (glutationa e ascorbato) (Halliwell and Gutteridge, 2007).

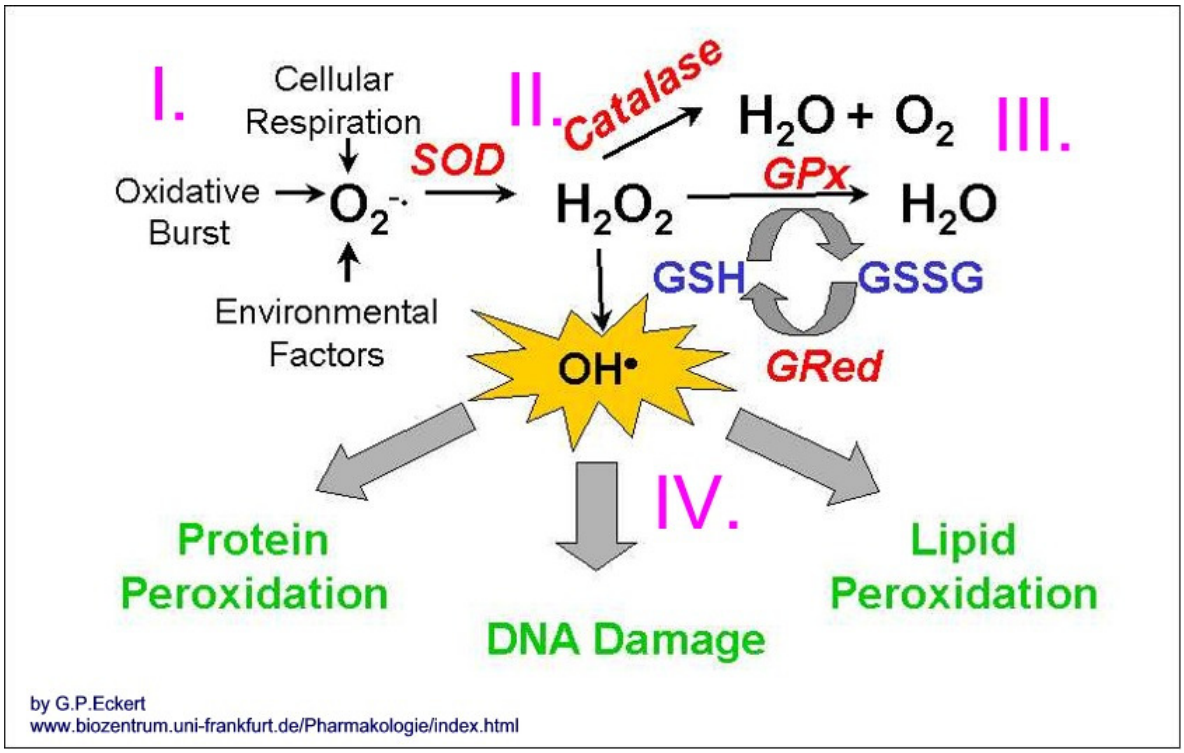

Figura 5: Representação esquemática dos mecanismos de defesa antioxidante enzimáticos e não enzimáticos. **Fonte:** www.biozentrum.uni-frankfurt.de/Pharmakologie/index.html.

2.4 Selênio

O selênio (Se) foi descoberto em 1817, pelo químico sueco J. J. Berzelius. O Se é um elemento do grupo 16 da tabela periódica, podendo apresentar-se sob quatro estados de oxidação: selenato (Se^{+6}), selenito (Se^{+4}), selênio elementar (Se^{0}) e seleneto (Se^{-2}).

O Se compartilha propriedades químicas e físicas com o enxofre (S). Esta similaridade permite que o Se substitua o S, promovendo interações Se-S nos sistemas biológicos. Por outro lado, as diferenças nas propriedades fisico-químicas entre Se e S constituem a base de seus papéis biológicos específicos (Stadtman, 1980).

Os selenóis (R-SeH) são as formas correspondentes aos tióis (R-SH), onde ocorre a substituição do átomo de S pelo átomo de Se (Klayman and Günther,1973).

2.4.1 Atividade Biológica

O Se é um elemento traço, cuja essencialidade nutricional foi demonstrada em 1957 por Schwartz and Foltz (1957). O Se apresenta um grande número de funções biológicas, sendo a mais importante como um antioxidante. Já é conhecido que o Se está presente como resíduo de selenocisteína no sítio ativo das enzimas glutationa peroxidase (Wingler and Brigelius-Flohé, 1999), tioredoxina redutase (Holmgren, 1985), 5'-deiodinase (Behné and Kyriakopoulos, 1990) e selenoproteina P (Ursini et al., 1990). A atividade redox do Se tem fundamental importância para o sítio catalítico dessas enzimas (Wingler and Brigelius-Flohé, 1999). Além disso, vários relatos demonstraram que compostos de Se mimetizam a atividade da enzima glutationa peroxidase.

Nos últimos anos, tem sido descrito que baixos níveis de Se podem levar à pré-disposição para o desenvolvimento de algumas doenças, tais como câncer, esclerose, doença cardiovascular, cirrose e diabetes (Navarro-Alarcón and López-Martinez, 2000). Neste contexto, a suplementação de dietas com Se, tanto para animais quanto para humanos, tem sido aceita pela comunidade científica. A Junta de Alimentação e Nutrição da Academia de Ciências dos Estados Unidos propõe uma ingestão diária de 50-200 μg de Se, a qual é considerada segura para indivíduos adultos (Food and Nutrition Board, 1989).

2.4.2 Toxicologia

O homem é sucetível à intoxicação por selênio, principalmente devido ao seu amplo uso na indústria (Wilber, 1980), particularmente na síntese de compostos, uma vez que este elemento constitui um importante intermediário em síntese orgânica (Comasseto et al., 1997).

Quando a ingestão diária do selênio excede a capacidade corporal de eliminação, algum tipo de intoxicação pode ocorrer e os sintomas crônicos mais comuns são: severa irritação das vias respiratórias, gosto tipicamente metálico na boca, edema pulmonar e característico odor gárlico (Bedwall et al., 1993; Diaz et al., 1997).

Os mecanismos envolvidos na toxicidade do selênio ainda não estão bem definidos. Painter (1941) propôs que a toxicidade do selenito era devido à sua interação com tióis. Yan and Spalallhoz (1991) evidenciaram danos oxidativos como a provável causa da

toxicidade do selênio, enquanto Schwarz (1961) demonstrou que o excesso de Se causaria inativação de enzimas sulfidrílicas.

2.5 Disseleneto de Difenila

A partir da década de 30, os organocalcogênios têm sido alvos de interesse para os químicos orgânicos em virtude da descoberta de suas aplicações sintéticas (Comasseto, 1983), suas propriedades biológicas e aplicações farmacológicas (Parnham & Graf, 1991; Kanda et al., 1999; Nogueira et al., 2004).

O disseleneto de difenila [$(PhSe)_2$ – **Figura 6**] é um composto orgânico que contém Se, portanto um organocalcogênio, largamente utilizado como intermediário em reações de síntese orgânica (Paulmier, 1986; Braga et al., 1996).

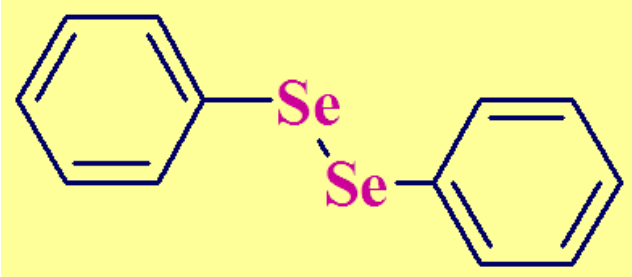

Figura 6. Estrutura química do disseleneto de difenila

2.5.1 Propriedades Farmacológicas

Nosso grupo de pesquisa tem demonstrado que o $(PhSe)_2$ possui inúmeras propriedades farmacológicas, tais como antioxidante (Rossato et al., 2002; Meotti et al., 2004), anti-úlcera (Savegnago et al., 2006), neuroprotetor (Ghisleni et al., 2003), hepatoprotetor (Borges et al., 2005), anti-inflamatório e antinociceptivo (Savegnago et al., 2007), do tipo antidepressiva (Savegnago et al., 2008), do tipo ansiolítica (Ghisleni et al., 2008), anti-hiperglicêmico (Barbosa et al., 2006) e pode apresentar atividade complexante em animais expostos ao cádmio (Santos et al., 2005). Além disso, o $(PhSe)_2$ apresenta

efeitos antioxidantes em diversos modelos de toxicidade induzida por estresse oxidativo (Meotti et al. 2004; Santos et al., 2004, 2005; Luchese et al., 2007, Prigol et al., 2009).

2.5.2 Toxicologia

O $(PhSe)_2$ também possui efeitos tóxicos *in vitro* e *in vivo*. O $(PhSe)_2$ pode causar efeitos nocivos para proteínas ou enzimas de vários tecidos de mamíferos, tais como a enzima δ-aminolevulinato desidratase (δ-ALA-D) (Barbosa et al., 1998; Farina et al., 2003; Nogueira et al., 2003b), Na^+, K^+-ATPase (Borges et al., 2005), e lactato desidrogenase (Kade et al., 2009). Além disso, pode causar toxicidade renal (Meotti et al, 2003) para roedores adultos.

A exposição prolongada a doses altas do $(PhSe)_2$ é causa de neurotoxicidade em roedores (Nogueira et al., 2003a). Estudos em camundongos demonstraram que, devido a sua lipofilicidade, o $(PhSe)_2$ atravessa facilmente a barreira cérebro-sangue após tratamento agudo ou prolongado, aumentando os níveis de selênio no cérebro dos animais (Jacques Silva et al., 2001; Maciel et al., 2003). Portanto, esses trabalhos dão suporte para a hipótese que o cérebro é um alvo potencial para as ações farmacológicas e também tóxicas do composto orgânico $(PhSe)_2$.

Estudos prévios demonstraram que a exposição ao $(PhSe)_2$ modula a funcionalidade do sistema glutamatérgico *in vivo* e *in vitro* (Nogueira et al., 2001), e também causa modificações no sistema neural que podem ocasionar convulsões (Nogueira et al., 2001, 2003). A modulação de grupos tiólicos de transportadores e receptores de glutamato no tecido cerebral pode representar a causa da toxicidade provocada por organocalcogênios em ratos (Nogueira et al., 2001; 2004).

2.5.3. Disseleneto de Difenila e Convulsão

Foi demonstrado que o $(PhSe)_2$ pode induzir convulsões e que estas dependem da via de administração (intraperitoneal, i.c.v ou subcutânea) e da espécie considerada (ratos ou camundongos). O $(PhSe)_2$ não causa convulsões e nem morte em camundongos quando administrado pela via subcutânea e intracerebroventricular, porém, quando administrado

pela via intraperitoneal, este composto causa convulsão em camundongos, mas, não em ratos adultos, demonstrando ser mais neurotóxico nessa espécie (Nogueira et al., 2003a).

Mudanças na estrutura dos disselenetos, como a introdução de grupos funcionais (doadores ou retiradores de elétrons) reduzem ou abolem o aparecimento de episódios convulsivos. Além disso, disselenetos dialquílicos são mais tóxicos em comparação a disselenetos diarílicos, em ratos e camundongos, causando episódios convulsivos em camundongos, sendo que a latência para o aparecimento de episódios convulsivos aumenta com o aumento no número de carbonos da cadeia ligados ao disseleneto (Nogueira et al., 2003a).

Demonstrou-se também que moduladores alostéricos GABAérgicos (diazepam, fenobarbital e muscimol) e antagonistas muscarínicos competitivos são hábeis em abolir ou reduzir as convulsões induzidas pelo $(PhSe)_2$, sugerindo que ocorra a modulação de mais de um sistema de neurotransmissão nas convulsões induzidas por este composto (Nogueira et al., 2003a).

CAPÍTULO II

2.6 Absorção, Distribuição, Biotransformação e Excreção de Drogas

Os conhecimentos da disposição cinética e da biotransformação das drogas no organismo são de grande importância no processo de desenvolvimento e estudo de suas propriedades farmacológicas e toxicológicas (Martín-Liménz et al., 2008).

Um fármaco precisa estar presente em concentrações adequadas nos seus locais de ação para produzir seus efeitos característicos. Embora seja obviamente uma função da quantidade da droga administrada, as concentrações atingidas também dependem da absorção, distribuição, ligação às proteínas plasmáticas ou localização nos tecidos, da sua biotransformação e da sua excreção (**Figuras 7 e 8**) (Goodman and Gilman, 2007).

A absorção representa a taxa na qual uma droga deixa seu local de administração e o grau em que isto ocorre. A absorção de uma droga depende de um grande número de fatores, os dois mais importantes que se pode ressaltar são as propriedades físico-químicas da droga (solubilidade, lipofilicidade, grau de ionização) e as vias de administração, cada um destes fatores, separadamente ou em conjunto, podem ter efeitos acentuados na eficácia clínica e na toxicidade de uma droga (Avdeef, 2001). A absorção pelo trato gastrointestinal é regulada pelo estado físico da droga e a área de superfície de absorção. O fármaco é melhor absorvido na forma não ionizada e mais lipofílica, sofrendo metabolismo de primeira passagem. Em contrapartida, a cavidade intraperitoneal oferece uma grande superfície de absorção a partir da qual os fármacos entram rapidamente na circulação, mas em especial através da veia porta, assim é possível a ocorrência de perdas na primeira passagem pelo fígado. Já a velocidade de absorção após a injeção subcutânea de uma droga é, com freqüência, suficientemente constante e lenta para proporcionar a manutenção do efeito (Mannhold, 2005; Bergstrom, 2005).

Após o fármaco ser absorvido, ele chega à circulação sistêmica, o que é chamado de biodisponibilidade sistêmica. Na circulação, ele atinge uma concentração plasmática máxima obtida após administração extra vascular (Cmáx). A importância do Cmáx está

relacionada à eficácia e à segurança. É representado, geralmente, pelas unidades mcg/mL, ng/mL ou μg/mL e é diretamente proporcional à fração absorvida do fármaco (Consiglieri and Storpirtis, 2000).

Em relação à velocidade de absorção, tem-se o Tmáx, que corresponde ao tempo em que a concentração plasmática do fármaco atinge a concentração máxima após a administração. A absorção do fármaco continua após se atingir o Tmáx, mas em uma velocidade menor. A unidade empregada é tempo (horas ou minutos) (Consiglieri and Storpirtis, 2000).

Uma vez que as moléculas alcançam a circulação sanguínea sistêmica elas são transportadas para todos os compartimentos do organismo. Os padrões de distribuição das drogas refletem alguns fatores fisiológicos e suas propriedades físico-químicas (van de Waterbeemd, 2005). A fase inicial de distribuição reflete o débito cardíaco e o fluxo sanguíneo, onde o coração, fígado, rim, cérebro e outros órgãos bem perfundidos recebem grande parte da droga nos primeiros minutos após a sua absorção, e a oferta aos músculos, a maioria das vísceras, à pele e a gordura é mais lenta. A segunda fase da distribuição é limitada pelo fluxo sanguíneo (Lin et al., 2003; Roberts, 2003). Os fatores que determinam a taxa de difusão das drogas nos tecidos superpõem-se aos padrões de distribuição do fluxo sanguíneo. A difusão para o compartimento intersticial é rápida por causa da natureza permeável das membranas do endotélio capilar (exceto no cérebro) (Roberts, 2003; van de Waterbeemd, 2005). A distribuição pode ser limitada pela ligação da droga às proteínas do plasma em especial a albumina (Kratochwil et al., 2004).

Muitas drogas ligam-se a proteínas do plasma, essa ligação costuma ser reversível. A fração da quantidade do fármaco no plasma que está ligado é determinada pela concentração da droga, afinidade das drogas com as proteínas e pela concentração protéica (número de locais de ligação) (Huang and Zhang, 2004). A ligação de uma droga às proteínas plasmáticas limita a sua concentração nos tecidos e seu local de ação, visto que apenas o fármaco livre está em equilíbrio estável com as membranas. A ligação com as proteínas plasmáticas não é seletiva, muitas drogas com características físico-químicas semelhantes podem competir entre si e com substâncias endógenas por esses locais de ligação (Kratochwil et al., 2004; Huang Y and Zhang, 2004).

As drogas fortemente ligadas a proteínas possuem volume de distribuição pequeno, pois permanecem principalmente no plasma. As drogas hidrossolúveis permanecem principalmente no plasma e no líquido intersticial enquanto as drogas lipossolúveis chegam a todos os compartimentos, podem acumular-se nos tecidos adiposos (Mannhold, 2005).

(geralmente atravessam barreiras hematoencefálica e placentária), apresentando assim um alto volume de distribuição (Pardridge, 2005). A droga que se acumula em determinado tecido pode atuar como reservatório que prolonga sua ação nesse mesmo tecido ou em um local distante atingido pela circulação (Mannhold, 2005).

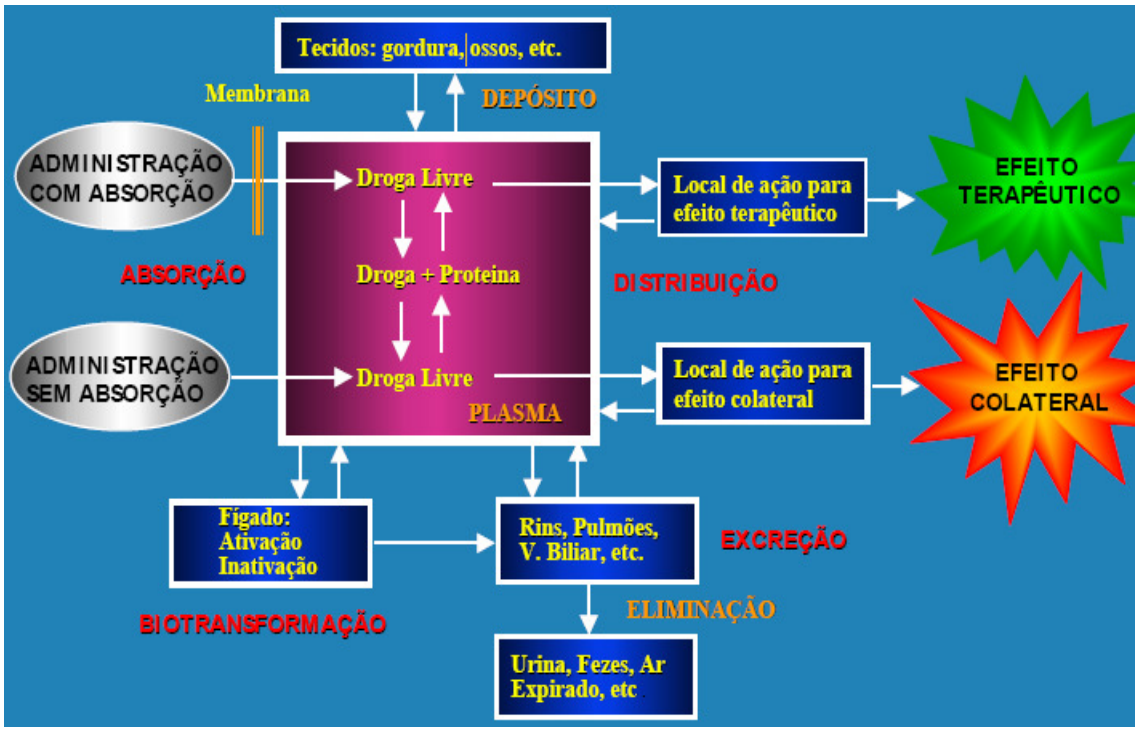

Figura 7: Absorção, distribuição, ligação ou localização nos tecidos, biotransformação e excreção de drogas.
Fonte:http://www.portalesmedicos.com/images/publicaciones/1107_farmacocinetica/farmacocinetica_17.jpg

As características lipofílicas das drogas que atravessam membranas biológicas e depois têm acesso a seu local de ação prejudicam sua eliminação pelo organismo (Mannhold, 2005). Por isso a biotransformação de drogas e outros agentes xenobióticos em metabólitos mais hidrófilos é essencial para o término de sua atividade biológica e para a eliminação destes compostos do organismo (Goodman and Gilman, 2007). Em geral as reações de biotransformação geram metabólitos inativos mais polarizados que são prontamente excretados do organismo. Contudo, em alguns casos são produzidos metabólitos com atividade biológica potente ou propriedades tóxicas (Robert, 2003; Alavijeh and Palmer, 2004; Nassar et al., 2009).

As reações de biotransformação de um fármaco são classificadas como reações de funcionalização da fase I e reações biossintéticas da fase II. As reações da fase I introduzem ou expõem um grupo funcional no composto original. Em geral a conversão

metabólica das drogas tem natureza enzimática, e os sistemas enzimáticos participantes da biotransformação dos fármacos localizam-se principalmente no fígado. A família de enzimas do citocromo P450 é o principal catalisador das reações de biotransformação de fármacos (Alavijah and Palmer, 2004; Nassar et al., 2009).

As reações de conjugação da fase II determinam a formação de uma ligação covalente entre um grupo funcional no composto original e o ácido glicurônico, o sulfato, a glutationa, aminoácidos ou o acetato. Esses conjugados altamente polares costumam ser inativados e são excretados com rapidez na urina e nas fezes (Nassar et al., 2009).

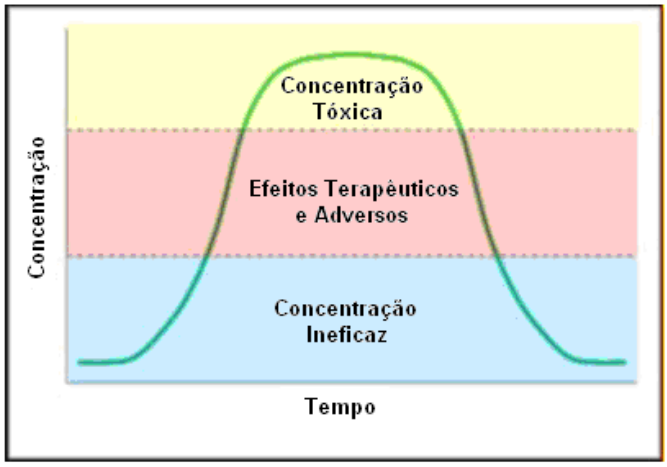

Figura 8: Representação da curva dose-resposta: Parâmetro utilizado na farmacocinética e farmacodinâmica para correlacionar a concentração plasmática das drogas aos seus efeitos clínicos.
Fonte:http://www.saludmentalinfantojuvenil.com/smij/psiquiatria/psicofarmacologia/generalidadesfarmacologia.html

2.6.1 Absorção, Distribuição, Metabolismo e Excreção de Drogas em Bebês

Mudanças profundas no desenvolvimento e maturação de órgãos e sistemas em neonatos, potencializam os riscos ou submetem os mesmos a diferentes riscos quando comparados aos adultos em relação à exposição a agentes farmacêuticos. As doses utilizadas em adultos, mesmo quando ajustadas as diferenças de peso corporal, podem levar a drásticas consequências em pacientes neonatos (Alcorn and McNamara., 2003).

As diferenças entre crianças e adultos podem ser agrupadas em duas diferentes categorias: fatores farmacocinéticos: os que influenciam a chegada da droga ao tecido alvo à partir da administração externa, e os fatores farmacodinâmicos: aqueles que influenciam

a resposta no tecido alvo à partir da dose que chega neste tecido (Choonara and Conroy, 2002).

As diferenças de absorção relacionadas a idade se devem a uma série de fatores anatômicos e fisiológicos que influenciam a taxa e a quantidade de absorção gastrointestinal. Dentre estes fatores podemos citar a redução no tempo de esvaziamento gástrico, no trânsito intestinal, na superfície de absorção do intestino delgado e ainda a redução no metabolismo de primeira passagem. Além disso a função pancreática e biliar está muito pouco desenvolvida (Besunder et al., 1988; Alcorn and McNamara., 2003).

A composição corporal afeta diretamente o volume de distribuição das drogas. Neste sentido, os bebês apresentam um maior volume corporal de água no organismo, em contrapartida apresentam uma menor quantidade de proteínas plasmáticas e lipídios corporais quando comparados a adultos. Em geral, bebês e crianças apresentam uma grande fração das drogas livre na circulação, devido a menor quantidade de proteínas plasmáticas, o que aumenta a distribuição nos tecidos (Choonara and Conroy., 2002; Alcorn and McNamara., 2003).

O metabolismo de drogas também sofre profunda transformação no período de desenvolvimento. O sistema citocromo P450, principal catalizador das reações de biotransformação de fase I, sofre um significante impacto na eficácia terapêutica e na suceptibilidade tóxica em bebês e crianças. Diferenças interindividuais, polimorfimo genético, e potencial de indução/inibição, associados a maturação do sistema enzimático P450, causam alterações farmacocinéticas no período de desenvolvimento (Alcorn and McNamara., 2003).

As reações de conjugação ou de fase II, as quais contribuem para a eliminação de uma variedade de compostos endógenos e exógenos também sofrem alterações durante o período de desenvolvimento. Em geral ocorrem mudanças na expressão de diversas enzimas responsáveis pelas reações metabólicas de fase II ou ainda na concentração dos substratos responsáveis pela conjugação. Estas mudanças podem resultar em uma significante redução na habilidade dos bebês em eliminar compostos endógenos ou exógenos, podendo levar a um aumento na toxicidade (McCarver and Hine., 2002).

Os mecanismos de eliminação renal das drogas também são diferentes em bebês. Em geral, a eliminação das drogas em recém nascidos é menor se comparado com adultos e crianças mais velhas, isso é atribuído a imaturidade anatômica e funcional do rim, que apresenta uma reduzida filtração glomerular (Anker et al., 1996; Alcorn and McNamara., 2003).

Além disso, a barreira hemato-encefálica, que exerce um controle sobre a entrada de drogas no cérebro não está completamente desenvolvida no sistema nervoso central de recém nascidos (Mares et al., 2000).

2.7 Absorção, Distribuição, Biotransformação e Excreção do Selênio

Vários estudos têm demonstrado a biotransformação do selênio oriundo de compostos inorgânicos de selênio e selenoaminoácidos provindos de alimentos em um intermediário comum, o seleneto, para a síntese de selenoproteínas para utilização e posterior excreção na urina na forma de seleno-açúcar (Schomburg et al., 2004; Park et al., 2004; Suzuki et al., 2005a,b).

Os compostos de selênio parecem ser rapidamente absorvidos no duodeno, seguido pelo jejuno e íleo. Após absorvidas, as formas inorgânicas de selênio são diretamente reduzidas para assumir um intermediário, o seleneto (Park et al., 2004). A administração de selênio inorgânico na forma de selenito é reduzido a seleneto nos eritrócitos pela abundante quantidade de glutationa e este é transportado no plasma ligado a albumina (Shiobara et al., 1998). Por outro lado, as formas orgânicas de selênio na forma de aminoácidos são transformadas em seleneto por diversas rotas: a selenocisteína é tranformada em seleneto por reações com a β-lyase, enquanto selenometionina passa primeiro por reações de trans-selenação (Spallholz et al., 2004).

O intermediário seleneto é transportado para vários órgãos, principalmente ao fígado para utilização na biosíntese de selenoproteínas via selenofosfato e selenocisteína.

Por fim, o seleneto é excretado em maior parte na urina na forma de 1 β-metilseleno-N-acetil-D-galactosamina, também chamado de selenoaçúcar. O seleno-açúcar é formado à partir da transferência de uma forma ativada do selênio (glutationa conjugada ao seleneto) a uma forma ativa de açúcar, para produzir o GSH conjugado ao selenoaçúcar (seleno-açúcar A), este é metilado para poduzir um seleno-açúcar urinário (seleno-açúcar B). Entretanto, nos casos de doses excessivas de selênio, o seleneto é excretado na forma de dimetilselenônio e trimetilselenônio **(Figura 9)** (Suzuki and Itoh, 1997; Kobayashi et al., 2002; Suzuki et al., 2005a, 2006).

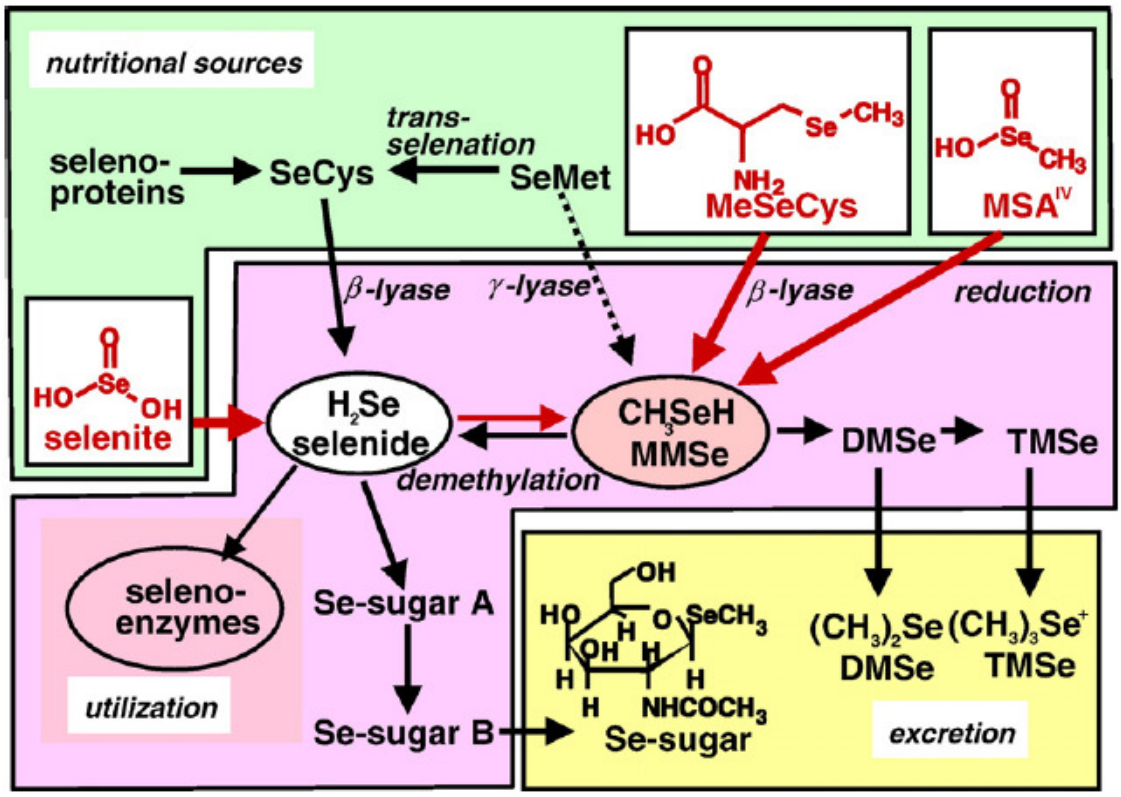

Figura 9: Esquema metabólico proposto para os compostos de selênio metilselenocisteína (MeSeCys), ácido metil selenínico (MSAIV), e selenito. Abreviações: dimetilseleneto (DMSe), metilselenol (MMSe), ácido metilselenínico (MSAIV), metilselenocisteína (MeSeCys), selenocisteína (SeCys), selenometionina (SeMet) e trimetilselenonio (TMSe). Fonte: Suzuki et al., 2008.

Estudos demonstram ainda que as reservas de açúcar que se conjugam ao selenito para formar o selenosugar são mais abundantes em ratos adultos do que em ratos jovens e que a fonte de açúcar é de origem endógena e que aumenta com a idade (Suzuki and Itoh, 1997; Suzuki et al., 2005b). Com isso o TMSe é muito mais aumentado na urina de ratos jovens após uma ingestão excessiva de Se, sendo sempre um metabólito menor em ratos adultos (Suzuki et al., 2005b).

2.7.1 Absorção, Distribuição, Biotransformação e Excreção do Ebselen

O ebselen é um composto orgânico heterocíclico de selênio que apresenta propriedades farmacológicas conhecidas como mimético da enzima glutationa peroxidase *in vitro* e atividade antiinflamatória *in vivo* (Schewe, 1995). Por ser uma droga utilizada na clínica médica, a farmacocinética do ebselen já foi amplamente estudada e serve de parâmetro para o estudo da farmacocinética ou toxicocinética de outros compostos de selênio, como é o caso do $(PhSe)_2$.

O ebselen apresenta uma rápida e extensa ligação à albumina, e esta representa seu mecanismo fisiológico de transporte na circulação sanguínea. Wagner et al (1994), em um estudo utilizando camundongos, demonstrou que mais de 90% do [^{14}C] ebselen, administrado pela via intravenosa, apresenta-se ligado a albumina 30 minutos após a administração. A ligação do ebselen a albumina ocorre principalmente de forma covalente, e se deve a rápida reação do ebselen com o grupo sulfidrila da cisteína 34, o único grupo tiol reativo da albumina (Wagner et al., 1994).

Varios estudos *in vitro* e *in vivo* demonstraram que o ebselen é metabolizado no fígado. É característica em todos os metabólitos detectados a abertura do anel isoselenoazol, devido a uma clivagem da ligação Se-N (Kamigata et al., 1986; Fisher and Dereu., 1987). No fígado o ebselen é reduzido por tióis a 2-selenilbenzenoanilida (Ziegler et al., 1992) ou reage com grupos de tióis endógenos de proteínas para formar selenosulfetos em proteínas (Dimmeler et al., 1991). Após a transformação a 2-selenilbenzenoanilida, o ebselen sofre glucuronidação ou metilação, formando metabólitos mais polares, para serem excretados pela urina (Muller et al., 1988). Trabalhos demonstram que o ebselen pode causar inativação no citocromo P450 *in vitro*, provavelmente por interagir com o resíduo de cisteína presente no sítio ativo da enzima (Vendel et al., 1986; Kuhn-Velten and Sies., 1988).

A distribuição do ebselen no organismo está relacionada com a presença de grupamentos tiólicos. Segundo Wagner et al., (1994) e Ullrich et al (1996), o ebselen encontra-se em equilíbrio dinâmico com todos os grupos tióis acessíveis, portanto, apesar da maioria do composto estar ligada covalentemente à albumina, a entrada do mesmo nas células ocorre rapidamente, devido a trocas com outras proteínas-alvo nos tecidos (Ullrich et al. 1996) (Figura 10).

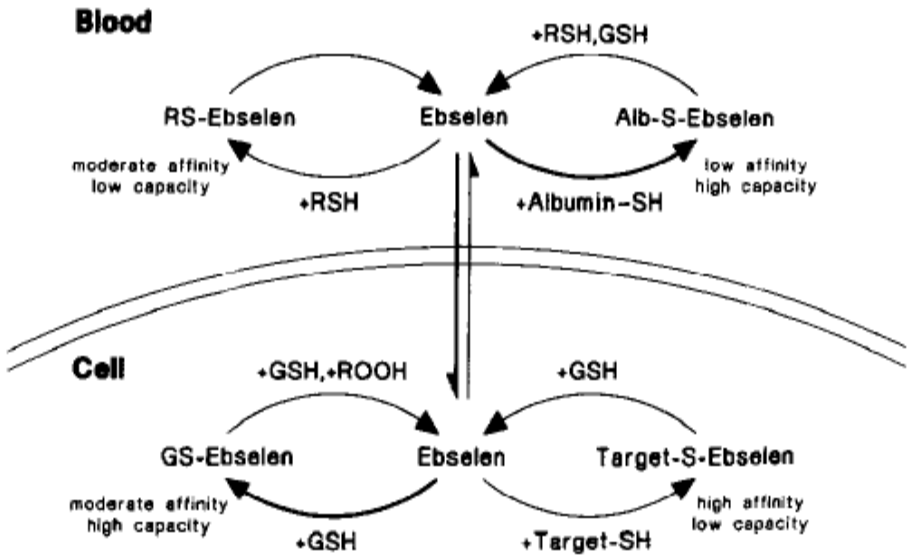

Figura 10: Esquema demostrando o equilíbrio da ligação do ebselen entre as proteínas plasmáticas e as proteínas alvo nas células.
Fonte: Ullrich et al., 1996.

2.7.2 Absorção, Distribuição e Excreção do Disseleneto de Difenila

Pouco se conhece sobre a absorção, distribuição e excreção do $(PhSe)_2$ até o momento. Sabe-se que o $(PhSe)_2$ pode causar efeitos farmacológicos e toxicológicos e que estes podem variar de acordo com a dose, espécie animal, via de administração ou modelo experimental utilizado (Brito et al., 2006; Ghisleni et al., 2008; Ineu et al., 2008; Nogueira et al., 2002; Nogueira et al., 2003a; Savegnago et al., 2008). O que se tem em comum é que estudos farmacológicos usando curvas de dose-resposta demonstraram que o efeito máximo do composto em modelos de dor, inflamação, nocicepção e memória ocorrem 30 minutos após sua administração pela via oral (Rosa et al., 2003; Savegnago et al. 2007a,b), sugerindo que haja uma rápida absorção do composto.

Em relação à distribuição do $(PhSe)_2$, Maciel et al. (2003) demonstraram que o tratamento agudo e crônico com $(PhSe)_2$ aumentou significantemente as concentrações de selênio no fígado, no rim e no cérebro de camundongos. Sendo que a deposição foi dependente da dose; e maior no fígado, seguida pelo rim e cérebro. No entanto, nada se sabe em relação ao metabolismo do composto. Nogueira et al. (2004) sugeriram que o

$(PhSe)_2$ é biotransformado à selenol, e que esta poderia ser a forma ativa, mas não há dados experimentais concretos demonstrando isto.

Em relação à excreção, Adams Jr et al (1989) em estudo usando $(PhSe)_2$ marcado com ^{14}C, demonstraram que o conteúdo de selênio do $(PhSe)_2$ parece ser totalmente eliminado em camundongos até 120 horas após a administração intraperitoneal do composto. Kostyniak (1984) demonstrou dados semelhantes para outro composto orgânico de selênio, o bis, beta-(*n,n*-dimorpholino) etilseleneto. O $(PhSe)_2$ é excretado na urina, aparentemente em uma forma que não possui relação à forma química administrada. Cerca de um terço do selênio biotransformado contém o grupamento orgânico ^{14}C, que pode ser resultado de glicuronido-conjugados do selênio, contendo análogos de fenol e catecol; a outra via pode resultar em metabolitos contendo selênio porém não identificados (Adams Jr et al., 1989).

3. OBJETIVOS

Considerando que o $(PhSe)_2$ apresenta inúmeras propriedades farmacológicas, que o tornam potencial candidato a fármaco, o estudo da toxicologia do composto torna-se importante. Desta forma, os objetivos deste estudo foram delineados em duas linhas gerais de investigação:

(I) Estudo da neurotoxicidade aguda induzida por $(PhSe)_2$ em ratos bebês, avaliando o aparecimento de convulsões induzidas pelo $(PhSe)_2$**,** o envolvimento do estresse oxidativo e dos sistemas glutamatérgico e GABAérgico no efeito convulsivante do composto;

(II) Determinação e quantificação dos níveis plasmáticos de $(PhSe)_2$ em ratos e camundongos**,** verificando o envolvimento de diferentes vias de administração, veículos e espécie animal nos níveis plasmáticos do composto e no aparecimento de convulsões induzidas pelo mesmo; determinação e quantificação dos níveis de $(PhSe)_2$ no plasma, fígado e cérebro de ratos bebês e correlacão destes níveis à latência para o aparecimento de convulsões; investigação as propriedades cinéticas do $(PhSe)_2$ *in vitro*, tais como: estabilidade, solubilidade, absorção, distribuição, ligação às proteínas plasmáticas e biotransformação;

4. ARTIGOS CIENTÍFICOS E MANUSCRITOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos e manuscritos, os quais encontram-se assim organizados. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas encontram-se nos próprios artigos e manuscritos. Os artigos estão dispostos da mesma forma que foram publicados nas revistas científicas (artigos 1, 2, 3, 4 e 5). Os manuscritos 1 e 2 estão dispostos da mesma forma que serão submetidos para avaliação.

4.1 Envolvimeto do estresse oxidativo nas convulsões induzidas por disseleneto de difenila em ratos bebês

4.1.1 Artigo 1

INVOLVEMENT OF OXIDATIVE STRESS IN SEIZURES INDUCED BY DIPHENYL DISELENIDE IN RAT PUPS

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BRAIN RESEARCH

Research Report

Involvement of oxidative stress in seizures induced by diphenyl diselenide in rat pups

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ABSTRACT

In the present study the potential neurotoxicity of diphenyl diselenide, as measured by the manifestation of seizures in rat pups (postnatal days, PND, 12–14) was evaluated. The results suggest that the latency for the appearance of tonic–clonic seizures, characterized by rearing and falling of rat pups body, was dependent of the dose tested. Diphenyl diselenide at high doses induced seizure episodes in rat pups. The highest dose of diphenyl diselenide (500 mg/kg) increased the levels of lipid peroxidation and catalase activity as well as decreased δ-ALA-D (δ-aminolevulinate dehydratase) and Na^+, K^+ ATPase activity in the brain of rat pups. Our results indicate the possible involvement of free radical oxygen injury in diphenyl diselenide-induced seizures. The data obtained with the dose of 150 mg/kg in the brain of rats that exhibited seizures are: an increase in lipid peroxidation levels; the lack of effect on catalase activity; an inhibition of δ-ALA-D activity, supporting that the enzyme activity is more sensitive than other parameters analyzed as an indicator of oxidative stress. The lowest dose of diphenyl diselenide emphasizes the relationship between the appearance of seizures and the latency for the onset of the first episode. Taken together, this paper could add to our understanding of diphenyl diselenide neurotoxic effect demonstrated by the appearance of seizures which are, at least in part, related to the oxidative stress.

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1. Introduction

Epilepsy is a major health problem, affecting 0.5–1.0% of the world's population (Hauser et al., 1996). This condition is characterized by prolonged or repetitive epileptic discharges, resulting clinically in persistent alterations of the normal brain function and cognitive state (Treiman, 1995). While seizures can occur at any age, they are far more common in children than adults, seizure incidence is highest in the first year of life, and decreases with age throughout childhood and adolescence (Cowan, 2002). Prolonged seizures in the early developmental period can cause brain damage and lead to serious consequences later in life (Meldrum, 2002).

There are points of evidences suggesting that oxidative stress is important in brain tissue damage following seizure induction (Kaneko et al., 2002) and the activation of excitatory amino acid receptor can also trigger the formation of reactive oxygen species (ROS) (Said et al., 2000). The brain is a preferential target for the peroxidative process because it has a high content of polyunsaturated fatty acids (Halliwell and Gutteridge, 1984). Many cerebral enzymes which contain sulfhydryl groups, such as δ-aminolevulinate dehydratase (δ-ALA-D) and Na^+, K^+ ATPase,

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are sensitive to oxidizing agents (Borges et al., 2005) and to situations associated with oxidative stress (Demasi et al., 1996). To circumvent the oxidative stress organisms have systems that prevent hazardous effects of free radicals such as superoxide dismutase (SOD) and catalase (CAT). Accordingly, differences have been reported in free radical scavenging enzyme levels during the convulsive process (Freitas et al., 2004).

Diphenyl diselenide is an organoselenium compound, highly lipophilic, with many pharmacological properties such as antiulcer (Savegnago et al., 2006) anti-inflammatory and anti-nociceptive (Savegnago et al., 2007), neuroprotector (Ghisleni et al., 2003), anti-hyperglycemic (Barbosa et al., 2006) and antioxidant (Meotti et al., 2003). On the other hand, studies performed by our group demonstrated that prolonged exposure to high doses of diphenyl diselenide increased three times the total selenium content in the brain. There is also evidence that diphenyl diselenide crosses the blood barrier and brain selenium levels increase in mice after acute and chronic exposure to diphenyl diselenide (Maciel et al., 2003).

It is important to mention that this compound when administrated by i.p. route induced seizure and death in mice, but did not display any overt sign of neurotoxicity in adult rats (Nogueira et al., 2003), probably because species differences in toxicity are often related to differences in the liver metabolism and disposition of a compound (Caldwell, 1982). In addition, a direct injection of diphenyl diselenide, at high doses (1000 μmol), into the brain of rodents did not cause any overt sign of neurotoxicity (Nogueira et al., 2003) indicating that this compound must be first metabolized to cause seizures in mice, and that the site for entry into the body of a toxic compound is important in determining the final toxic effect (Timbrell, 2000). Reinforcing this idea are the data on oral administration of diphenyl diselenide which demonstrated that this organoselenium compound, at high doses (312 mg/kg), did not induce toxicological effects in rats and mice (Savegnago et al., 2007). Therefore, these results have supported the hypothesis that the brain is a potential target for the toxicity of diphenyl diselenide and possibly for its pharmacological and therapeutic actions.

Based on the considerations above, the aims of the present study were: (a) to investigate the acute toxicity induced by diphenyl diselenide in rat pups using oral route of administration with the purpose of offering safety in the possible therapeutic use of this compound; (b) to evaluate the appearance of seizure behavior in rat pups since diphenyl diselenide does not induce this neurotoxic effect in adult rats, this is an important topic from an academic point of view; and (c) to examine if oxidative stress, investigated by measuring lipid peroxidation, antioxidant enzymes and enzymes sensitive to oxidative conditions, is involved in seizures induced by diphenyl diselenide in rat pups.

2. Results

2.1. Diphenyl diselenide-induced seizures

The convulsion behavior was classified as generalized seizures (tonic–clonic) or stage 5 seizures. As shown in Table 1 diphenyl diselenide at 500 mg/kg induced seizures in 100% of rat pups, showing the latency for the first episode of 19 min. Diphenyl diselenide at 50 and 150 mg/kg induced seizure episodes in 30% and 66% of rat pups, respectively. Therefore, the appearance of seizures and the latency for the first episode were dependent on the dose used. At the lowest dose (5 mg/kg), diphenyl diselenide did not cause seizures in rat pups.

2.1.1. *Lipid peroxidation*

One-way ANOVA of TBARS demonstrated that diphenyl diselenide at 500 mg/kg increased lipid peroxidation in brain of pups. TBARS levels were also increased in rat pups which had seizure episodes induced by 150 mg/kg diphenyl diselenide. Diphenyl diselenide did not alter lipid peroxidation at all other doses tested (Fig. 1).

In the liver of rat pups, TBARS levels were not changed by diphenyl diselenide (data not shown).

2.1.2. *Antioxidant defenses*

One-way ANOVA of CAT activity revealed that diphenyl diselenide at 50 mg/kg inhibited enzyme activity in the brain of pups which had seizure episodes. The highest dose of diphenyl diselenide stimulated catalase activity in the brain of pups. CAT activity was not altered in all other groups (Fig. 2).

There was no change of catalase activity in the liver of rat pups (data not shown). SOD activity and ascorbic acid levels were also unaltered by diphenyl diselenide in liver and brain of rat pups (data not shown).

2.1.3. *δ-ALA-D activity*

δ-ALA-D activity was significantly inhibited in the brain of pups which had seizure episodes independent of the dose of diphenyl diselenide administered. The enzyme activity was not altered in the brain of rat pups which did not have seizures (Fig. 3a).

In the liver, diphenyl diselenide at the highest dose inhibited δ-ALA-D activity. Hepatic δ-ALA-D activity was inhibited in rat pups which received diphenyl diselenide at 150 mg/kg and did not have seizures. All other groups had the enzyme activity unaltered (Fig. 3b).

Table 1 – Diphenyl diselenide-induced seizures in rat pups

Groups $(PhSe)_2$ (mg/kg)	Appearance of seizures [a]	Latency [b] (min)
Control	0/8	ns
5	0/9	ns
50	6/20**	44.97 ± 0.68*,#
150	12/18**	33.81 ± 9.50*,#
500	9/9**	19.09 ± 2.30*,#

"ns"—Animals which did not present seizure. Data are reported as mean ± SEM. **Denoted $p < 0.05$ as compared to the control group (χ^2 method and Fischer's Exact Probability Test). *Denoted $p < 0.05$ as compared to the control group (one-way ANOVA/Duncan). #Denoted $p < 0.05$ as compared to the other groups (one-way ANOVA/Duncan).

[a] Number of animals which presented seizures/Number of animals per group.

[b] Time to the appearance for the first seizure episode.

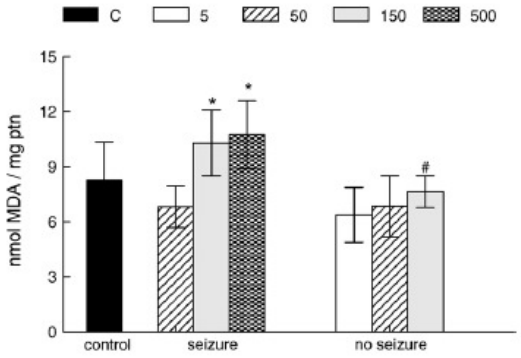

Fig. 1 – Effect of diphenyl diselenide on TBARS levels in brain of rat pups. Data are reported as mean ± SEM of six to fourteen animals per group and expressed as nmol MDA (malondialdehyde)/mg ptn. *Denoted $p<0.05$ as compared to the control group (one-way ANOVA/Duncan). Abbreviations: C—control; diphenyl diselenide at the dose of: 5—5 mg/kg; 50—50 mg/kg; 150—150 mg/kg; 500—500 mg/kg.

2.1.4. *Na⁺, K⁺ ATPase activity*

One-way ANOVA of Na^+, K^+ ATPase activity revealed that diphenyl diselenide at 500 mg/kg significantly inhibited the enzyme activity in the brain of pups. Na^+, K^+ ATPase activity was also significantly reduced in rat pups which received diphenyl diselenide (50 mg/kg) and had seizure episodes (Fig. 4).

3. Discussion

In the present study we have demonstrated that diphenyl diselenide administered at high doses induced seizure episodes in rat pups. The latency for the appearance of tonic–clonic seizures characterized by rearing and falling of rat pup body was dependent on the dose tested. This work is the first evidence indicating that diphenyl diselenide is capable of inducing seizures in pups when administered by oral route. It is important to point out that diphenyl diselenide did not display any overt sign of neurotoxicity when administered by i.p, s.c., oral and i.c.v. routes in adult rats (Nogueira et al., 2003; Savegnago et al., 2007). In addition, the doses of diphenyl diselenide which induced seizures in rat pups are about 10 times higher than the doses that have pharmacological properties (Meotti et al., 2003; Ghisleni et al., 2003; Nogueira et al., 2003; Savegnago et al., 2007).

Several authors have reported that the oxidative stress has been associated with seizure-induced neuronal death (Walz et al., 2000). In accordance, all pups treated with the highest dose of diphenyl diselenide (500 mg/kg) had seizures and the parameters evaluated in the brain of pups support the involvement of oxidative stress in this neurotoxic effect.

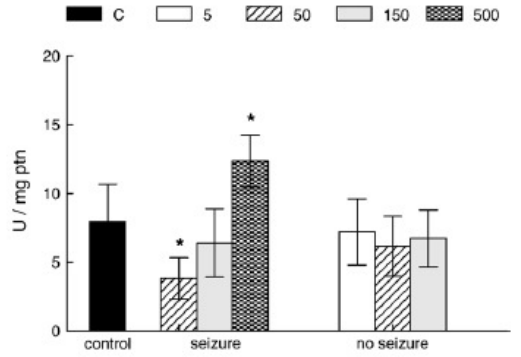

Fig. 2 – Effect of diphenyl diselenide on catalase activity in the brain of rat pups. Data are reported as mean ± SEM of six to fourteen animals per group and express U/mg ptn. *Denoted $p<0.05$ as compared to the control group (one-way ANOVA/Duncan). Abbreviations: C—control; diphenyl diselenide at the dose of: 5—5 mg/kg; 50—50 mg/kg; 150—150 mg/kg; 500—500 mg/kg.

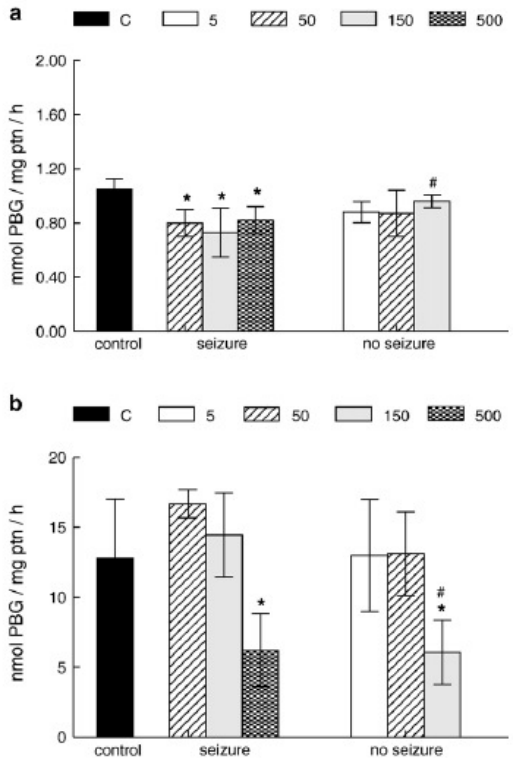

Fig. 3 – Effect of diphenyl diselenide on δ-ALA-D activity in brain (a) and liver (b) of rat pups. Data are reported as mean ± SEM of six to fourteen animals per group and expressed as nmol of porphobilinogen/mg protein/h. *Denoted $p<0.05$ as compared to the control group (one-way ANOVA/Duncan). #Denoted $p<0.05$ as compared to the 150 mg/kg (with seizure) group (one-way ANOVA/Duncan). Abbreviations: C—control; diphenyl diselenide at the dose of: 5—5 mg/kg; 50—50 mg/kg; 150—150 mg/kg; 500—500 mg/kg.

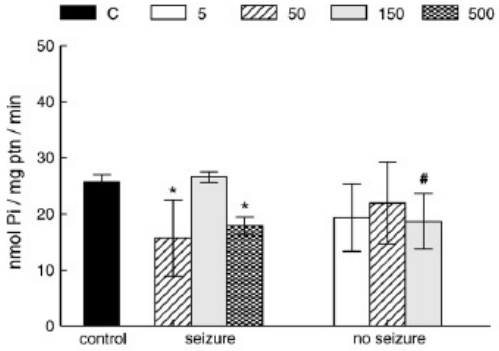

Fig. 4 – Effect of diphenyl diselenide on Na^+, K^+ ATPase activity in brain of rat pups. Data are reported as mean ± SEM of six to fourteen animals per group and express nmol Pi/mg ptn/min. *Denoted $p<0.05$ as compared to the control group (one-way ANOVA/Duncan). #Denoted $p<0.05$ as compared to the 150 mg/kg (with seizure) group. Abbreviations: C—control; diphenyl diselenide at the dose of: 5—5 mg/kg; 50—50 mg/kg; 150—150 mg/kg; 500—500 mg/kg.

Consistent with this hypothesis are the observations that diphenyl diselenide: (1) increased lipid peroxidation, a marker of oxidative stress; (2) increased catalase activity, confirming the defensive role of catalase and indicating a cellular response to increased reactive oxygen species; (3) reduced the activity of δ-ALA-D and Na^+, K^+ ATPase, sulfhydryl containing enzymes, which are sensitive to oxidizing agents (Meotti et al., 2003; Nogueira et al., 2004; Borges et al., 2005). The inhibition of sulfhydryl enzymes could also be attributed to the ability of organoselenium in oxidizing SH groups (Spallholz et al., 2001). Moreover, compounds that oxidize –SH groups have long been known as potent δ-ALA-D inhibitors (Pappas et al., 1995). SOD activity and ascorbic acid levels were not altered by diphenyl diselenide, suggesting that these markers of oxidative stress are not related to neurotoxic effects induced by diphenyl diselenide. Regarding the data obtained in the liver, only δ-ALA-D activity was altered by the highest dose of diphenyl diselenide. In fact, diphenyl diselenide inhibited hepatic δ-ALA-D activity, which is consistent with the site of xenobiotic metabolism. The fact that none of the other parameters evaluated were changed in the liver reinforces δ-ALA-D as an enzyme very sensitive to diphenyl diselenide.

The results obtained in brain with the two medium doses (50 and 150 mg/kg) also emphasize the relationship between the appearance of seizures and the latency for the onset of the first convulsive episode. Regarding the parameters evaluated, different effects occurred between rat pups exhibiting seizures and those not seizing and these effects were dependent of the dose of diphenyl diselenide. In fact, there is a significant difference between rat pups with and without seizures after 150 mg/kg of diphenyl diselenide in TBARS levels, δ-ALA-D and Na^+, K^+ activity. Conversely, there is no difference between rat pups with and without seizures after 50 mg/kg of diphenyl diselenide in the same parameters evaluated. These results allow us to suggest that oxidative stress, in the brain, is related to the dose of diphenyl diselenide administered to rat pups and at least in part to the seizure episode.

Taking these data together the oxidative stress in brain seems to be directly related to the dose of diphenyl diselenide administered. In addition, the inhibition of catalase activity on rat pups after 50 mg/kg diphenyl diselenide suggests that the time to onset for the first seizure episode (about 45 min) allowed that catalase act against reactive oxygen species, demonstrated by the lack of alteration in lipid peroxidation. Similar to the data obtained with the highest dose of diphenyl diselenide, SOD activity and ascorbic acid levels were not altered by the medium doses.

Another finding of this study is that diphenyl diselenide, at doses which induced seizures in rat pups, inhibited brain δ-ALA-D activity. The significant difference between rat pups with and without seizures in δ-ALA-D activity after the dose of 150 mg/kg, but the lack of significance after 50 mg/kg, could reflect that δ-ALA-activity is only, in part, related to the seizure episode induced by diphenyl diselenide. The fact that the dose of diphenyl diselenide is an important factor to δ-ALA-inhibition is another interpretation that emerges from this comparison. Accordingly, a number of evidence suggest the direct involvement of δ-ALA, the substrate of δ-ALA-D, in neurological manifestations such as seizures (Kappas et al., 1995). Important, ALA accumulation has been reported in tissues of animals and patients which δ-ALA-D activity is inhibited (Juknat et al., 1995). ALA can undergo autooxidation generating reactive oxygen species and the ALA enoyl radical (Bechara et al., 1993). These reactive species increased lipid peroxidation (Emanuelli et al., 2003), induced oxidative damage and increased activity of antioxidant enzymes (Demasi et al., 1996). Therefore, the inhibition of δ-ALA-D activity is probably a consequence of oxidative stress induced by seizure episodes.

Na^+, K^+ ATPase activity in rats presenting seizures revealed distinct effects dependent on the dose of diphenyl diselenide administered and the time to onset for the first seizure episode. The lowest (50 mg/kg) and the highest (500 mg/kg) doses decreased the ATPase activity, but at 150 mg/kg enzyme activity was not altered. These results are very hard to explain but tentatively could be related to the latency for the onset for the first seizure episode (~45 and 30 min) for 50 and 150 mg/kg, respectively. Since Na^+, K^+ ATPase is sensitive to oxidizing agents (Borges et al., 2005), the enzyme inhibition after the highest dose could be explained by the very high dose of diphenyl diselenide administered.

Concerning hepatic tissue, some controversial results which are dependent on the dose studied were found. Oxidative parameters were unaltered by 50 mg/kg of diphenyl diselenide independent of seizing or not. Conversely, δ-ALA-D was inhibited in rat pups after 150 mg/kg diphenyl diselenide, which did not induce seizing. δ-ALA-D inhibition could be explained by the fact that these animals were sacrificed after 60 min of diphenyl diselenide administration, allowing that the organoselenium compound remained in the liver and by oxidizing sulfhydryl groups inhibited enzyme activity.

In conclusion, this paper could add to our understanding of diphenyl diselenide neurotoxic effect demonstrated by the appearance of seizures which are, at least in part, related to the oxidative stress. Our data provide interesting indications,

since diphenyl diselenide has pharmacological properties, mainly when administered by oral route, and can be a useful compound for therapeutic use in a near future.

4. Experimental procedure

4.1. Chemicals

δ-Aminolevulinic acid (δ-ALA), *p*-dimethylaminobenzaldehyde, epinephrine, ATP and ouabain were purchased from Sigma (St. Louis, MO, USA).

Diphenyl diselenide was prepared in our laboratory according to literature methods Paulmier (1986). Analysis of the ^{1}H NMR and ^{13}C NMR spectro showed that $(PhSe)_2$ obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of compound (99.9%) was determined by GC/HPLC. This drug was dissolved in canola oil, which was obtained from a standard commercial supplier. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

4.2. Animals

Young Wistar rats (postnatal days, PND, 12–14) of both sexes were obtained from a local breeding colony. Postnatal days 12–14 was chosen based on Mikulecká et al. (2004). The animals were kept in separate animal rooms, on a 12-h light/12-h dark cycle, in an air-conditioned room (22±2 °C). Commercial diet (GUABI, RS, Brazil) and tap water were supplied ad libitum. The dams were allowed to deliver and wean their pups until postnatal days (PND) 12–14. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil.

4.3. In vivo experiments

4.3.1. Diphenyl diselenide-induced seizures

Experimenters were blind to the drug given to rat pups. Diphenyl diselenide was administered per oral route (p.o., 10 ml/kg) and the animals were immediately observed. Rats were divided into groups as follows: Group 1—control (canola oil, p.o.), Group 2—$(PhSe)_2$ (5 mg/kg, p.o.), Group 3—$(PhSe)_2$ (50 mg/kg, p.o.), Group 4—$(PhSe)_2$ (150 mg/kg, p.o.) and Group 5—$(PhSe)_2$ (500 mg/kg, p.o.).

Appearance of seizures was quantified as previously described by Racine (1972) and Sperk et al. (1985) as follows: Stage 0: no changes; Stage 0.5: wet dog shakes; Stage 1: mouth and facial movements; Stage 2: head nodding; Stage 3: forelimb clonus; Stage 4: rearing; Stage 5: rearing and falling. The animals were observed for 1 h in Plexiglas chambers for the appearance of tonic–clonic seizures lasting more than 5 s. The latency for the onset of the first tonic–clonic seizure episode was also recorded.

Subsequently to the seizure episode, rat pups were decapitated. Animals which did not display seizures were considered protected and sacrificed 1 h after the compound administration. The liver and whole brain of all animals were removed and used for ex vivo assays.

4.4. Ex vivo experiments

The samples of liver and whole brain were homogenized in 50 mM Tris–HCl, pH 7.5 (1/10, w/v) or (1/5, w/v), respectively, and centrifuged at 2400×*g* for 15 min.

4.4.1. Lipid peroxidation

The reaction product was determined using an aliquot (200 ml) of homogenized tissue, 500 μl thiobarbituric acid (0.8%), 200 μl SDS (sodium dodecil sulfate, 8.1%) and 500 μl acetic acid and 500 ml TBA (thiobarbituric acid 0.8%), the mixture was incubated at 95 °C for 2 h. TBARS (thiobarbituric acid reactive species) were determined as described by Ohkawa et al. (1979).

4.4.2. Ascorbic acid determination

Ascorbic acid determination was performed as described by Jacques-Silva et al. (2001). Proteins were precipitated in 10 volumes of a cold 4% trichloroacetic acid solution. An aliquot of the sample at a final volume of 1 ml of the solution was incubated for 3 h at 38 °C then 1 ml H_2SO_4 65% (v/v) was added to the medium. The reaction product was determined using a color reagent containing 4.5 mg/ml dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/ml) at 520 nm. The content of ascorbic acid is related to tissue amount (μmol ascorbic acid/g wet tissue).

4.4.3. δ-Aminolevulinic dehydratase (δ-ALA-D) activity

δ-ALA-D activity was assayed according to the method described by Sassa (1982) by measuring the rate of product porphobilinogen (PBG) formation except that 45 mM sodium phosphate buffer and 2.2 mM δ-ALA were used. An aliquot of 200 ml of the homogenized tissue was incubated for 1 h at 37 °C (liver) and for 3 h at 37 °C (brain). Enzymatic reaction was initiated by adding the substrate (δ-ALA) to a final concentration of 2.2 mM in a medium containing 45 mM phosphate buffer, pH 6.8. The incubation was stopped by adding trichloroacetic acid solution (10% TCA) with 10 mM $HgCl_2$. The reaction product was determined using modified Erlich's reagent at 555 nm. The reaction was linear in relation to protein and time of incubation.

4.4.4. Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity was assayed spectrophotometrically as described by Misra and Fridovich (1972). This method is based on the capacity of SOD to inhibit autooxidation of epinephrine to adrenochrome. Enzymatic reaction was initiated by adding an aliquot (20–60 ml) of the homogenized tissue and the substrate (epinephrine) to a concentration of 60 mM in a medium containing 50 mM glycine buffer, pH 10.3. The color reaction was measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autooxidation by 50% at 26 °C.

4.4.5. Catalase activity

Catalase activity was assayed spectrophotometrically by the method of Aebi (1984), which involves monitoring the disappearance of H_2O_2 in the homogenate presence at 240 nm. Enzymatic reaction was initiated by adding an aliquot

of 20 ml of the homogenized tissue and the substrate (H_2O_2) to a concentration of 0.3 mM in a medium containing 50 mM phosphate buffer, pH 7.0. The enzymatic activity was expressed in units (1 U decomposes 1 μmol of H_2O_2 per minute at pH 7 at 25 °C).

4.4.6. *Na^+, K^+ ATPase activity*

The homogenate was centrifuged at 4000×*g* at 4 °C for 10 min and supernatant was used for assay of protein Na^+, K^+ ATPase. The reaction mixture for Na^+, K^+ ATPase activity assay contained 3 mM MgCl, 125 mM NaCl, 20 mM KCl and 50 mM Tris–HCl, pH 7.4, in a final volume of 500 μl. The reaction was initiated by the addition of ATP to a final concentration of 3.0 mM. Control samples were carried out under the same conditions with the addition of 0.1 mM ouabain. The samples were incubated for 30 min at 37 °C, the incubation was stopped by adding trichloroacetic acid solution (10% TCA) with 10 mM $HgCl_2$. Na^+, K^+ ATPase activity was calculated by the difference between the two assays. Released inorganic phosphate (Pi) was measured by the method of Fiske and Subbarow (1925).

4.4.7. *Protein quantification*

Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin as the standard.

4.5. *Statistical analysis*

Data are expressed as means±SEM. Statistical analysis was performed using a one-way analysis of variance (ANOVA), followed by the Duncan's multiple range test when appropriate. Values of $p<0.05$ were considered statistically significant. Seizure incidence was analyzed statistically by the χ^2 method and Fisher's Exact Test.

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REFERENCES

Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.

Barbosa, N.B.V., Rocha, J.B.T., Wondracek, D.C., Perottoni, J., Zeni, G., Nogueira, C.W., 2006. Diphenyl diselenide reduces temporarily hyperglycemia: possible relationship with oxidative stress. Chem. Biol. Interact. 163, 230–238.

Bechara, E.J.H., Medeiros, M.H.G., Monteiro, H.P., Hermes-Lima, M., Pereira, B., Demasi, M., Costa, C.A., Abdalla, D.S.P., Onuki, J., Wendel, C.M.A., Di Mascio, P., 1993. A free radical hypothesis of lead poisoning and inborn porphyries associated with 5-aminolevulinic acid overload. Quím. Nova 16, 391–685.

Borges, V.C., Rocha, J.B.T., Nogueira, C.W., 2005. Effect of diphenyl diselenide, diphenyl ditelluride and Ebselen on cerebral Na^+ K^+-ATPase activity in rats. Toxicology 215, 191–197.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal. Biochem. 72, 248–254.

Caldwell, J., 1982. Conjugation reactions in foreign compound metabolism: definition, consequences and species variations. Drug Metab. Rev. 13, 745–777.

Cowan, L.D., 2002. The epidemiology of the epilepsies in children. Ment. Retard. Dev. Disabil. Res. Rev. 8, 171–181.

Demasi, M., Penatti, C.A.A., De Lucia, R., Bechara, E.J.H., 1996. The prooxidant effect of 5-aminolevulinic acid in the brain tissue of rats: implications in neuropsychiatric manifestations in porphyrias. Free Radical Biol. Med. 20, 291–299.

Emanuelli, T., Pagel, F.W., Porciúncula, L.O., Souza, D.O., 2003. Effects of 5-aminolevulinic acid in the glutamatergic neurotransmission. Neurochem. Int. 42, 115–121.

Fiske, C.H., Subbarow, Y.J., 1925. The colorimetric determination of phosphorus. Biol. Chem. 66, 375–381.

Freitas, R.M., Souza, F.C.F., Vasconcelos, S.M.M., Viana, G.S.B., Fonteles, M.M.F., 2004. Pilocarpine-induced status epilepticus in rats: lipid peroxidation levels, nitrite formation, GABAergic and glutamatergic receptor alterations in the hippocampus, striatum and frontal cortex. Pharmacol. Biochem. Behav. 78, 327–332.

Ghisleni, G., Porciúncula, L.O., Cimarosti, H., Rocha, J.B.T., Salbego, C.G., Souza, D.O., 2003. Diphenyl diselenide protects rat hippocampal slices submitted to oxygen-glucose deprivation and diminishes inducible nitric oxide synthase immunocontent. Brain Res. 986, 196–199.

Halliwell, B., Gutteridge, J.M.C., 1984. Oxygen toxicity, oxygen radical, transition metals and diseases. Biochem. J. 219, 1–14.

Hauser, W.A., Annegers, J.F., Rocca, W.A., 1996. Descriptive epidemiology of epilepsy: contributions of population-based studies from Rochester, Minnesota. Mayo Clin. Proc. 71, 576–886.

Jacques-Silva, M.C., Nogueira, C.W., Broch, L.C., Rocha, J.B.T., 2001. Diphenyl diselenide and ascorbic acid changes deposition of selenium and ascorbic acid in brain of mice. Pharmacol. Toxicol. 88, 119–125.

Juknat, A.A., Kotler, M.L., Batlle, A.M.C., 1995. High δ-aminolevulinic acid uptake in rat cerebral cortex: effect on porphyrin biosynthesis. Comp. Biochem. Physiol. 111C, 143–150.

Kaneko, K., Itoh, K., Berliner, L.J., Miyasaka, K., Fujii, H., 2002. Consequences of nitric oxide generation in epileptic-seizure rodent models as studied by in vivo EPR. Magn. Reson. Med. 48, 1051–1056.

Kappas, A., Sassa, S., Galbraith, R.A., Nordmann, Y., 1995. The porphyrias, In: Scriver, C.R., Beaudet, A.l., Sly, W.S., Valle, D. (Eds.), The Metabolic Bases of Inherited, 7th ed. McGraw Hill, New York, pp. 2103–2160.

Maciel, E.N., Flores, E.M., Rocha, J.B.T., Folmer, V., 2003. Comparative deposition of diphenyl diselenide in liver, kidney, and brain of mice. Bull. Environ. Contam. Toxicol. 70, 470–476.

Meldrum, B.S., 2002. Concept of activity-induced cell death in epilepsy: historical and contemporary perspectives. Prog. Brain Res. 135, 3–11.

Meotti, F.C., Borges, V.C., Zeni, G., Rocha, J.B.T., Nogueira, C.W., 2003. Potential renal and hepatic toxicity of diphenyl diselenide, diphenyl ditelluride and Ebselen for rats and mice. Toxicol. Lett. 143, 9–16.

Mikulecká, Anna, Kubová, Hana, Mares, Pavel, 2004. Lamotrigine does not impair motor performance and spontaneous behavior in developing rats. Epilepsy Behav. 5, 464–471.

Misra, H.P., Fridovich, I., 1972. The role of superoxide anion in the autoxidation of epinephrine and simple assay for superoxide dismutase. J. Biol. Chem. 247, 3170–3175.

Nogueira, C.W., Meotti, F.C., Curte, E., Pilissão, C., Zeni, G., Rocha, J.B.T., 2003. Investigations into the potential neurotoxicity induced by diselenides in mice and rats. Toxicology 183, 29–37.

Nogueira, C.W., Zeni, G., Rocha, J.B.T., 2004. Organoselenium and organotellurium compounds: toxicology and pharmacology. Chem. Rev. 104, 6255–6286.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.

Pappas, J.B., Ahlquist, J.T., Allen, E.M., Banner, J.W., 1995. Oral dimercaptosuccinic acid and ongoing exposure to lead: effects on heme synthesis and lead distribution in a rat model. Toxicol. Appl. Pharmacol. 133, 121–129.

Paulmier, C., 1986. Selenoorganic functional groups, In: Paulmier, C. (Ed.), Selenium Reagents and Intermediates in Organic Synthesis, 1st ed. Pergamon Press, Oxford, England, pp. 25–51.

Racine, R.J., 1972. Modification of seizures activity by electrical stimulation: II. Motor seizure. Electroencephalogr. Clin. Neurophysiol. 32, 281–294.

Said, S.J., Pakbaz, H., Berisha, H.I., Raza, S., 2000. NMDA receptor activation: critical role in oxidant tissue injury. Free Radical Biol. Med. 28, 1300–1302.

Sassa, S., 1982. Delta-aminolevulinic acid dehydratase assay. Enzyme 28, 133–145.

Savegnago, L., Trevisan, M., Alves, D., Rocha, J.B.T., Nogueira, C. W., Zeni, G., 2006. Antisecretory and antiulcer effects of diphenyl diselenide. Environ. Toxicol. Pharmacol. 21, 86–92.

Savegnago, L., Pinto, L.G., Jesse, C.R., Alves, D., Rocha, J.B.T., Nogueira, C.W., Zeni, G., 2007. Antinociceptive properties of diphenyl diselenide: evidences for the mechanism of action. Eur. J. Pharmacol 555, 129–138.

Spallholz, J.E., Shriver, J.E., Reid, T.W., 2001. Dimethyldiselenide and methylselenide acid generates superoxide in an in vitro chemiluminescence assay in the presence of glutathione: implications for the anticarcinogenic activity of L-selenomethionine and L-Se-methylselenocysteine. Nutr. Cancer 40, 34–41.

Sperk, G.H., Lassmamm, H., Baran, H., Seitelberger, F., Hornykiewicz, O., 1985. Kainic acid-induced seizures: dose-relationship of behavioural, neurochemical and histopathological changes. Brain Res. 338, 289–295.

Treiman, D.M., 1995. Electroclinical features of status epilepticus. J. Clin. Neurophysiol. 12, 343–362.

Timbrell, J., 2000. Factors affecting toxic responses: disposition, In: Timbrell, J. (Ed.), Principles of Biochemical Toxicology, 3rd ed. Taylor and Francis Ltd., Philadelphia, USA, pp. 25–62.

Walz, R., Moreira, J.C.F., Benfato, M.S, Quevedo, J., Schorer, N., Vianna, M.M.R., 2000. Lipid peroxidation in hippocampus early and late after status epilepticus induced by pilocarpine of kainic acid in Wistar rats. Neurosci. Lett. 291, 179–182.

4.2 Convulsões induzidas pelo disseleneto de difenila em ratos bebês: Possível interação com o sistema glutamatérgico

4.2.1 Artigo 2

DIPHENYL DISELENIDE-INDUCED SEIZURES IN RAT PUPS: POSSIBLE INTERACTION WITH GLUTAMATERGIC SYSTEM

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ORIGINAL PAPER

Diphenyl Diselenide-Induced Seizures in Rat Pups: Possible Interaction with Glutamatergic System

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Abstract The aims of the present study were to investigate the possible involvement of glutamatergic system in seizures induced by diphenyl diselenide in rat pups (postnatal day, 12-14) and to evaluate the role of oxidative stress in seizures induced by diphenyl diselenide/glutamate. Glutamate (4 g/kg of body weight) administered in association with diphenyl diselenide (500 mg/kg of body weight) increased the latency for the appearance of the first seizure episode, reduced lipid peroxidation levels and catalase, Na^+,K^+-ATPase and δ-ALA-D activities. At the lowest dose (5 mg/kg of body weight), diphenyl diselenide reduced the appearance of seizure episodes induced by glutamate but did not alter the latency for the onset of the first episode. Glutamate uptake was inhibited in glutamate, diphenyl diselenide (the highest dose) and in the association of diphenyl diselenide (both doses) and glutamate groups. Pre-treatment with a N-methyl-D-aspartate (NMDA) receptor antagonist, MK-801 (5*S*,10*R*-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate), significantly prolonged the latency for the onset for the first convulsive episode. A non-NMDA receptor antagonist, DNQX (6,7-dinitroquinoxaline-2,3-dione), did not protect seizures induced by diphenyl diselenide. The results of the present study demonstrated that: (a) when diphenyl diselenide and glutamate were administered concomitantly in pups, glutamate was the main responsible for the neurotoxic effects; (b) oxidative stress was not involved in glutamate-induced seizures; (c) NMDA glutamatergic receptors, were at least in part, involved in diphenyl diselenide- induced seizures; and (d) diphenyl diselenide, at the lowest dose, protected seizures induced by glutamate.

Keywords Diphenyl diselenide · Selenium · Glutamate · Seizures · Brain · Oxidative stress

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Introduction

During the last decades, the interest in organoselenium biochemistry has been intensified, mainly due to the fact that a variety of these compounds possess pharmacological properties. In this context, diphenyl diselenide is an organoselenium compound, highly lipophilic, with many pharmacological properties such as antiulcer [1] anti-inflammatory and anti-nociceptive [2], neuroprotector [3], hepatoprotector [4], anti-hyperglycemic [5], and antioxidant [6]. Beside to its pharmacological properties, diphenyl diselenide has toxic effects. In fact, intraperitoneal administration of high doses of this organoselenium compound caused seizure and death in mice but did not induce any overt sign of neurotoxicity in rats. These ambiguous data are probably explained by the fact that differences in toxicity among species and age are often related to differences in the liver metabolism and disposition of a compound [7, 8]. Accordingly, diphenyl diselenide injected directly into the brain of rodents did not cause any overt sign of neurotoxicity [9] indicating that this compound must be first metabolized, and, subsequently, crosses the blood-brain barrier to cause seizures [10]. Recently, we have reported that diphenyl diselenide, administrated by oral route, induced seizure and death in rat pups and reduced the activity of δ-ALA-D and Na^+,K^+-ATPase [11], sulfhydryl containing enzymes, which are sensitive to

oxidizing agents. These results reiforce the idea that neurotoxicity induced by diphenyl diselenide in adult rats differs from that of reported in pups.

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system (CNS), involved in essential brain functions, such as neural development, learning and memory [12]. Altered excitatory amino acid neurotransmission, mediated primarily by glutamate, is a major cause of the imbalance of excitation and inibition that contributes to hiperexcitability in the immature brain [13]. Glutamate exerts its toxicological effects by acting on both metabotrophic and ionotrophic receptors. Ionotrophic glutamate receptors have been categorized as N-methyl-D-aspartate (NMDA) and non-NMDA receptor subtypes. Studies indicate that alterations in glutamate transporters can lead to epileptic phenotypes and that impairment in glutamate uptake may play an important role in epileptogenesis [14, 15]. Glutamate does not cross the blood-brain barrier in adult rats but induces seizures in rat pups when given systemically [16].

It is well recognized that the imature brain differs from mature brain in the basic mechanisms of epileptogenesis. The imature brain is more prone to seizures than the adult brain due to an imbalance between the development of excitation and inhibition [17]. The activation of excitatory amino acid receptors can also trigger the formation of reactive oxygen species (ROS). Previous studies suggest that oxidative stress is important in brain tissue damage following seizure induction [18]. Seizures can induce neurological damage in the imature brain and lead to serious consequences later in life [19, 20].

In the previous study we have demonstrated that diphenyl diselenide induced seizure episodes in rat pups, depending on the dose of diphenyl diselenide administered [11]. Based on the considerations above, the specific aims of the present study were to investigate: (a) the possible involvement of glutamatergic system in seizures induced by diphenyl diselenide; and (b) the role of oxidative stress in seizures induced by diphenyl diselenide/glutamate.

Materials and methods

Chemicals

Monosodium glutamate, MK-801 (5*S*,10*R*-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate), DNQX (6,7-dinitroquinoxaline-2,3-dione), δ-ALA (δ-aminolevulinic acid), *p*-dimethylaminobenzaldehyde, epinephrine, ATP (adenosine triphosphate) and ouabain were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Monosodium glutamate and MK 801 were dissolved in 0.9% physiological saline. DNQX was dissolved in a minimum amount of DMSO (dimetilsulfoxide) and adjusted to the appropriate volume with 0.9% physiological saline.

Diphenyl diselenide was prepared in our laboratory according to the literature method [21]. Analysis of the ^{1}H NMR and ^{13}C NMR spectro showed that diphenyl diselenide obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of compound (99.9%) was determined by GC/HPLC. This drug was dissolved in canola oil.

Animals

Young Wistar rats (postnatal day 12–14) of both sexes were obtained from a local breeding colony. PND (postnatal day) 12–14 was chosen based on Mikuleckà et al. [22]. The animals were kept in separate animal rooms, on a 12 h light/dark cycle, in an air conditioned room (22 ± 2°C). Commercial diet (GUABI, RS, Brazil) and tap water were supplied ad libitum. The dams were allowed to deliver and wean their pups until PND 12–14. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, the Federal University of Santa Maria, Brazil.

In vivo experiments

Effect of glutamate on diphenyl diselenide-induced seizures

Diphenyl diselenide was administered by oral route (p.o., 10 ml/kg of body weight). The doses of diphenyl diselenide used in this study (5 mg/kg, a subeffective dose and 500 mg/kg, a dose which induces seizures in 100% of rat pups) were based on Prigol et al. [11]. To address the role played by glutamatergic system in seizures induced by diphenyl diselenide distinct groups of animals were treated with diphenyl diselenide and glutamate. Glutamate (4 g/kg) was administered by intraperitonial route (i.p., 10 ml/kg of body weight) based on Mares et al. [16], with intent to investigate if seizures induced by diphenyl diselenide are potentiated by glutamate.

Experimenters were blind to the drugs given to rat pups. Diphenyl diselenide and glutamate were concomitantly administered (groups 5 and 6). Rats were divided into groups as follows: Group 1—control (canola oil, p.o.) + (saline i.p), Group 2- Diphenyl diselenide (5 mg/kg, p.o.) + (saline i.p), Group 3- Diphenyl diselenide (500 mg/kg, p.o.) + (saline i.p), Group 4- Glutamate (4 g/kg, i.p) + (canola oil p.o), Group 5- Diphenyl diselenide (5 mg/kg, p.o.) + Glutamate

(4 g/kg, i.p), Group 6- Diphenyl diselenide (500 mg/kg, p.o.) + Glutamate (4 g/kg, i.p).

The appearance of seizures was quantified as previously described by Racine [23] and Sperk et al. [24] as follows: Stage 0: no changes; Stage 0.5: wet dog shakes; Stage 1: mouth and facial movements; Stage 2: head nodding; Stage 3: forelimb clonus; Stage 4: rearing; Stage 5: rearing and falling. The animals were observed for 1 h in Plexiglas chambers for the appearance or not of seizures lasting more than 5 s. The latency for the onset of the first seizure episode was also recorded. Animals belonging to all groups, which presented seizures, were classified at the stage 5 of the Racine scale and presented tonic-clonic seizures [11].

Subsequently to the seizure episode, rat pups were anesthetized and decapitated. Animals which did not display seizures were considered protected and sacrificed 1 h after the administration of compounds. The liver and whole brain of all animals were removed and used for ex vivo assays.

Involvement of NMDA receptor on diphenyl diselenide-induced seizures

To evaluate the role played by NMDA receptor on seizures induced by diphenyl diselenide distinct groups of animals were treated with distinct classes of drugs. For this purpose, rat pups were pretreated with MK801, a NMDA receptor antagonist (0.5 mg/kg, i.p., 10 ml/kg of body weight), or DNQX, a non-NMDA receptor antagonist (10 mg/kg of body weight, i.p., 10 ml/kg). Thirty minutes after MK 801 administration, or 20 min after DNQX, diphenyl diselenide (500 mg/kg of body weight, p.o.) or canola oil was injected.

The appearance of seizures was quantified as described on 2.3.1 Sect. Subsequently to the seizure episode, rat pups were anesthetized and decapitated. Animals which did not display seizures were considered protected and sacrificed 1 h after drug administration.

Ex vivo experiments

The samples of liver and whole brain were homogenized in 50 mM Tris–HCl, pH 7.5 (1/10, w/v) or (1/5, w/v), respectively, and centrifuged at 2,400*g* for 15 min. The supernatants (S1) were separated and used for biochemical assays.

Lipid peroxidation

The reaction product was determined using an aliquot (200 µl) of the S1, 500 µl thiobarbituric acid (0.8%), 200 µl SDS (sodium dodecil sulfate, 8.1%), and 500 µl acetic acid and 500 µl TBA (0.8% thiobarbituric acid), the mixture was incubated at 95°C for 2 h. TBARS (thiobarbituric acid reactive species) were determined as described by Ohkawa et al. [25].

Ascorbic acid determination

Ascorbic acid determination was performed as described by Jacques-Silva et al. [26]. Proteins were precipitated in 10 Vol. of a cold 4% trichloroacetic acid solution. An aliquot of the sample at a final volume of 1 ml of the solution was incubated for 3 h at 38°C then 1 ml H_2SO_4 65% (v/v) was added to the medium. The reaction product was determined using a color reagent containing 4.5 mg/ml dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/ml) at 520 nm. The content of ascorbic acid is related to tissue amount (µmol ascorbic acid/g wet tissue).

Superoxide dismutase (SOD) activity

Superoxide dismutase activity was assayed spectrophotometrically as described by Misra and Fridovich [27]. This method is based on the capacity of SOD to inhibit autoxidation of epinephrine to adrenochrome. Enzymatic reaction was initiated by adding an aliquot (20–60 µl) of the S1 and the substrate (epinephrine) to a concentration of 60 mM in a medium containing 50 mM glycine buffer, pH 10.3. The color reaction was measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autooxidation by 50% at 26°C.

Catalase activity

Catalase activity was assayed spectrophotometrically by the method of Aebi [28], which involves monitoring the disappearance of H_2O_2 in the S1 presence at 240 nm. Enzymatic reaction was initiated by adding an aliquot of 20 µl of the S1 and the substrate (H_2O_2) to a concentration of 0.3 mM in a medium containing 50 mM phosphate buffer, pH 7.0. The enzymatic activity was expressed in Units (1 U decomposes 1 µmole of H_2O_2 per min at pH 7 at 25°C).

δ-Aminolevulinate dehydratase (δ-ALA-D) activity

δ-Aminolevulinate dehydratase activity was assayed according to the method described by Sassa [29] by

measuring the rate of product porphobilinogen (PBG) formation except that 45 mM sodium phosphate buffer and 2.2 mM δ-ALA were used. An aliquot of 200 µl of the S1 was incubated for 1 h (liver) and for 3 h (brain) at 37°C. Enzymatic reaction was initiated by adding the substrate (δ-ALA) to a final concentration of 2.2 mM in a medium containing 45 mM phosphate buffer, pH 6.8. The incubation was stopped by adding 250 µl trichloroacetic acid solution (10% TCA) with 10 mM $HgCl_2$. The reaction product was determined using modified Erlich's reagent at 555 nm. The reaction was linear in relation to protein and time of incubation.

Na^+, K^+-ATPase activity

The homogenate was centrifuged at 4,000*g* at 4°C for 10 min and the S1 was used for assay of protein Na^+, K^+-ATPase. The reaction mixture for Na^+, K^+-ATPase activity assay contained 3 mM MgCl, 125 mM NaCl, 20 mM KCl, and 50 mM Tris–HCl, pH 7.4, in a final volume of 500 µl. The reaction was initiated by addition of ATP to a final concentration of 3.0 mM. Control samples were carried out under the same conditions with the addition of 0.1 mM ouabain. The samples were incubated for 30 min at 37°C, the incubation was stopped by adding 250 µl trichloroacetic acid solution (10% TCA) with 10 mM $HgCl_2$. Na^+, K^+-ATPase activity was calculated by the difference between the two assays. Released inorganic phosphate (Pi) was measured by the method of Fiske and Subbarow [30].

Preparation of synaptosomes

The rat whole brain was used to prepare synaptosomes on a discontinuous Percoll gradient according to Dunkley et al. [31]. In group 5, only animals which had seizure episodes were used for [^{3}H] glutamate uptake and release.

[^{3}H]Glutamate release by synaptosomes. Determination of [^{3}H]glutamate release was accomplished according to the method described by Migues et al. [32]. The synaptosomal preparation was loaded with 0.25 µCi [^{3}H]glutamate (Amersham, specific activity 53 mCi/mmol, final concentration 5 µM) by pre-incubation in Tris–HCl buffered salt solution (composition in mM: Tris–HCl 27, NaCl 133, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, Glucose 12, $CaCl_2$ 1.0) pH 7.4 (adjusted with HCl), for 15 min at 37°C. Aliquots of labeled synaptosomes (1.4 mg protein) were centrifuged at 16,000*g* for 1 min. Supernatants were discarded, and the pellets were washed four times in Tris–HCl buffer by centrifugation at 16,000*g* for 1 min (at 4°C). To assess the basal release of [^{3}H]glutamate, the final pellet was resuspended in Tris–HCl buffer and incubated for 60 s at 37°C. Incubation was terminated by immediate centrifugation (16,000*g*, 1 min, 4°C). Radioactivity present in supernatants and pellet was separately determined in a scintillation counter. The released [^{3}H]glutamate was calculated as a percentage of the total amount of radioactivity present in the synaptosomes at the start of the incubation period. K^+-stimulated [^{3}H]glutamate release was assessed as described for basal release, except for the fact that the incubation medium contained 40 mM KCl to induce synaptosomal depolarization.

[^{3}H]Glutamate uptake by synaptosomes. The synaptosomal preparation was washed twice by suspending in 3 Vol. of 0.3 M sucrose, in 15 mM Tris-acetate buffer (pH 7.4) and centrifuging at 35,000*g* for 15 min. The final pellet was suspended in 0.3 M sucrose, 15 mM Tris-acetate buffer (pH 7.4), and incubated in Tris–HCl buffer (composition in mM: Tris–HCl 27, NaCl 133, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, Glucose 12, $CaCl_2$ 1.0) pH 7.4 (adjusted with HCl), in the presence of [^{3}H]glutamate (final concentration 100 nM) for 1 min at 37°C. The reaction was stopped by centrifugation (16,000*g*, 1 min, 4°C), and the pellets were washed three times in Tris–HCl buffer by centrifugation at 16,000*g* for 1 min (at 4°C). Radioactivity present in pellet was measured in a scintillation counter. Specific [^{3}H]glutamate uptake was calculated as the difference between the uptake obtained in the incubation medium described above, and the uptake obtained with a similar incubation medium in which NaCl was replaced by choline chloride.

Protein quantification

Protein concentration was measured by the method of Bradford [33], using bovine serum albumin as the standard.

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed using a one-way analysis of variance (ANOVA), followed by the Duncan's multiple range test when appropriate. Values of $p < 0.05$ were considered statistically significant. Seizure incidence was statistically analyzed by the χ^2 method and Fisher's exact test.

Results

Effect of glutamate on diphenyl diselenide-induced seizures

As shown in Table 1, the administration of diphenyl diselenide at the highest dose together with glutamate

Table 1 Influence of treatment with diphenyl diselenide and glutamate in rat pups

Groups	Appearance of seizures[a]	Latency[b] (min)
Control	0/8	ns
$(PhSe)_2$ 5	0/8	ns
$(PhSe)_2$ 500	8/8**	18.73 ± 2.79*,#
Glutamate 4	8/8**	36.36 ± 12.64*
$(PhSe)_2$ 5 + Glutamate 4	10/16**,##	36.86 ± 14.00*
$(PhSe)_2$ 500 + Glutamate 4	9/9**	33.64 ± 8.80*

[a] Number of animals which presented seizures/N of animals per group

[b] Time (min) to the appearance for the first seizure episode. "ns" animals which did not present seizure (in 60 minutes of observation). $(PhSe)_2$ (mg/kg); Glutamate (g/kg). Data are reported as mean ± S.D

* $p < 0.05$ as compared to the control group (one-way ANOVA/Duncan)

# $p < 0.05$ as compared to the other groups (one-way ANOVA/Duncan)

** $p < 0.05$ as compared to the control group (χ^2 method and Fischer's exact probability test)

$p < 0.05$ as compared to the other groups (χ^2 method and Fischer's exact probability test)

induced seizures in 100% of rat pups. Glutamate administered concomitantly with diphenyl diselenide increased the latency for the appearance of the first seizure episode, when compared to the diphenyl diselenide group.

At the lowest dose, diphenyl diselenide reduced (about 37%) the appearance of seizure episodes induced by glutamate but did not alter the latency for the onset of the first episode (Table 1).

Table 2 Influence of pretreatment with MK 801 and DNQX in $(PhSe)_2$ induced seizures in rat pups

Groups	Appearance of Seizures [a]	Latency [b] (min)
Control	0/7	ns
$(PhSe)_2$ 500	7/7**	22.06 ± 2.1*
MK 801 0.5 + $(PhSe)_2$ 500	10/10**	31 ± 7.58*,#
DNQX 10 + $(PhSe)_2$ 500	8/8**	23.03 ± 2.25*

[a] Number of animals which presented seizures/N of animals per group

[b] Time (min) to the appearance for the first seizure episode. "ns" animals which did not present seizure (in 60 minutes of observation). $(PhSe)_2$, MK 801 and DNQX (mg/kg). Data are reported as mean ± S.D

* $p < 0.05$ as compared to the control group (one-way ANOVA/Duncan)

# $p < 0.05$ as compared to the other groups (one-way ANOVA/Duncan)

** $p < 0.05$ as compared to the control group (χ^2 method and Fischer's exact probability test)

Involvement of NMDA receptor on diphenyl diselenide-induced seizures

MK-801 significantly prolonged the latency for the onset of the first convulsive episode but did not reduce the number of animals convulsing (Table 2).

Pretreatment with DNQX neither prolonged the latency for the onset of the first convulsive episode nor reduced the number of animals convulsing (Table 2).

MK-801 or DNQX, given alone, did not cause any alteration in animal behavior (data not shown).

Lipid peroxidation

Diphenyl diselenide (5 mg/Kg) and glutamate administered alone or in association did not alter lipid peroxidation levels in brain of rat pups (Fig. 1). Glutamate reduced an increase in lipid peroxidation levels induced by 500 mg/kg diphenyl diselenide (Fig. 1).

In the liver of rat pups, one-way ANOVA of TBARS levels revealed that diphenyl diselenide (5 and 500 mg/kg) and the association of them with glutamate did not alter lipid peroxidation levels (data not shown).

Antioxidant defenses

One-way ANOVA of catalase activity revealed that glutamate bloked the stimulation of enzyme activity induced by 500 mg/kg diphenyl diselenide in brain of rat pups. The enzyme activity was not altered in the brain of pups that received all other treatments (Fig. 2).

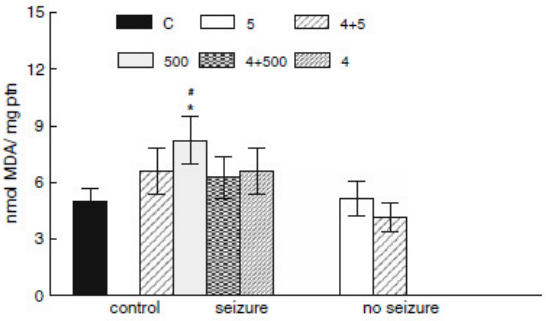

Fig. 1 Effect of diphenyl diselenide and glutamate on TBARS levels in brain of rat pups. Data are reported as mean ± SD of six to ten animals per group and expressed as nmol MDA (malondialdehyde)/mg protein. (*) denoted $p < 0.05$ as compared to the control group, (#) denoted $p < 0.05$ as compared to the Glutamate + 500mg/kg diphenyl diselenide group (one-way ANOVA/Duncan). *Abbreviations:* ***C*** control, *5* diphenyl diselenide (5 mg/kg), *4* glutamate (4 g/kg), *4 + 5* glutamate (4 g/kg) plus diphenyl diselenide (5 mg/kg), 500 diphenyl diselenide (500 mg/kg), *4 + 500* glutamate (4 g/kg) plus diphenyl diselenide (500 mg/kg)

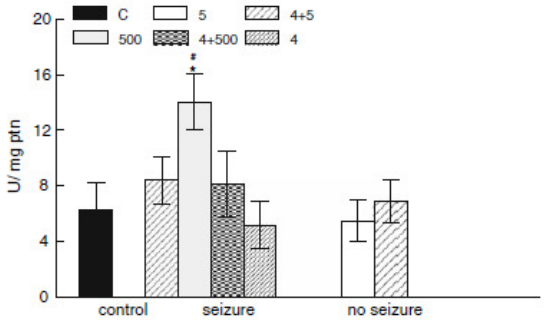

Fig. 2 Effect of diphenyl diselenide and glutamate on catalase activity in brain of rat pups. Data are reported as mean ± SD of six to ten animals per group and express U/mg protein. (*) denoted $p < 0.05$ as compared to the control group, (#) denoted $p < 0.05$ as compared to the Glutamate + diphenyl diselenide 500 mg/kg group (one-way ANOVA/Duncan). *Abbreviations: C* control, *5* diphenyl diselenide (5 mg/kg), *4* glutamate (4 g/kg), *4 + 5* glutamate (4 g/kg) plus diphenyl diselenide (5 mg/kg), *500* diphenyl diselenide (500 mg/kg), *4 + 500* glutamate (4 g/kg) plus diphenyl diselenide (500 mg/kg)

In the liver, there was no change in catalase activity independent of the treatment evaluated (data not shown).

One-way ANOVA of SOD activity demonstrated that diphenyl diselenide and glutamate did not alter the enzyme activity in liver and brain of rat pups. Ascorbic acid data showed that diphenyl diselenide and glutamate did not alter this parameter in liver and brain of rat pups (data not shown).

δ-ALA-D activity

Diphenyl diselenide (500 mg/kg) or glutamate inhibited δ-ALA-D activity in brain of pups (Table 3). The association of diphenyl diselenide and glutamate avoided the inhibitory effect induced by these compounds alone on δ-ALA-D activity. Cerebral δ-ALA-D activity was inhibited in rat pups which received concomitantly diphenyl diselenide (5 mg/Kg) and glutamate and did not present seizure episodes (Table 3).

In the liver of rat pups, glutamate ameliorated δ-ALA-D activity inhibition induced by 500 mg/kg diphenyl diselenide (Table 3).

Na^+,K^+-ATPase activity

Glutamate ameliorated Na^+,K^+-ATPase activity inhibited by 500 mg/kg diphenyl diselenide in brain of pups (Fig. 3). Diphenyl diselenide (5 mg/kg), glutamate and the association of these compounds did not alter Na^+,K^+-ATPase activity in brain of rat pups (Fig. 3).

Table 3 Effect of diphenyl diselenide and glutamate on δ-ALA-D activity in the brain and liver of rat pups

Groups	δ-ALA-D activity (nmol PBG/mg/protein/h)	
	Brain	Liver
Control	1.23 ± 0.14	13.27 ± 1.07
$(PhSe)_2$ 5 ns	1.15 ± 0.13	12.74 ± 1.75
$(PhSe)_2$ 500 s	0.82 ± 0.10*	4.50 ± 1.65*
Glutamate 4 s	0.77 ± 0.10*	14.19 ± 2.07
$(PhSe)_2$ 5 + Glutamate 4 ns	0.87 ± 0.07*	14.22 ± 0.86
$(PhSe)_2$ 5 + Glutamate 4 s	1.05 ± 0.19	13.06 ± 1.76
$(PhSe)_2$ 500 + Glutamate 4 s	1.00 ± 0.19	8.90 ± 1.90*

"s" animals which presented seizures

"ns" animals which did not present seizures (in 60 min of observation)

Diphenyl diselenide $(PhSe)_2$, dose (mg/Kg), Glutamate, dose (g/kg)

* $p < 0.05$ as compared to the control group (one-way ANOVA/Duncan)

Evaluation of synaptosomal [^{3}H]glutamate uptake and release

The [^{3}H]glutamate uptake by synaptosomes was significantly reduced in rats treated with diphenyl diselenide, glutamate and with the association of these compounds (Fig. 4).

The basal and K^+-stimulated [^{3}H]glutamate release by synaptosomes were similar in the control and all other groups tested (data not shown).

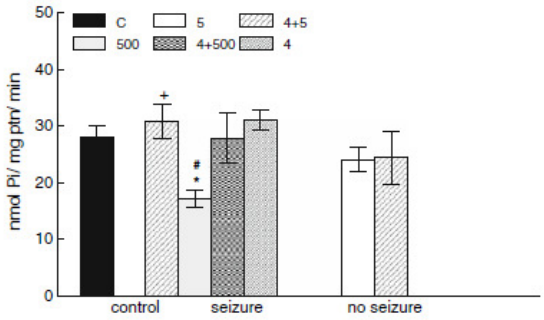

Fig. 3 Effect of diphenyl diselenide and glutamate on Na^+,K^+-ATPase activity in brain of rat pups. Data are reported as mean ± SD of six to ten animals per group and express nmolPi/mg protein/min. (*) denoted $p < 0.05$ as compared to the control group, (#) denoted $p < 0.05$ as compared to the Glutamate 4 g/kg + diphenyl diselenide 500 mg/kg group, (+) denoted $p < 0.05$ as compared to the Glutamate 4 g/kg + diphenyl diselenide 5 mg/kg group which no seizuring (one-way ANOVA/Duncan). *Abbreviations: C* control, *5* diphenyl diselenide (5 mg/kg), *4* glutamate (4 g/kg), *4 + 5* glutamate (4 g/kg) + diphenyl diselenide (5 mg/kg), *500* diphenyl diselenide (500 mg/kg), *4 + 500* Glutamate (4 g/kg) plus diphenyl diselenide (500 mg/kg)

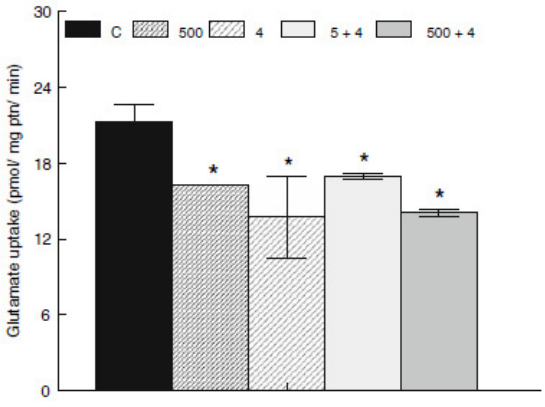

Fig. 4 Effect of diphenyl diselenide and glutamate on [^{3}H]glutamate uptake from rat brain synaptosomes. Data are reported as mean ± SD of three animals per group and glutamate uptake is expressed as pmol of glutamate uptake/mg protein/min. (*) denoted $p < 0.05$ as compared to the control group (one-way ANOVA/Duncan). *Abbreviations: C* control, *5* diphenyl diselenide (5 mg/kg), *4* glutamate (4 g/kg), *4 + 5* glutamate (4 g/kg) plus diphenyl diselenide (5 mg/kg), *500* diphenyl diselenide (500 mg/kg), *4 + 500* glutamate (4 g/kg) plus diphenyl diselenide (500 mg/kg)

Discussion

In the present study, we investigated the possible involvement of the glutamatergic system in seizures induced by diphenyl diselenide. Our results demonstrated that the diphenyl diselenide (500 mg/kg) + glutamate association increased the latency for diphenyl diselenide-induced seizures. In fact, there was an increase in the latency for the first seizure episode from 18.73 min (diphenyl diselenide group) to 33.64 min (diphenyl diselenide + glutamate group), suggesting that diphenyl diselenide could be more slowly metabolized in the liver in view of the high dose of glutamate administered concomitantly with this organoselenium compound. In agreement, previous results from our research group clearly indicate that seizures induced by diphenyl diselenide are dependent of the hepatic metabolism [9]. However, if we consider the similarity between the latency for the first seizure episode found for the diphenyl diselenide (500 mg/kg) + glutamate group (~33 min) and the glutamate group (~36 min) the idea that glutamate is the major responsible for seizures induced by concomitant administration of these compounds, is reinforced. Taken together these data lead us to exclude the hypothesis that seizures induced by diphenyl diselenide could be potentiated by glutamate.

Recently, our research group has reported that oxidative stress is, at least in part, involved in seizures-induced by diphenyl diselenide [11]. Therefore, in this study we explored the possible role of oxidative stress in seizures induced by glutamate/diphenyl diselenide. The levels of lipid peroxidation and the activity of catalase and Na^+, K^+-ATPase, altered by the highest dose of diphenyl diselenide in brain of animals, were found unaltered in those administered with glutamate and diphenyl diselenide. Consequently, we assume that the effects observed on the brain are most likely caused by glutamate than by diphenyl diselenide, when these compounds were administered in association. A plausible explanation for this fact is that, possibly, low levels of diphenyl diselenide metabolites reached brain. This hypothesis is supported by the evidence that diphenyl diselenide injected directly into the brain of rodents did not cause any overt sign of neurotoxicity [9] indicating that this compound must be first metabolized. Therefore, the results obtained with diphenyl diselenide in brain parameters reinforce a relationship between the dose of compound administered and the levels of diphenyl diselenide which reached brain.

It is important to point out that δ-ALA-D activity was found inhibited in the brain of rat pups which received glutamate concomitantly with diphenyl diselenide. If the assumption that glutamate delays diphenyl diselenide metabolism is correct, δ-ALA-D inhibition could be explained by the high sensitivity of this enzyme to the prooxidative damage induced by low levels of diphenyl diselenide.

In order to provide evidence for glutamatergic system involvement in seizures, we attempted to determine whether or not diphenyl diselenide and glutamate were capable of inhibiting glutamate uptake by synaptosomes. Uptake is one of the mechanisms by which glutamate is removed from the synaptic cleft, and its inhibition contributes for an increase in extracellular glutamate concentrations, which ultimately leads to over stimulation of the glutamatergic system [12, 34]. In the present study, the inhibition of glutamate uptake was observed in glutamate, diphenyl diselenide (highest dose) and the association of diphenyl diselenide (both doses) and glutamate groups, i.e., in all animals which had seizure episodes. Therefore, glutamate uptake seems to be related, at least in part, to the mechanisms by which these compounds induced seizures.

Taken the data above reported together, it is hard complex to explain the interaction of diphenyl diselenide (at the highest dose) and the glutamatergic system in an integrative way. Although all groups had some convulsant effect, the glutamate administration or association of diphenyl diselenide and glutamate caused similar effect on the latency for the onset to the first seizure episode, but the latencies were different from that of obtained after administration with diphenyl diselenide. While TBARS levels and catalase activity were increased only in the diphenyl diselenide group, cerebral δ-ALA-D activity was inhibited by glutamate and diphenyl diselenide groups and

these effects disappeared when both compounds were administered together. When the parameter analyzed was the activity of hepatic δ-ALA-D or cerebral Na^+,K^+-ATPase glutamate group presented no effect, but the effect on the diphenyl diselenide group was attenuated by concomitant administration of glutamate and diphenyl diselenide. The inhibition in glutamate uptake was clearly demonstrated in all animals which presented seizure episodes independent of the group analyzed.

Based on the idea that the high dose of glutamate used to induce seizures could be delaying diphenyl diselenide metabolism and consequently hiding the possible effect of diphenyl diselenide on the glutamatergic system, we performed experiments using NMDA and non-NMDA receptor antagonists. Glutamate exerts its toxicological effects by acting, at least in part, in ionotropic glutamate receptors [35]. In this study, pretreatment with MK-801, a NMDA receptor antagonist, increased the onset for the first seizure episode (22–31 min), suggesting that NMDA receptors play a role in diphenyl diselenide induced seizures. Conversely, pretreatment with DNQX, a non-NMDA receptor antagonist, did not alter the onset for the first seizure episode, excluding the involvement of non-NMDA receptors in this event.

In sharp contrast to our expectation the lowest dose of diphenyl diselenide protected against seizures induced by glutamate. In fact, diphenyl diselenide reduced about 37% the appearance of seizure episodes induced by glutamate. Although we did not focus on mechanisms by which diphenyl diselenide was capable of protecting seizures induced by glutamate, our results are in accordance with previous data reported by us [2] and others [36]. In fact, diphenyl diselenide produced pharmacological effect in several models of pain through mechanisms that involve an interaction with redox modulatory sites of glutamate receptors [2]. Accordingly, Herin et al. [36] have reported that ebselen, an organoselenium compound, may be neuroprotective in part due to its actions as a modulator of the NMDA receptor redox modulatory site. It is important to highlight that the doses in which diphenyl diselenide has pharmacological properties [2, 3, 6, 37] are about ten times lower than those that cause seizures.

In ex vivo experiments, the effects found with diphenyl diselenide (5 mg/kg) and glutamate association were analogous to those obtained for the glutamate group, i.e.: no change in lipid peroxidation, catalase, SOD, δ-ALA-D and Na^+, K^+ ATPase activities, and ascorbic acid levels. These results clearly point out that oxidative stress was not related to seizures induced by glutamate.

Another point that must be discussed here is that in general groups which had seizure episodes, excepting diphenyl diselenide (500 mg/kg) and glutamate group, showed glutamate uptake and brain δ-ALA-D activity inhibited. Supporting these data ALA has been reported as an inhibitor of glutamate uptake and a stimulator of the release of this excitatory neurotransmitter from rat synaptosomes [38, 39]. Emanuelli et al. [40, 41] have demonstrated that ionotropic glutamatergic antagonists, at doses which had no effect per se, antagonized ALA-induced seizures, supporting the involvement of the glutamatergic system.

Collectively, the results of the present study demonstrated that: (a) when diphenyl diselenide and glutamate are administered concomitantly in pups, glutamate is the main responsible for the neurotoxic effects; (b) oxidative stress was not involved in glutamate-induced seizures; (c) NMDA glutamatergic receptors, are at least in part, involved in diphenyl diselenide- induced seizures; and (d) diphenyl diselenide, at the lowest dose, protected seizures induced by glutamate.

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References

1. Savegnago L, Trevisan M, Alves D, et al (2006) Antisecretory and antiulcer effects of diphenyl diselenide. Environ Toxicol Pharmacol 21:86–92
2. Savegnago L, Pinto LG, Jesse CR, et al (2007) Antinociceptive properties of diphenyl diselenide: evidences for the mechanism of action. Eur J Pharmacol 555:129–138
3. Ghisleni G, Porciúncula LO, Cimarosti H, et al (2003) Diphenyl diselenide protects rat hippocampal slices submitted to oxygen–glucose deprivation and diminishes inducible nitric oxide synthase immunocontent. Brain Res 986:196–199
4. Borges LP, Nogueira CW, Panatieri RB, et al (2006) Acute liver damage induced by 2-nitropropane in rats: effect of diphenyl diselenide on antioxidant defenses. Chem Biol Interact 160:99–107
5. Barbosa NBV, Rocha JBT, Wondracek DC, et al (2006) Diphenyl diselenide reduces temporarily hyperglycemia: possible relationship with oxidative stress. Chem Biol Interact 163:230–238
6. Meotti F, Stangherlin EC, Zeni G, et al (2004) Protective role of aryl and alkyl diselenides on lipid peroxidation. Environ Res 94:276–282
7. Caldwell J (1981) The current status of attempts to predict species differences in drug metabolism. Drug Metab Rev 12:221–237
8. Caldwell J (1982) Conjugation reactions in foreign compound metabolism: definition, conseqüences and species variations. Drug Metab Rev 13:745–777
9. Nogueira CW, Meotti FC, Curte E, et al (2003) Investigations into the potential neurotoxicity induced by diselenides in mice and rats. Toxicology 183:29–37
10. Timbrell J (2000) Factors affecting toxic responses: disposition. In: Timbrell J (ed) Principles of biochemical toxicology, 3rd edn. Taylor & Francis, Philadelphia, USA, pp 25–62
11. Prigol M, Wilhelm EA, Schneider CC, et al (2007) Involviment of oxidative stress in seizures induced by Diphenyl Diselenide in rat pups. Brain Res 1147:226–232

12. Ozawa S, Kamiya H, Tsuzuki K, et al (1998) Glutamate receptors in the mammalian central nervous systen. Prog Neurobiol 54:581–618
13. Raol YH, Lynch DR, Brooks-Kayal A, et al (2001) Role of exitatory aminoacids in developmental epilepsies. Ment Retard Dev Disabil Res Rev 7:254–260
14. Rothstein JD, Dykes-Hoberg M, Pardo CA, et al (1996) Knockout of glutamate transporters reveals a major role for astroglial transports in exitotoxicity and clearance of glutamate. Neuron 16:675–686
15. Tanaka K, Watase K, Manabe T, Yamada K, et al (1997) Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporters GLT-1. Science 276:1699–1702
16. Mares P, Chino M, Kubová H, Mathern P, et al (2000) Convulsant action of systemically administered glutamate and bicuculline methiodide in immature rats. Epilepsy Res 42:183–189
17. Holmes GL, Gairsa JL, Chevassus-Au-louis N, et al (1998) Consequences of neonatal seizures in the rat: morphological and behavioral effects. Ann Neurol 44:845–857
18. Kaneko K, Itoh K, Berliner LJ, et al (2002) Consequences of nitic oxide generation in epileptic-seizure rodent models as studied by in vivo EPR. Magn Redon Med 48:1051–1056
19. Grunewald R (2002) Childhood seizures and their consequences for the hippocampus. Brain 125:1935–1936
20. Meldrum BS (2002) Concept of activity-inducet cell death in epilepsy: historical and contemporany perspectives. Prog Brain Res 135:3–11
21. Paulmier C (1986) Selenoorganic functional groups. In: Paulmier C (ed) Selenium reagents and intermediates in organic synthesis, 1st edn. Pergamon Press, Oxford, England, pp 25–51
22. Mikulecká A, Kubová H, Mares P, et al (2004) Lamotrigine does not impair motor performance and spontaneous behavior in developing rats. Epilepsy Behav 5:464–471
23. Racine RJ (1972) Modificationof seizures activity by electrical stimulation:II. Motor seizure. Electroencephalogr Clin Neurophysiol 32:281–294
24. Sperk GH, Lassmamm H, Baran H, et al (1985) Kainic acid-inducet seizures: dose-relationship of behavioural, neurochemical amd histopathological changes. Brain Res 338:289–295
25. Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358
26. Jacques-Silva MC, Nogueira CW, Broch LC, et al (2001) Diphenyl diselenide and acorbic acid changes deposition of selenium and ascorbic acid in brain of mice. Pharmacol Toxicol 88:119–125
27. Misra HP, Fridovich I (1972) The role of superoxide anion in the autoxidation of epinephrine and simple assay for superoxide dismutase. J Biol Chem 247:3170–3175
28. Aebi H (1984) Catalase in vitro. Meth Enzymol 105:121–126
29. Sassa S (1982) Delta-aminolevulinic acid dehydratase assay. Enzyme 28:133–145
30. Fiske CH, Subbarow YJ (1925) The calorimetic determination of phosphorus. Biol Chem 66:375–381
31. Dunkley PR, Heath J, Harrison SM, et al (1988) A rapid gradient procedure for isolation of synaptosomes directly from an S-1 fraction-homogeneity and morphology of subcellular fractions. Brain Res 441:59–71
32. Migues PV, Leal RB, Mantovani M, et al (1999) Synaptosomal glutamate release induced by the fraction Bc2 from the venon of the sea anemone Bunodosoma caissarum. Neuroreport 10:67–70
33. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal Biochem 72:248–254
34. Oliveira DL, Horn JF, Rodrigues JM (2004) Quinolinic acid promotes seizures and decreases glutamate uptake in youn rats: reversal by orally administered guanosine. Brain Res 1018:48–54
35. Danbolt NC (1994) The high affinity uptake system for excitatory amino acids in the brain. Prog Neurobiol 44:377–396
36. Herin GA, Du S, Aizenman E (2001) The neuroprotective agent ebselen modifies NMDA receptor function via the redox modulatory site. J Neorchem 78:1307–1314
37. Nogueira CW, Zeni G, Rocha JBT (2004) Organoselenium and organotellurium compounds: toxicology and pharmacology. Chem Rev 104:6255–6286
38. Brennan MJW, Cantrill RC (1979) The effect of delta-aminolevulinic acid on the uptake and efflux of amino acid is a potent agonist of GABA autoreceptors. Nature (London) 280:514–515
39. McLoughlin JL, Cantrill RC (1984) The effect of delta-aminolaevulinic acid on the high affinity uptake of aspartic acid byrat brain synaptosomes. Gen Pharmacol 15:553–555
40. Emanuelli T, Prauchner CA, Dacanal J (2000) Intrastriatal administration of 5-aminolevulinic acid induces convulsions and body asymmetry through glutamatergic mechanisms. Brain Res 868:88–94
41. Emanuelli T, Pagel FW, Porciúncula LO (2003) Effects of 5-aminolevulinic acid in the glutamatergic neurotansmission. Neurochem Int 42:115–121

4.3 Disseleneto de difenila induz convulsões em ratos bebês: Possível interação com o sistema GABAérgico

4.3.1 Artigo 3

DIPHENYL DISELENIDE-INDUCED SEIZURES IN RAT PUPS: POSSIBLE INTERACTION WITH GABAERGIC SYSTEM

Diphenyl diselenide-induced seizures in rat pups: possible interaction with GABAergic system

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Objectives: The involvement of the GABAergic system in seizures induced by diphenyl diselenide $(PhSe)_2$ in rat pups was investigated.
Methods: To this end, the effect of aminooxyacetic acid hemihydrochloride (AOAA, 20 mg/kg; by intraperitoneal route, i.p.), a GABA-T inhibitor; DL-2,4-diamino-n-butyric acid hydrochloride (DABA, 16 mg/kg; i.p.), an inhibitor of GABA uptake; and γ-aminobutyric acid (GABA, 10 and 40 mg/kg; i.p.), diazepam (3 mg/kg; i.p.) and phenobarbital (40 mg/kg; i.p.), GABAergic agonists as well as picrotoxin (1 mg/kg; i.p.), a $GABA_A$ receptor antagonist on $(PhSe)_2$ (50 and 500 mg/kg, by oral route, p.o.)-induced seizures, were studied.
Results: The [^{3}H]GABA uptake levels by cortical and hippocampal slices in rat pups exposed to $(PhSe)_2$ were also carried out. Pre-treatment with GABA (40 mg/kg), diazepam, phenobarbital, AOAA and DABA abolished the appearance of seizures induced by 50 mg/kg $(PhSe)_2$ in rat pups. Picrotoxin increased the percentage of convulsing rat pups from 42 to 100% and reduced significantly the onset for the first convulsive episode induced by $(PhSe)_2$ at the dose of 50 mg/kg. Diazepam and phenobarbital prolonged significantly the latency for the onset of the first convulsive episode caused by 500 mg/kg $(PhSe)_2$ in rat pups. [^{3}H]GABA uptake levels were stimulated in cerebral cortical and hippocampal slices of convulsing rat pups administered with both doses of $(PhSe)_2$.
Discussion: Our findings demonstrated that seizures induced by $(PhSe)_2$ are mediated, at least in part, by an interaction with GABAergic system.

Keywords: Diphenyl diselenide, GABA, Pups, Seizures, Selenium

Introduction

Epilepsy is a collection of diverse disorders that together affect approximately 1–2% of the world population.[1] Although epilepsy can manifest itself in a number of different ways, each type shares the common feature of increased neuronal excitability, culminating in seizures.[2] The immature brain is more prone to seizures than the adult brain due to an imbalance between the development of excitation and inhibition.[3] Studies suggest that seizure-induced effects in the immature brain compared with the adult brain are different and that the deleterious effects of seizures may not solely be a consequence of neuronal damage and loss *per se*, but could be due to the fact that seizures interfere with the highly regulated developmental processes in the immature brain.[4,5]

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Evidence suggests that oxidative stress, due to an increase in reactive oxygen species (ROS) production, is an important factor involved in seizure induced neuronal damage.[6] The involvement of ROS in seizures is supported by the fact that overactivation of excitatory amino-acid receptors can trigger the ROS formation, resulting in excitotoxic process and neuronal damage.[7] In this view, recent reports have indicated that diphenyl diselenide $(PhSe)_2$, an organoselenium compound with several pharmacological proprieties, such as anti-inflammatory,[8] anti-nociceptive,[9] and antidepressant-like,[10] induced seizure and death in rat pups, increased oxidative stress and reduced the activity of sulfhydryl containing enzymes, ALA-D and Na^+,K^+-ATPase.[11] Moreover, the involvement of *N*-methyl-D-aspartic acid (NMDA) glutamatergic receptor and glutamate uptake in $(PhSe)_2$-induced seizures has been reported.[12]

An increase in glutamatergic transmission and a decrease in GABAergic inhibitory responses seem to be important mechanisms in the genesis of convulsions

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which induces neurological damage in the immature brain and lead to serious consequences later in life.[13,14] In this context, γ-aminobutyric acid (GABA) is one of the most important inhibitory neurotransmitters in the central nervous system. GABA biosynthesis occurs in GABAergic neurons through glutamate decarboxylase (GAD) enzyme, and it is transported to vesicles and plasma membrane. Degradation of GABA takes place via GABA-transaminase (GABA-T), which are not only characteristics of GABAergic neurons.[15] Upon release into the synaptic cleft, GABA binds to post-synaptic $GABA_A$ receptors and elicits a neuroinhibitory response by activating Cl^- channels. This ionophore complex is widely implicated in epilepsy.[16,17] There are various modulators of the $GABA_A$ receptor–Cl^- channel complex, including barbiturates and benzodiazepines. These drugs are standard antiepileptic by stimulating their respective receptors in the ionophore complex to increase chloride flux and enhance GABAergic functions.[17]

Based on the considerations above and knowing that changes in GABAergic neurotransmission occur due to different mechanisms, the aim of the present study was to investigate the role of GABAergic system in $(PhSe)_2$-induced seizures. The [^{3}H]GABA uptake levels by cortical and hippocampal slices in rat pups exposed to $(PhSe)_2$ was also carried out.

Material and Methods

Chemicals

GABA, phenobarbital, diazepam, aminooxyacetic acid hemihydrochloride (AOAA), DL-2,4-diamino-n-butyric acid hydrochloride (DABA), and picrotoxin were purchased from Sigma (St Louis, MO, USA). 4-amino-n-[2.3-3]butyric acid ([^{3}H]GABA) (specific activity: 20 Ci/mmol) were purchased from Amersham International (Berkingshamshire, UK). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Phenobarbital and diazepam were dissolved in a minimum amount of 1 N NaOH and polyethylene glycol, respectively. These solutions were adjusted to the appropriate volumes with 0.9% physiological saline. Picrotoxin, GABA, AOAA and DABA were dissolved in 0.9% physiological saline.

$(PhSe)_2$ was prepared in our laboratory according to the literature method.[18] Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that $(PhSe)_2$ obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of compound (99.9%) was determined by GC/HPLC. This compound was dissolved in canola oil.

Animals

Wistar rats (postnatal day, PND, 12–14), weighing 24–30 g, of both sexes, were obtained from a local breeding colony. PND 12–14 was chosen based on Mikuleckà *et al.* (2004). The animals were kept in a separate animal room, on a 12 hour light/dark cycle, in an air conditioned room (22±2°C). Commercial diet (Guabi, Santa Maria, RS, Brazil) and tap water were supplied *ad libitum*. The dams were allowed to deliver and wean their pups until PND 12–14. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil.

Drug treatments

$(PhSe)_2$ was administered by oral route (p.o., 10 ml/kg body weight). The doses of $(PhSe)_2$ used in this study (50 and 100 mg/kg, a dose which induces seizures in 30 and 100% of rat pups, respectively) were based on Prigol *et al.*.[11]

To study the involvement of the GABAergic system in seizures induced by $(PhSe)_2$, AOAA, an inhibitor of GABA-T, DABA, an inhibitor of GABA uptake, and GABA, diazepam and phenobarbital, GABAergic agonists, as well as picrotoxin, an antagonist of $GABA_A$ receptor, were used. The drug dosages by intraperitoneal route (i.p., 10 ml/kg body weight) and pre-treatment times before the injection of $(PhSe)_2$ were: 10 or 40 mg/kg GABA (20 minutes), 40 mg/kg phenobarbital (30 minutes), 3 mg/kg diazepam (30 minutes), 1 mg/kg picrotoxin (15 minutes), 20 mg/kg AOAA (20 minutes) and 16 mg/kg DABA (30 minutes). The pre-treatment, times and doses of the drugs were established from preliminary studies in our laboratory.

Assessment of seizure activity

Appearance of seizures was quantified as previously described by Racine (1972)[19] and Sperk *et al.*.[20] The animals were observed for 1 hour in Plexiglas chambers for the appearance of tonic–clonic seizures lasting more than 5 seconds. The latency for the onset of the first tonic–clonic seizure episode was recorded. The convulsion behavior induced by $(PhSe)_2$ was classified as generalized seizures (tonic–clonic). Subsequently to the seizure episode, rat pups were decapitated. Animals which did not display seizures were considered protected and euthanized 1 hour after the compound administration.

[^{3}H]GABA uptake by cerebral cortex and hippocampus slices

To prove the involvement of the GABAergic system in seizures induced by $(PhSe)_2$, [^{3}H]GABA uptake was carried out in slices of hippocampus and cortex from rat pups.

The adequate [^{3}H]GABA concentration and incubation time for uptake assays, slices of hippocampus and parietal cortex were accomplished according to the method described by Schweigerta *et al.*.[21] The animals were divided into four groups: C, control; 500, $(PhSe)_2$ 500 mg/kg; 50 s, $(PhSe)_2$ 50 mg/kg, rats which presented seizure episode; 50 ns, $(PhSe)_2$ 50 mg/kg, rats which did not present seizure episode.

After the observation of the appearance of tonic–clonic seizures, the brains of rat pups were immedi-

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Table 1 Influence of pre-treatment with diazepam and phenobarbital in $(PhSe)_2$-induced seizures in rat pups

Groups	Appearance of seizures*	Latency† (minute)
Control	0/11	ns
$(PhSe)_2$ 50	5/12‡	49.00±7.14¶
$(PhSe)_2$ 500	7/7‡	22.28±2.75¶#
$(PhSe)_2$ 50+diazepam 3	0/11‡§	ns
$(PhSe)_2$ 500+diazepam 3	6/10‡	46.83±7.88¶
$(PhSe)_2$ 50+phenobarbital 40	0/11‡§	ns
$(PhSe)_2$ 500+phenobarbital 40	8/10‡	43.25±8.01¶

Note: *No. of animals which presented seizures/no. of animals per group.
†Time (minute) to the appearance for the first seizure episode. ns: animals which did not present seizure (in 60 minutes of observation). $(PhSe)_2$: diazepam and phenobarbital (mg/kg). Data are reported as mean±SD.
‡$P < 0.05$ as compared to the control group.
§$P < 0.05$ as compared to the $(PhSe)_2$ 50 group (χ^2 method and Fischer's exact probability test).
¶$P < 0.05$ as compared to the control group (one-way ANOVA/Duncan).
#$P < 0.05$ as compared to the other groups (one-way ANOVA/Duncan).

ately removed and submerged in Hank's balanced salt solution (HBSS), pH 7.2. Parietal cerebral cortices were dissected and coronal slices (0.4 mm) were obtained from the parietal area using a McIlwain tissue chopper. Slices were transferred to multiwell dishes and washed with 1.0 ml HBSS. The same procedure was undertaken for the hippocampal [^{3}H]GABA uptake assay, The uptake assay was assessed by adding 50 µM [^{3}H]GABA in 300 µl HBSS at 37°C. Incubation was stopped after 15 minutes by three ice-cold washes with 1 ml HBSS immediately followed by addition of 0.5 N NaOH, which was kept overnight. Aliquots of lysate were taken for determination of intracellular content of [^{3}H]GABA trough scintillation counting. Sodium independent uptake was determined using choline instead of sodium chloride, being subtracted from the total uptake to obtain the sodium-dependent uptake. The experiments were carried out in duplicate.

Protein quantification

Protein concentration was measured by the method of Bradford[22], using bovine serum albumin as the standard.

Statistical analysis

Data are expressed as mean±SD. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test when appropriate. Values of $P<0.05$ were considered statistically significant. The seizure incidence was analysed statistically by the χ^2 method and Fisher's exact test.

Results

As shown in Table 1, pre-treatment with diazepam and phenobarbital completely avoided the appearance of seizures induced by $(PhSe)_2$ (50 mg/kg). Diazepam and phenobarbital prolonged significantly the latency for the onset of the first convulsive episode induced by $(PhSe)_2$ (500 mg/kg) in approximately 24 and 21 minutes, respectively (Table 1).

Effects of GABA on diphenyl diselenide-induced seizures

Pre-treatment with GABA (10 mg/kg) did not increase significantly the onset of the first seizure episode and did not reduce the number of convulsing animals at both doses of $(PhSe)_2$ (Table 2).

Pre-treatment with GABA (40 mg/kg) completely protected animals against seizures induced by $(PhSe)_2$ (50 mg/kg) (Table 2). GABA did not protect animals against seizures, but it significantly prolonged the latency for the onset of the first convulsive episode (about 14 minutes) induced by $(PhSe)_2$ (500 mg/kg) (Table 2).

Effects of picrotoxin on $(PhSe)_2$-induced seizures

Picrotoxin (1 mg/kg), at a sub-effective dose, did not cause enhance of the exploratory behaviour of

Table 2 Influence of pre-treatment with GABA in $(PhSe)_2$ induced seizures in rat pups

Groups	Appearance of seizures*	Latency† (minute)
Control	0/11	ns
$(PhSe)_2$ 50	5/12‡	49.00±7.14¶
$(PhSe)_2$ 500	6/6‡	18.20±1.64¶
$(PhSe)_2$ 50+GABA 10	4/8	55±3.00¶
$(PhSe)_2$ 500+GABA 10	6/6‡	24.25±3.87¶
$(PhSe)_2$ 50+GABA 40	0/11§	57.00±0.00¶
$(PhSe)_2$ 500+GABA 40	6/6‡	32.00±3.08¶#

Note: *No. of animals which presented seizures/no. of animals per group.
†Time (minute) to the appearance for the first seizure episode. ns: animals which did not present seizure (in 60 minutes of observation). $(PhSe)_2$ and GABA (mg/kg). Data are reported as mean±SD.
‡$P < 0.05$ as compared to the control group (χ^2 method and Fischer's exact probability test).
§$P < 0.05$ as compared to the $(PhSe)_2$ 50 group (χ^2 method and Fischer's exact probability test).
¶$P < 0.05$ as compared to the control group (one-way ANOVA/Duncan).
#$P < 0.05$ as compared to the $(PhSe)_2$ 500 (one-way ANOVA/Duncan).

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animals. Prior administration of picrotoxin increased the percentage of convulsing rat pups from 42 to 100% and reduced significantly the onset for the first convulsive episode (about 20 minutes) induced by $(PhSe)_2$ (50 mg/kg) (Table 3).

Pre-treatment with picrotoxin reduced the latency for the first seizure episode induced by $(PhSe)_2$ (500 mg/kg) (about 7 minutes) (Table 3).

Effects of DABA and AOAA on $(PhSe)_2$-induced seizures

Pre-treatment with AOAA (20 mg/kg) and DABA (16 mg/kg) completely protected animals against seizures induced by $(PhSe)_2$ (50 mg/kg) (Table 4).

AOAA and DABA did not protect seizures induced by $(PhSe)_2$ 500 mg/kg, but the onset for the first seizure episode induced by $(PhSe)_2$ (500 mg/kg) was significantly delayed by AOAA and DABA, about 9 and 10 minutes, respectively (Table 4).

[^{3}H]GABA uptake

[^{3}H]GABA uptake was significantly increased in cerebral cortex and hippocampus slices of seizing rats treated with both doses of $(PhSe)_2$. Rat pups administered with 50 mg/kg $(PhSe)_2$, which did not display seizure episode, showed [^{3}H]GABA uptake in cerebral and hippocampus slices similar to the control group (Fig. 1).

Discussion

The roles of GABA, a major inhibitory neurotransmitter, and its receptor $GABA_A$ in epilepsy, have been widely reported.[23] In this study, we have evidence that the GABAergic system is, at least in part, involved in seizures induced by $(PhSe)_2$ in rat pups, since agonists of $GABA_A$ receptor and inhibitors of GABA transaminase and GABA uptake completely protected animals against seizure episodes or prolonged the latency for the onset of the first convulsive episode induced by $(PhSe)_2$. The current study demonstrated, for the first time, that $(PhSe)_2$ stimulated [^{3}H]GABA uptake in cortex and hippocampal slices from seizing rat pups.

The toxicological and pharmacological effects of $(PhSe)_2$ depend on many factors that include chemical form, specie and age of animal, route, and regime of administration.[11,24] Clarifying this point, it was earlier demonstrated that $(PhSe)_2$ administered to mice increased the latency for seizures and prevented death induced by 4-aminopyridine. In the reported study, adult mice received $(PhSe)_2$ at doses lower than 50 mg/kg through the subcutaneous route.[25] The results reported by Brito and collaborators[25] clearly differ from those demonstrated here; however, these discrepancies can be related to differences in the experimental protocol, such as the animal species, the route of administration, age of the animals, and dose employed. In the present study, rat pups showed seizures after oral administration of both doses of $(PhSe)_2$ (50 and 500 mg/kg). It was previously demonstrated that $(PhSe)_2$ administered by the oral route produced seizure and death in rat pups.[11,12]

The current study demonstrated that pre-treatment with diazepam and phenobarbital abolished convulsion induced by $(PhSe)_2$ at the dose of 50 mg/kg and increased the latency for the first seizure episode caused by the highest dose of $(PhSe)_2$ in rat pups. The principal therapeutic mechanisms of diazepam and phenobarbital are thought to stimulate their respective receptors in the ionophore complex, increasing the chloride flux through chloride channels at $GABA_A$ receptor sites, enhancing GABAergic functions.[26] Central $GABA_A$ receptor synaptic function has been associated with epilepsy and stimulation of $GABA_A$ receptors by GABA has been shown to overcome seizures.[27] Regarding seizures induced by $(PhSe)_2$, GABAergic modulators, diazepam, and phenobarbital have been reported to prevent seizures in adult mice.[28] Since $(PhSe)_2$ oxidizes sulfhydryl groups from proteins and of low-molecular weight thiol containing molecules such as glutathione,[21,29] this compound could potentially oxidize the $GABA_A$ receptor redox site, diminishing $GABA_A$ receptor activity. In accordance, Brito *et al.*[30] demonstrated that $(PhSe)_2$ increased pentylenetetrazol-induced chemical seizure and mortality in mice, suggesting that depletion of reduced thiols by $(PhSe)_2$ is one

Table 3 Influence of pre-treatment with picrotoxin in $(PhSe)_2$ induced seizures in rat pups

Groups	Appearance of seizures*	Latency† (minute)
Control	0/11	ns
$(PhSe)_2$ 50	5/12‡	49.00±7.14§¶
$(PhSe)_2$ 500	6/6‡	21.20±2.48¶
Picrotoxin 1	0/11	ns
$(PhSe)_2$ 50+picrotoxin 1	8/8‡	26.75±6.06§#
$(PhSe)_2$ 500+picrotoxin 1	6/6‡	14.00±2.60§¶@

Note: *No. of animals which presented seizures/no. of animals per group.
†Time (minute) to the appearance for the first seizure episode. ns: animals which did not present seizure (in 60 minutes of observation). $(PhSe)_2$ and picrotoxin (mg/kg). Data are reported as mean±SD.
‡$P < 0.05$ as compared to the control, $(PhSe)_2$ 500 and picrotoxin 1 groups (χ^2 method and Fischer's exact probability test).
§$P < 0.05$ as compared to the control group (one-way ANOVA/Duncan).
¶$P < 0.05$ as compared to the other groups (one-way ANOVA/Duncan).
#$P < 0.05$ as compared to the $(PhSe)_2$ 50 group (one-way ANOVA/Duncan).
@$P < 0.05$ as compared to the $(PhSe)_2$ 500 group (one-way ANOVA/Duncan).

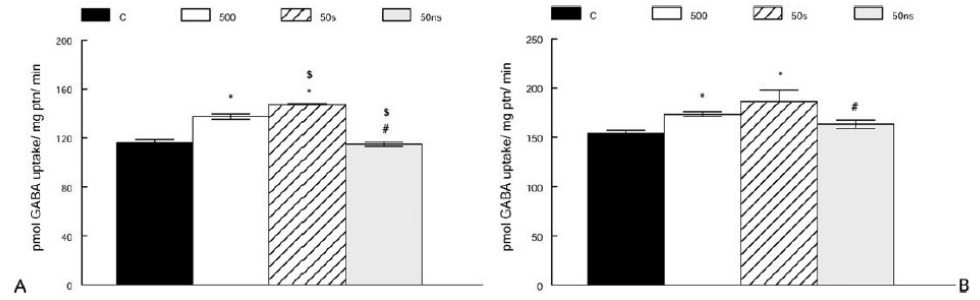

Figure 1 Effect of diphenyl diselenide on [^{3}H]GABA uptake by cerebral cortex (A) and hippocampus slices (B). Data are reported as mean±SD of three animals per group. [^{3}H]GABA uptake is expressed as pmol of GABA uptake/mg protein/min. *$P < 0.05$ as compared to the control group (one-way ANOVA/Duncan). C: control; 500: $(PhSe)_2$ (500 mg/kg); 50 s: $(PhSe)_2$ (50 mg/kg) rat pups which presented seizure episodes; 50 ns: $(PhSe)_2$ (50 mg/kg) rat pups which did not present seizure

mechanism by which this compound induced neurotoxicity.

Another evidence for the GABAergic system action in seizures induced by $(PhSe)_2$ was found with the experiments in which rat pups were pre-treated with GABA. The highest dose of GABA (40 mg/kg) completely protected animals against seizures induced by 50 mg/kg $(PhSe)_2$. In seizing animals treated with $(PhSe)_2$ at the dose of 500 mg/kg, pre-treatment with GABA (40 mg/kg) reduced the latency for the first convulsive episode. One explanation for the lack of GABA protection against the appearance of seizures caused by $(PhSe)_2$ would be a high dose of $(PhSe)_2$ administered in rat pups. At the lowest dose used, GABA (10 mg/kg) did not protect animals against seizures induced by $(PhSe)_2$ at both doses used. The plausible explanation for this fact is that GABA is distributed in every region of the brain and enzymatic degradation for GABA-T is possible, producing glutamate, reducing its quantity in synaptic cleft and the new synthesis of GABA, which occurs by GAD only in GABAergic neurons.

Picrotoxin, a potent $GABA_A$ receptor antagonist,[31] increased the neurotoxic effect induced by $(PhSe)_2$. In fact, picrotoxin significantly shortened the onset for the first seizure episode caused by $(PhSe)_2$ at both doses. This fact reinforces the idea that $GABA_A$ receptor modulation is involved in seizures induced by $(PhSe)_2$. In addition, picrotoxin acts by blocking the chloride channels linked to $GABA_A$ receptors. Taken together the data on GABA and picrotoxin, we suggest that seizures induced by $(PhSe)_2$ entails, at least in part, a direct effect on chloride channels linked to $GABA_A$ receptors.

Although $GABA_A$ receptors stimulation is an important mechanism involved in seizures, it is well known that other mechanisms can increase GABA levels in synaptic cleft, increasing the GABAergic inhibitory transmission. In this context, AOAA is a potent inhibitor of GABA-T, an enzyme which metabolizes GABA, thereby leading to an increase in brain GABA levels. In the present study, pre-treatment with AOAA protected rat pups against seizures induced by 50 mg/kg $(PhSe)_2$ and increased significantly the latency for the first seizure episode caused by the highest dose of $(PhSe)_2$. The increase in GABA levels, as a result of the activity of AOAA, may be the responsible for the AOAA protective effect against $(PhSe)_2$-induced seizures.

Similarly, rat pups pre-treated with DABA did not show seizure episodes when exposed to 50 mg/kg $(PhSe)_2$ and the latency for the first seizure episode was increased significantly when pups were exposed to the highest dose of $(PhSe)_2$. DABA is a potent and

Table 4 Influence of pre-treatment with DABA and AOAA in $(PhSe)_2$ induced seizures in rat pups

Groups	Appearance of seizures*	Latency† (minute)
Control	0/11	ns
$(PhSe)_2$ 50	5/12‡	49.00±7.14¶
$(PhSe)_2$ 500	6/6‡	18.20±1.64¶#
$(PhSe)_2$ 50+AOAA 20	0/11§	ns
$(PhSe)_2$ 500+AOAA 20	7/7‡	27.76±6.37¶
$(PhSe)_2$ 50+DABA 16	0/11§	ns
$(PhSe)_2$ 500+DABA 16	7/7‡	28.33±5.77¶

Note: *No. of animals which presented seizures/no. of animals per group.
†Time (minute) to the appearance for the first seizure episode. ns: animals which did not present seizure (in 60 minutes of observation). $(PhSe)_2$, AOAA and DABA (mg/kg). Data are reported as mean±SD.
‡$P < 0.05$ as compared to the control group (χ^2 method and Fischer's exact probability test).
§$P < 0.05$ as compared to the control, $(PhSe)_2$ 50 (χ^2 method and Fischer's exact probability test).
¶$P < 0.05$ as compared to the control group (one-way ANOVA/Duncan).
#$P < 0.05$ as compared to the other groups (one-way ANOVA/Duncan).

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selective inhibitor of GABA uptake which results in the accumulation of GABA in the brain. The termination of GABAergic synaptic transmission depends on rapid GABA re-uptake activity of specific transporters located on the membrane of pre-synaptic and glial cells, especially the predominant neuronal transporter subtype I (GAT1) which is the predominant neuronal transporter.[32] Higher GAT1 function would decrease the concentration of GABA in synaptic clefts.[33] Therefore, DABA effect may be due to an increase in the levels of free GABA available at the post-synaptic receptor sites.

Some studies demonstrated that cortex and hippocampus contain a high number of GABAergic neurons or projection neurons, areas that normally serve a gating function, restricting the propagation of epileptic activity.[34–36] In addition, GAT1 is continually expressed in the rat hippocampus and cortex.[37] To further support the involvement of GABA system in seizures induced by $(PhSe)_2$, we determined whether $(PhSe)_2$ alters [^{3}H]GABA uptake by cortical and hippocampal slices. The increase in [^{3}H]GABA uptake levels was demonstrated in all seizing animals exposed to $(PhSe)_2$ independent of the dose tested. The effect on [^{3}H]GABA uptake levels in hippocampal and cerebral cortex slices could be associated with the convulsing action of $(PhSe)_2$, since the stimulation of GABA uptake could decrease extracellular GABA levels, leading to observed enhancement in the sensibility of rat pups to seizure episodes. This finding helps to explain the protective effect of DABA against seizures induced by $(PhSe)_2$. Therefore, GABA uptake seems to be related to the one of the mechanisms by which $(PhSe)_2$ induced seizure in rat pups.

We reported that pre-treatment with MK-801 increased the onset for the first seizure episode induced by $(PhSe)_2$ 500 mg/kg, suggesting that glutamatergic NMDA receptor play a role in $(PhSe)_2$ induced seizures; also the inhibition in [^{3}H]glutamate uptake, with increasing glutamate in synaptic cleft, was demonstrated as a mechanism which contributes to hyperexcitability and seizures in rat pups.[12] In this research, we demonstrated that $GABA_A$ receptor agonists as well as GABA transaminase and GABA uptake inhibitors completely protected animals against seizures induced by $(PhSe)_2$, suggesting that $GABA_A$ receptors, GABA-T and neuronal transporter play a role in $(PhSe)_2$-induced seizures. In addition, $(PhSe)_2$ stimulated [^{3}H]GABA uptake in cortex and hippocampal slices from seizing rat pups, with decreasing GABA level in the synaptic cleft. In this respect, Gale[27] demonstrated that the direct or indirect interference with GABA-mediated neurotransmission results in convulsive seizure activity in humans and animals. These results together demonstrated that seizure induced by $(PhSe)_2$ is caused by its interaction with GABA and glutamate systems, and consequent imbalance between the excitatory and inhibitory neurotransmission.

In conclusion, the results of the present study show that seizures induced by $(PhSe)_2$ are mediated, at least in part, by interaction with the GABAergic system demonstrated by the following results: modulation of $GABA_A$ receptors; GABA-T and neuronal GABA transporter, and stimulation of [^{3}H]GABA uptake in cortex and hippocampal slices from seizing rat pups.

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References

1 Mcnamara JO. Emerging insights into the genesis of epilepsy. Nature. 1999; 399: A15–A22.
2 Lothman EW, Bertram EH, Stringer JL. Functional anatomy of hippocampal seizures. Prog Neurobiol. 1991; 37: 1–82.
3 Holmes GL, Gairsa JL, Chevassus-Au-louis N, Ben-Ari Y. Consequences of neonatal seizures in the rat: morphological and behavioral effects. Ann Neurol. 1998; 44: 845–57.
4 Zupanc ML. Neonatal seizures. Pediatr Clin N Am. 2004; 51: 961–78.
5 Wasterlain CG, Niquet J, Thompson KW, Baldwin R, Liu H, Sankar R, *et al.* Seizure-induced neuronal death in the immature brain. Prog Brain Res. 2002; 135: 335–53.
6 Yu X, Shao XG, Sun H, Li YN, Yang J, Deng YC, *et al.* Activation of cerebral peroxisome proliferator-activated receptors gamma exerts neuroprotection by inhibiting oxidative stress following pilocarpine-induced status epilepticus. Brain Res. 2008; 1200: 146–58.
7 Mueller-Burke D, Koehler RC, Martin LJ. Rapid NMDA receptor phosphorylation and oxidative stress precede striatal neurodegeneration after hypoxic ischemia in newborn piglets and are attenuated with hypothermia. Int J Dev Neurosci. 2008; 26: 67–76.
8 Savegnago L, Jesse CR, Pinto LG, Rocha JBT, Nogueira CW. Diphenyl diselenide attenuates acute thermal hyperalgesia and persistent inflammatory and neuropathic pain behavior in mice. Brain Res. 2007; 1175: 54–59.
9 Savegnago L, Pinto LG, Jesse CR, Alves D, Rocha JB, Nogueira CW, *et al.* Antinociceptive properties of diphenyl diselenide: evidences for the mechanism of action. Eur J Pharmacol. 2007; 555: 129–38.
10 Savegnago L, Pinto LG, Jesse CR, Rocha JB, Nogueira CW, Zeni G. Monoaminergic agents modulate antidepressant-like effect caused by diphenyl diselenide in rats. Prog Neuropsychopharmacol Biol Psychiatry. 2007; 31: 1261–69.
11 Prigol M, Wilhelm EA, Schneider CC, Rocha JB, Nogueira CW, Zeni G. Involvement of oxidative stress in seizures induced by diphenyl diselenide in rat Pups. Brain Res. 2007; 1147: 226–32.
12 Prigol M, Wilhelm EA, Stangherlin EC, Barancelli DA, Nogueira CW, Zeni G. Diphenyl diselenide-induced seizures in rat pups: possible interaction with glutamatergic system. Neurochem Res. 2008; 33: 996–1004.
13 Grunewald R. Childhood seizures and their consequences for the hippocampus. Brain. 2002; 125: 1935–36.
14 Meldrum BS. Concept of activity-induced cell death in epilepsy: historical and contemporany perspectives. Prog Brain Res. 2002; 135: 3–11.
15 Chaudhry FA, Reimer RJ, Bellocchio EE, Danbolt NC, Osen KK, Edwards RH, *et al.* The vesicular GABA transporter, VGAT, localizes to synaptic vesicles in sets of glycinergic as well as GABAergic neurons. J Neurosci. 1998; 18: 9733–50.
16 Amabeoku G, Chikuni O, Bwakura E. Gamma aminobutyric acid mediation of the anticonvulsivant effect of clonidine on pentylenetetrazol-induced seizures in mice. Pharmacol Res. 1994; 29: 273–80.

17 Johnston GA. $GABA_A$ receptor pharmacology. Pharmacol Ther. 1996; 69: 173–98.
18 Paulmier C. Selenoorganic functional groups. In: Paulmier, C. (ed.). Selenium reagents and intermediates in organic synthesis, 1st ed. Oxford: Pergamon Press; 1986. pp. 25–51.
19 Racine RJ. Modification of seizures activity by electrical stimulation: II. Motor seizure Electroencephalogr. Clin Neurophysiol. 1972; 32: 281–94.
20 Sperk GH, Lassmamm H, Baran H, Seitelberger F, Hornykiewicz O. Kainic acid-inducet seizures: dose-relationship of behavioural, neurochemical amd histopathological changes. Brain Res. 1985; 338: 289–95.
21 Schweigerta ID, de Oliveira DL, Scheibela F, da Costa F, Wofchuk ST, Souza DO, *et al.* Gestational and postnatal malnutrition affects sensitivity of young rats to picrotoxin and quinolinic acid and uptake of GABA by cortical and hippocampal slices. Brain Res Dev Brain Res. 2005; 154: 177–85.
22 Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principles of protein-dye binding. Anal Biochem. 1976; 72: 248–54.
23 Leidenheimer NJ, Browning MD, Harris RA. $GABA_A$ receptor phosphorylation: multiple sites, actions and artifacts. Trends Pharmacol Sci. 1991; 12: 84–87.
24 Prigol M, Schumacher RF, Nogueira CW. Convulsant effect of diphenyl diselenide in rats and mice and its relationship to plasma levels. Toxicol Lett. 2009; 189: 35–39.
25 Brito VB, Rocha JB, Folmer V, Erthal F. Diphenyl diselenide and diphenyl ditelluride increase the latency for 4-aminopyridine-induced chemical seizure and prevent death in mice. Acta Biochim Pol. 2009; 56: 125–34.
26 Meldrum BS. Why and when are seizures bad for de brain? Trend Pharmacol Sci. 2001; 22: 445–46.
27 Gale K. Role of GABA in the genesis of chemoconvulsant seizures. Toxicol Lett. 1992; 64: 417–28.
28 Nogueira CW, Meotti FC, Curte E, Pilissão C, Zeni G, Rocha JB. Investigations into the potential neurotoxicity induced by diselenides in mice and rats. Toxicology. 2003; 183: 29–37.
29 Chaudhry FA, Reimer RJ, Bellocchio EE, Danbolt NC, Osen KK, Edwards RH, *et al.* The vesicular GABA transporter, VGAT, localizes to synaptic vesicles in sets of glycinergic as well as GABAergic neurons. J Neurosci. 1998; 18: 9733–50.
30 Brito VB, Folmer V, Puntel GO, Fachinetto R, Soares JC, Zeni G, *et al.* Diphenyl diselenide and 2,3-dimercaptopropanol increase the PTZ-induced chemical seizure and mortality in mice. Brain Res Bull. 2006; 68: 414–18.
31 Newland CF, Cull-Candy SG. On the mechanism of action of picrotoxin on GABA receptor channels in dissociated sympathetic neurones of the rat. J Physiol. 1992; 447: 191–213.
32 Gadea A, Lopez-Colome AM. Glial transporters for glutamate, glycine, and GABA: II. GABA transporters, J Neurosci Res. 2001; 63: 461–68.
33 Devaud LL. Ethanol dependence has limited effects on GABA or glutamate transporters in rat brain. Alcohol Clin Exp Res. 2001; 25: 606–11.
34 Löscher W, Ebert U. Basic mechanisms of seizure propagation: targets for rational drug design and rational polypharmacy. Epilepsy Res Suppl. 1996; 11: 17–44.
35 Meyer DA, Carter JM, Johnstone AF. Pyrethroid modulation of spontaneous neuronal excitability and neurotransmission in hippocampal neurons in culture, Neurotoxicology. 2008; 29: 213–25.
36 Yu ZY, Wang W, Fritschy JM, Witte OW, Redecker C. Changes in neocortical and hippocampal $GABA_A$ receptor subunit distribution during brain maturation and aging. Brain Res. 2006; 1999: 73–81.
37 Sabau H, Frahm C, Pfeiffer M, Breustedt JM, Piechotta A, Numberger M, *et al.* Age-dependence of the anticonvulsant effects of the GABA uptake inhibitor tiagabine *in vitro*. Eur J Pharmacol. 1999; 383: 259–66.

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4.4 Efeito convulsivante do disseleneto de difenila em ratos e camundongos e sua relação com os níveis plasmáticos

4.4.1 Artigo 4

CONVULSANT EFFECT OF DIPHENYL DISELENIDE IN RATS AND MICE AND ITS RELATIONSHIP TO PLASMA LEVELS

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Convulsant effect of diphenyl diselenide in rats and mice and its relationship to plasma levels

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ABSTRACT

Diphenyl diselenide [$(PhSe)_2$], an organoselenium compound, presents pharmacological and toxicological properties in rodents. The aim of this study was to carry out the determination and quantification of $(PhSe)_2$ in plasma after oral administration (p.o.) of this compound (500 mg/kg), dissolved in canola oil, in rats and mice. The second objective was to verify the involvement of different routes of administration ((p.o.), intraperitoneal (i.p.) and subcutaneous (s.c.)) and vehicle solutions (canola oil and dimethyl sulfoxide (DMSO)) in the appearance of seizure episodes and in the plasmatic levels of $(PhSe)_2$ in rats and mice. Analysis of $(PhSe)_2$ in blood samples was performed by gas chromatography/flame ionized detector system (GC/FID). Rat and mouse peak plasma $(PhSe)_2$ levels were 13.13 and 10.11 μg/ml (C_{max}), respectively, and occurred at 0.5 h (T_{max}) post-dosing. The use of different administration routes (p.o., i.p. and s.c.) and vehicle solutions (canola oil or DMSO) in rats and mice indicated that the appearance of seizures and $(PhSe)_2$ plasmatic levels are dependent of administration routes (i.p. > p.o. > s.c.), vehicle solutions (DMSO > canola oil) and animal species (mice > rat).

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1. Introduction

Selenium is an essential trace element nutritionally important to mammals, with physiological roles as a structural component of several antioxidant enzymes involved in the peroxide decomposition (Rayman, 2000; Ursini and Bindoli, 1987). Recent studies about selenium species distribution and metabolic transformation in biological tissues have been reported. These studies show the biological transformation of Se from inorganic Se compounds and selenoamino acids in foods, into excreted selenocompounds via key metabolic intermediates (Schomburg et al., 2004; Suzuki, 2005). The biotransformation involves diffusion of Se compounds into the intestine (Mc Connell and Cho, 1965), transport to organs and utilization to biosynthesize selenoproteins via selenophosphate and selenocysteine, and transformation into selenosugars to be excreted into the breath and urine (Kobayashi et al., 2002; Suzuki et al., 2005).

A number of novel pharmaceutical agents derived from selenium or designed to influence specific aspects of selenium metabolism are under development (Mugesh et al., 2001; Nogueira et al., 2004; Rosa et al., 2007). In spite of extensive literature describing pharmacological properties of organoselenium compounds, little is known about their mode of action (Machado et al., 2006; Prigol et al., 2008a; Rocha et al., 2005; Rosa et al., 2003). Organoselenium compounds are products with a double face due to their contrasting behaviour, which depends on the dose used (Nogueira et al., 2004). Recently the biological activities of diphenyl diselenide $(PhSe)_2$ have been reported and this compound has emerged as a candidate for therapeutic purposes. On one hand, $(PhSe)_2$ has been proven effective against neurotoxicity (Ghisleine et al., 2003), hyperglycemia (Barbosa et al., 2006), lipid peroxidation (Meotti et al., 2004; Luchese et al., 2007), inflammation (Savegnago et al., 2007a), nociception (Savegnago et al., 2007b), anxiety-like (Savegnago et al., 2007c) and depressant-like (Ghisleni et al., 2008; Savegnago et al., 2008) models using rats and mice. On the other hand, $(PhSe)_2$ has been reported to cause neurotoxicity in rodents (Nogueira et al., 2003a; Prigol et al., 2007, 2008b) and inhibition of sulfhydryl enzymes (Barbosa et al., 1998; Maciel et al., 2000; Meotti et al., 2003; Borges et al., 2005; Nogueira et al., 2003b; Prigol et al., 2007). In addition, a different toxicity across species induced by $(PhSe)_2$ has been documented, since the chemical failed to produce any adverse effects in rats, despite a much higher dose. In contrast, a similar dose of the compound induces seizures and death in mice (Nogueira et al., 2003a). The reasons for differential toxicity between mice and rats are presently unknown. Although Maciel et al. (2003) have reported that acute treatment with $(PhSe)_2$ caused a significant increase in Se concentration in liver, kidney and brain, information related to the analysis of $(PhSe)_2$ in biological matrices are limited. Therefore, the literature dealing with detection and quantification of $(PhSe)_2$ concentration in plasma of rats and mice

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is scarce.

The aim of this study was to carry out the determination and quantification of $(PhSe)_2$ after oral administration of this compound, dissolved in canola oil, in plasma of rats and mice. Since seizures induced by $(PhSe)_2$ are dependent of species and route of administration (Nogueira et al., 2003a) the second objective of this study was to verify the involvement of different routes of administration (oral (p.o.), intraperitoneal (i.p.) and subcutaneous (s.c.)) and vehicle solutions (canola oil and dimethyl sulfoxide (DMSO)) in the plasmatic levels of $(PhSe)_2$ and in the appearance of seizure episodes in rats and mice.

2. Materials and methods

2.1. Chemicals

$(PhSe)_2$ was prepared in our laboratory according to the literature method (Paulmier, 1986). Analysis of the ^1H NMR and ^{13}C NMR spectra showed that $(PhSe)_2$ obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of compound (99.9%) was determined by GC/HPLC and was stable under storage conditions at room temperature, humidity and light.

2.2. Animals

Male adult Wistar rats (200–250 g) and male adult Swiss mice (25–30 g) obtained from a local breeding colony were used. The animals were kept in a separate animal room, on a 12 h light/12 dark cycle, in an air conditioned room (22 ± 2 °C). Commercial diet (GUABI, RS, Brazil) and tap water were supplied *ad libitum*. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil.

2.3. Preparation of $(PhSe)_2$ solution

The solution was weekly prepared and the compound was dissolved in canola oil or DMSO. The concentration of $(PhSe)_2$ was analyzed prior to use. The solutions were stored at 2–8 °C and allowed to warm up to room temperature before use. The adult rats and mice were given a single p.o., i.p. or s.c. dosage of 500 mg/kg $(PhSe)_2$. The dosage of $(PhSe)_2$ used in this study is far above the therapeutic range (Savegnago et al., 2007a, 2008), because $(PhSe)_2$ causes neurotoxicity only at very high doses. Therefore, the choice of the $(PhSe)_2$ dosage was based on previous studies dealing with dose–response for appearance of seizures episodes (Nogueira et al., 2003; Prigol et al., 2007).

$(PhSe)_2$ solutions were administered to animals in the morning between 8:00 a.m. and 9:30 a.m. at a dosing volume of 10 ml/kg. The feed was available *ad libitum* and animals were not fasted prior to dosing.

2.4. Experimental design

2.4.1. Determination and quantification of $(PhSe)_2$ in plasma of rats and mice

$(PhSe)_2$ dissolved in canola oil was administered to animals once a day per oral route by gavage. Four animals per group (for each time-point) were anesthetized and blood samples (1 ml) were collected from each animal by heart puncture, in heparin tubes, at 0 (for blank blood sample), 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h after the gavage dose.

Blood samples were centrifuged at 3000 rpm for 5 min. The plasma was separated and stored chilled until assay.

2.4.2. Involvement of administration route and vehicle solution in the appearance of seizure episodes and in plasma concentration of $(PhSe)_2$ in rats and mice

The end point seizure was chosen to correlate the involvement of different routes of administration and vehicle solutions with plasmatic levels of $(PhSe)_2$ in mice and rats. To this end, $(PhSe)_2$ at the dose of 500 mg/kg dissolved in canola oil or DMSO was administered by i.p., s.c. or p.o. route in mice and rats.

Appearance of seizures was quantified as previously described by Prigol et al. (2007). In short, animals were observed for 1 h in Plexiglas chambers for the appearance of tonic-clonic seizures lasting more than 5 s. The latency for the onset of the first tonic-clonic seizures episode was also recorded. Animals which did not display seizures (1 h) were considered protected.

Three animals per group were anesthetized and blood samples were collected from each animal subsequently to the seizure episodes. Blood samples were obtained as above described in Section 2.4.1.

2.5. Determination and quantification of $(PhSe)_2$ in plasma samples

Determination of $(PhSe)_2$ concentrations in plasma were made in all samples, collected at the time of euthanasia. The plasma (200 μl) was mixed to ethyl acetate in the ratio of 1:1 (v/v) to each tube. The extraction was performed by vortex-mixing the tubes for 3 min. After, the samples were centrifuged at 3000 rpm for 5 min, the supernatants were separated and filtrated through a membrane (0.45 μm pore size) Millipore®. The filtrated was transferred to a clean test tube and 1 μl aliquot was injected into chromatographic system for analysis. Qualitative and quantitative analysis of $(PhSe)_2$ in plasma samples were conducted by gas chromatography (CG 2010 Shimadzu®) associated to flame ionized detector system (FID) and using a 5% diphenyl/95% dimethyl column, 30 m × 0.25 mm × 0.25 μm, from Restek®. The limit of detection (LOD) for $(PhSe)_2$ was 0.5 μg/ml, and the limit of quantification (LOQ) was 1 μg/ml. The standard curve for $(PhSe)_2$ was linear (R_2 = 0.99887) over the concentration range 1–100 μg/ml. The intra- and interday coefficients of variation for drug was <10%. Assay recovery for drug ranged between 90% and 110% of the target at all the concentrations tested with a coefficient of variation <10% to analysis.

The plasma concentration (C), the maximum plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were directly obtained from plasma data.

2.6. Statistical comparisons

Analysis of variance was performed using one-way (ANOVA) (latency to seizure and plasma $(PhSe)_2$ levels), followed by the Duncan's multiple range. Data are expressed as means ± S.E.M. Values of $p < 0.05$ were considered statistically significant.

3. Results

As shown in Table 1, the rat and mouse peak plasma $(PhSe)_2$ levels were 13.13 and 10.11 μg/ml (C_{max}), respectively and occurred at 0.5 h (T_{max}) post-dosing 500 mg/kg $(PhSe)_2$ p.o., dissolved in canola oil. Plasma levels of $(PhSe)_2$ for mice and rats decreased 1 h after

Table 1
Plasmatic parameters after oral administration $(PhSe)_2$ dissolved in canola oil in rats and mice.

	Rats	Mice
C_{max} (μg/ml)	13,13 ± 1,29	10,11 ± 1,26
T_{max} (h)	0,5 ± 0,00	0,5 ± 0,00

Data are reported as means ± S.E.D; $n = 4$.

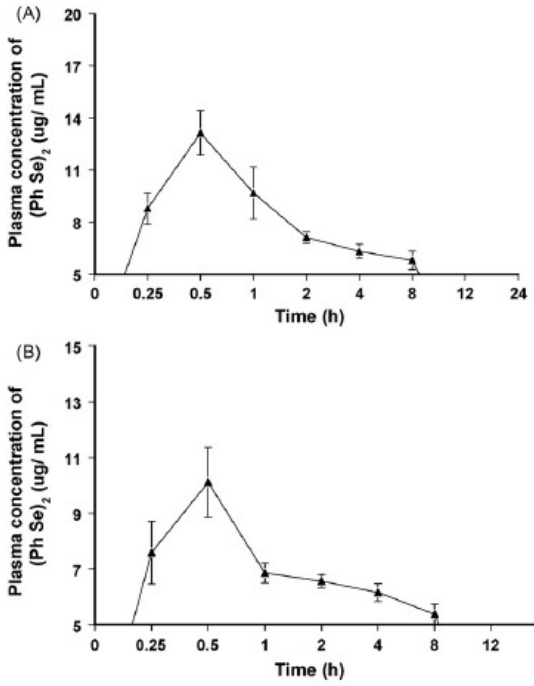

Fig. 1. Plasma concentration–time profile of $(PhSe)_2$ (μg/ml) in rats (a) and mice (b) after oral administration of $(PhSe)_2$ at the dose of 500 mg/kg (▲).

Table 2
Effect of different routes of administration, vehicle solutions and animal species on seizures induced by $(PhSe)_2$ and its plasma concentration (C).

Administration route	Vehicle solution	Species			
		Mice		Rats	
		C (μg/ml)	Latency to seizures (min)	C (μg/ml)	Latency to seizures (min)
i.p.	DMSO	$8.5 \pm 1.5^{\#}$	$5.5 \pm 0.7^{*}$	$22.5 \pm 0.1^{\$}$	$18.5 \pm 0.7^{*}$
i.p.	Canola oil	2.7 ± 1.6	$27 \pm 1.41^{*}$	1.70 ± 0.7	ns
p.o.	DMSO	$16.1 \pm 11.9^{\&}$	$12 \pm 2.0^{*}$	$5.0 \pm 1.3^{@}$	ns
p.o.	Canola oil	1.77 ± 0.5	ns	1.07 ± 0.4	ns
s.c.	DMSO	<0.5	ns	<0.5	ns
s.c.	Canola oil	<0.5	ns	<0.5	ns

Data are reported as means ± S.E.D; $n = 3$. Values of $p < 0.05$ were considered statistically significant. "ns" animals which did not present seizure (1 h of observation).
$^{\#}$ $p < 0.05$ as compared to $(PhSe)_2$ dissolved in canola oil, administered by i.p. route in mice (plasma concentration).
$^{\&}$ $p < 0.05$ as compared to $(PhSe)_2$ dissolved in canola oil, administered by p.o. route in mice (plasma concentration).
$^{\$}$ $p < 0.05$ as compared to $(PhSe)_2$ dissolved in canola oil, administered by i.p. route in rats (plasma concentration).
$^{@}$ $p < 0.05$ as compared to $(PhSe)_2$ dissolved in canola oil, administered by p.o. route in rats (plasma concentration).
* $p < 0.05$ as compared to all groups (latency to seizures).

dosing, although they remained at detectable levels for up to 8 h after dosing (Fig. 1a and b).

The concentration–time profiles of $(PhSe)_2$ (p.o., dissolved in canola oil) in plasma did not notably differ among two species (Table 1). All animals that receiving $(PhSe)_2$ by oral route exhibited a concentration–time profile characterized by an early peak concentration followed by a continuous decay in plasma concentration.

As shown in Table 2, different routes of administration, vehicle solutions and animal species produced different $(PhSe)_2$ plasma concentrations. The appearance of seizures and the latency for the onset of seizure episodes were also dependent of routes of administration, vehicle solutions and animal species.

$(PhSe)_2$ administered by s.c. route did not induce seizures in rats and mice independent of vehicle solutions (canola oil or DMSO) or species (rats or mice). $(PhSe)_2$ was not detected in plasma of rats and mice 1 h after s.c. administration (Table 2).

When administered by p.o. route, $(PhSe)_2$ did not cause seizures in rats, independent of vehicle solutions (canola oil or DMSO). In mice, $(PhSe)_2$ dissolved in DMSO induced seizures, while $(PhSe)_2$ solution made up in canola oil did not cause seizures. Rats and mice that received $(PhSe)_2$ p.o. in DMSO solution presented $(PhSe)_2$ plasmatic levels higher than those which received this compound in canola oil solution (Table 2).

Intraperitoneal administration of $(PhSe)_2$ dissolved in DMSO solution caused seizures in rats, when administered in canola oil solution $(PhSe)_2$ did not induce seizures episodes. $(PhSe)_2$ administered by i.p. route induced seizures in mice independent of vehicle solutions (canola oil or DMSO). Rats and mice that received $(PhSe)_2$ i.p. in DMSO solution presented $(PhSe)_2$ plasmatic levels higher than those which received this compound in canola oil solution (Table 2).

4. Discussion

The purpose of the current study was to determine and quantify $(PhSe)_2$ levels in plasma after p.o. administration of this compound, in canola oil, in rats and mice. The results indicated that the C_{max} for $(PhSe)_2$ in plasma of rats and mice occurs at 0.5 h (30 min) after compound administration. The animals exhibited a concentration–time profile characterized by an early peak concentration followed by a continuous decay in plasma concentration, which remained at detectable levels for up to 8 h after dosing. The use of different administration routes (p.o., i.p. and s.c.) and vehicle solutions (canola oil or DMSO) in rats and mice indicated that the appearance of seizures and $(PhSe)_2$ plasmatic levels are dependent of administration routes (i.p. > p.o. > s.c.), vehicle solutions (DMSO > canola oil) and animal species (mice > rat).

In the present study, it was demonstrated that $(PhSe)_2$ when administered by oral route in mice and rats exhibited a concentration–time profile characterized by an early peak concentration (30 min). The C_{max} could be responsible by the pharmacological or toxicological effects as well as the short time-effect exhibited by $(PhSe)_2$ in different experimental models. In this way, Savegnago et al. (2007a,b,c) using a time–response curve demonstrated that the maximal pharmacological effect of $(PhSe)_2$ was at 30 min of pre-administration in models of inflammation, antinociception and depression-like. In accordance, systemic administration of $(PhSe)_2$, 30 min before training in an object recognition task induces a facilitation of formation of long-term object recognition memory in mice (Rosa et al., 2003). Thus, the results reported here show that the C_{max}, that is associated to the maximal compound effect, can act as a parameter that correlates to compound efficacy in animal models (Stein, 1996).

Additionally, $(PhSe)_2$ plasma levels administered at the dose of 500 mg/kg were detectable for up to 8 after dosing. Among the factors that influence the plasma levels are the route of administration (Lipinski et al., 2008) and the chemical characteristics of the compound (Parker, 1996). The fact that $(PhSe)_2$ is a highly lipophilic compound could be related to the short concentration–time found in this study. It is well reported that lipophilic compounds present a lower bioavailability, probably due to limitations on dissolution in the gastrointestinal fluids and on absorption, that is a saturated capacity of incorporation into bile micelles (micelles are formed during lipolysis, and facilitate absorption of lipophilic compounds) seen at high doses (Parker, 1996).

The p.o. route of the administration dominates contemporary drug therapy and will most likely continue to do so as it is considered to be safe, efficient and easily accessible with minimal discomfort to the patient compared to other routes of administration (Lennernãs, 2007). The current study demonstrates that $(PhSe)_2$ administered by oral route, dissolved in canola oil, produces a C_{max} of 13.13 and 10.11 μg/ml for rats and mice, respectively. Also, the compound administration did not cause seizures in animals until a period of 24 h after compound administration. Comparison of mice with rats provide consistent evidence that the plasmatic levels of $(PhSe)_2$ are similar when this compound was administered by p.o. route.

In the second set of experiments the present study demonstrates that different routes of administration, vehicle solutions and animal species produced different $(PhSe)_2$ plasma concentrations. The appearance of seizures and the latency for the onset of seizure

episodes were also dependent of routes of administration, vehicle solutions and animal species.

In mice, the administration of $(PhSe)_2$ dissolved in canola oil by i.p. route caused seizure episodes, conversely when administered by p.o. or s.c. route $(PhSe)_2$ did not cause seizure episodes. The data are consistent with previous studies showing that the administration of $(PhSe)_2$ dissolved in canola oil by p.o. or s.c. route is more safe when compared to the i.p. route in mice (Nogueira et al., 2003a; Savegnago et al., 2007b). Accordingly, LD_{50} values were 62.4 and >312 mg/kg for mice that received $(PhSe)_2$ by i.p. and by p.o./s.c. routes, respectively (Nogueira et al., 2003a; Savegnago et al., 2007b). These data are supported by the Lipinski's study which demonstrates that different administration routes produce distinctive pharmacokinetic profiles with important experimental implications (Lipinski et al., 2008).

$(PhSe)_2$ has been investigated in different experimental protocols in mice and rats (Nogueira et al., 2002, 2003; Ghisleni et al., 2008; Ineu et al., 2008; Savegnago et al., 2008). These enveloped wide range of dosing regimens, routes of administration (including i.p., s.c. and p.o.) and different vehicle solutions (canola oil, DMSO, Tween 80 and ethanol) (Brito et al., 2006; Ghisleni et al., 2008; Ineu et al., 2008; Nogueira et al., 2002; Nogueira et al., 2003a; Savegnago et al., 2008). In this way, studies using $(PhSe)_2$ in a similar dose, but prepared in different vehicle solutions, exhibited different results (Nogueira et al., 2003a; Ghisleni et al., 2008). The present study shows an important difference in the occurrence of seizures and in the plasmatic levels of $(PhSe)_2$ when different vehicle solutions (DMSO or canola oil) were used.

$(PhSe)_2$ plasmatic levels were higher in mice treated with $(PhSe)_2$ by i.p. or p.o. route, dissolved in DMSO than those that received this compound dissolved in canola oil. The latency for the onset to the seizure episodes in mice was dependent of vehicle solution and administration route. In fact, $(PhSe)_2$ administered by p.o. route induced seizures only when the solution was made up in DMSO. Moreover, i.p. administration of $(PhSe)_2$ dissolved in DMSO reduced the latency for the onset of seizures in mice when compared to $(PhSe)_2$ dissolved in canola oil.

Experiments using rats demonstrated that only $(PhSe)_2$ dissolved in DMSO, administered by i.p. route, induced seizure episodes. This study is the first evidence indicating that $(PhSe)_2$ causes seizures in adult rats. It is important to point out that in previous studies $(PhSe)_2$ did not display any overt sign of neurotoxicity when administered by i.p., s.c., p.o. and i.c.v. routes in adult rats (Nogueira et al., 2003a; Savegnago et al., 2007b). These results could be attributed to a faster rate of absorption proportioned by the vehicle solution, since DMSO is rapidly and extensively distributed through tissues (Kaye et al., 1983) that is responsible to the larger magnitude of toxic effect. In fact, Jacob et al. (1971) demonstrated that DMSO has higher ability to pass through membranes and it can carry other drugs with it across membranes. Therefore, the variation in the occurrence of seizure episodes could be attributed to the use of different vehicle solutions that can be responsible to the distinctive rate of absorption and metabolism of the compound in the liver.

Moreover, mice are more likely to seizures episode than rats independent of the administration routes or the vehicle solutions. The fate of this highly hydrophobic compound in rodents is unknown but probably $(PhSe)_2$ can be either hydroxylated by liver cytochrome P450 enzymes or its selenide atoms can be oxidized by monooxygenases (Nogueira et al., 2004). If these transformations are faster in mice than in rats, the excretion and clearance of $(PhSe)_2$ might be higher in mice than in rats. This perhaps could at least, in part, explain the differences in toxicity. However, further detailed studies that prove the existence of an active metabolite of $(PhSe)_2$ will be necessary to clarify this matter.

In conclusion, the results provide consistent evidence that plasma levels of $(PhSe)_2$ dissolved in canola oil and administrated by p.o. route are similar in rats and mice. C_{max} for $(PhSe)_2$ occurred at 0.5 h (30 min) after compound administration in rats and mice. The use of different administration routes (p.o., i.p. and s.c.) and vehicle solutions (canola oil or DMSO) in rats and mice indicated that the appearance of seizures and $(PhSe)_2$ plasmatic levels are dependent of administration routes (i.p. > p.o. > s.c.), vehicle solutions (DMSO > canola oil) and animal species (mice > rat).

Conflict of interest

We have no competing interests.

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References

Barbosa, N.B.V., Rocha, J.B.T., Wondracek, D.C., Perottoni, J., Zeni, G., Nogueira, C.W., 2006. Diphenyl diselenide reduces temporarily hyperglycemia: possible relationship with oxidative stress. Chem. Biol. Interact. 163, 230–238.

Barbosa, N.B.V., Rocha, J.B.T., Zeni, G., Emanuelli, T., Beque, M.C., Braga, A.L., 1998. Effect of organic forms of selenium on delta-aminolevulinate dehydratase from liver, kidney, and brain of adult rats. Toxicol. Appl. Pharm. 149, 243–253.

Borges, V.C., Rocha, J.B.T., Nogueira, C.W., 2005. Effect of diphenyl diselenide, diphenyl ditelluride and ebselen on cerebral Na^+, K^+-ATPase activity in rats. Toxicology 215, 191–197.

Brito, V.B., Folmer, V., Puntel, G.O., Fachinetto, R., Soares, J.C.M., Zeni, G., Nogueira, C.W., Rocha, J.B.T., 2006. Diphenyl diselenide and 2,3-dimercaptopropanol increase the PTZ-induced chemical seizure and mortality in mice. Brain Res. Bull. 68, 414–418.

Ghisleine, G., Porciúncula, L.O., Cimarosti, H., Rocha, J.B.T., Salbego, C.G., Souza, D.O., 2003. Diphenyl diselenide protects rat hippocampal slices submitted to oxygen–glucose deprivation and diminishes inducible nitric oxide synthase immunocontent. Brain Res. 986, 196–199.

Ghisleni, G., Kazauckas, V., Both, F.L., Pagnussat, N., Mioranzza, S., Rocha, J.B.T., Souza, D.O., 2008. Diphenyl diselenide exerts anxiolytic-like effect in Wistar rats: putative roles of GABAA and 5HT receptors. Prog. Neuro-Psychopharmacol. 32, 1508–1515.

Ineu, R.P., Pereira, M.E., Aschner, M., Nogueira, C.W., Zeni, G., Rocha, J.B.T., 2008. Diphenyl diselenide reverses gastric lesions in rats: involvement of oxidative stress. Food Chem. Toxicol. 46, 3023–3029.

Jacob, S.W., Rosenbaum, E.E., Wood, D.C., 1971. Dimethyl Sulfoxide (Basic Concepts). Marcel Dekker, Inc., New York.

Kaye, T., Egorin, M.J., Riggs Jr., C.E., Olman, E.A., Chou, F.E., Salcman, M., 1983. The plasma pharmacokinetics and tissue distribution of dimethyl sulfoxide in mice. Life Sci. 43, 1223–1230.

Kobayashi, Y., Ogra, Y., Ishiwata, K., Takayama, H., Aimi, N., Suzuki, K.T., 2002. Selenosugars are key and urinary metabolites for selenium excretion within the required to low-toxic range. Proc. Natl. Acad. U.S.A. 99, 15932–15936.

Lennernäs, H., 2007. Animal data: the contributions of the using chamber and perfusion systems to predicting human oral drug delivery *in vivo*. Adv. Drug Deliv. Rev. 59, 1103–1120.

Lipinski, R.J., Hutson, P.R., Hannam, P.W., Nydza, R.J., Washington, I.M., Moore, R.W., Girdaukas, G.G., 2008. Dose- and route-dependent teratogenicity, toxicity, and pharmacokinetic profiles of the hedgehog signaling antagonist cyclopamine in the mouse. Toxicol. Sci. 104, 189–197.

Luchese, C., Brandao, R., Oliveira de, R., Nogueira, C.W., Santos, F.W., 2007. Efficacy of diphenyl diselenide against cerebral and pulmonary damage induced by cadmium in mice. Toxicol. Lett. 173, 181–190.

Machado, M.S., Rosa, R.M., Dantas, A.S., Reolon, G.K., Appelt, H.R., Braga, A.L., Henriques, J.A.N., Roesler, R., 2006. An organic selenium compound attenuates apomorphine-induced stereotypy in mice. Neurosci. Lett. 410, 198–202.

Maciel, E.N., Flores, E.M.M., Rocha, J.B.T., Folmer, V., 2003. Comparative deposition of diphenyl diselenide in liver, kidney, and brain of mice B. Environ. Contam. Toxicol. 70, 470–476.

Maciel, E.N., Bolzan, R.C., Braga, A.L., Rocha, J.B.T., 2000. Diphenyl diselenide and diphenyl ditelluride differentially affect delta-aminolevulinate dehydratase from liver, kidney, and brain of mice. J. Biochem. Mol. Toxicol. 14, 310–319.

Mc Connell, K.P., Cho, G.J., 1965. Transmucosal movement of selenium. Am. J. Phys. 208, 1191.

Meotti, F.C., Borges, V.C., Zeni, G., Rocha, J.B.T., Nogueira, C.W., 2003. Potential renal and hepatic toxicity of diphenyl diselenide, diphenyl ditelluride and Ebselen for rats and mice. Toxicol. Lett. 143, 9–16.

Meotti, F.C., Stangherlin, E.C., Zeni, G., Rocha, J.B.T., Nogueira, C.W., 2004. Protective role of aryl and alkyl diselenides on lipid peroxidation. Environ. Res. 94, 276–282.
Mugesh, G., Du Mont, W.W., Sies, H., 2001. Chemistry of biologically important synthetic organoselenium compounds. Chem. Rev. 101, 2125–2179.
Nogueira, C.W., Rotta, L.N., Zeni, G., Souza, D.O., Rocha, J.B.T., 2002. Exposure to Ebselen changes glutamate uptake and release by rat brain synaptosomes. Neurochem. Res. 27, 283–288.
Nogueira, C.W., Meotti, F.C., Curte, E., Pilissao, C., Zeni, G., Rocha, J.B.T., 2003a. Investigations into the potential neurotoxicity induced by diselenides in mice and rats. Toxicology 183, 29–37.
Nogueira, C.W., Borges, V.C., Zeni, G., Rocha, J.B.T., 2003b. Organochalcogens effects on delta-aminolevulinate dehydratase activity from human erythrocytic cells in vitro. Toxicology 191, 169–178.
Nogueira, C.W., Zeni, G., Rocha, J.B.T., 2004. Organoselenium and organotellurium compounds: toxicology and pharmacology. Chem. Rev. 104, 6255–6285.
Parker, R.S., 1996. Absortion, metabolism and transport of carotenoids. FASEB J. 10, 542–551.
Paulmier, C., 1986. Selenoorganic functional groups. In: Paulmier, C. (Ed.), Selenium Reagents and Intermediates in Organic Synthesis, 1st ed. Pergamon Press, Oxford, England, pp. 25–51.
Prigol, M., Wilhelm, E.A., Schneider, C.C., Rocha, J.B.T., Nogueira, C.W., Zeni, G., 2007. Involvement of oxidative stress in seizures induced by diphenyl diselenide in rat pups. Brain Res. 1147, 226–232.
Prigol, M., Wilhelm, E.A., Schneider, C.C., Nogueira, C.W., 2008a. Protective effect of unsymmetrical dichalcogenide, a novel antioxidant agent, *in vitro* and an *in vivo* model of brain oxidative damage. Chem. Biol. Interact. 176, 129–136.
Prigol, M., Wilhelm, E.A., Stangherlin, E.C., Barancelli, D.A., Nogueira, C.W., Zeni, G., 2008b. Diphenyl diselenide-induced seizures in rat pups: possible interaction with glutamatergic system. Neurochem. Res. 33, 996–1004.
Rayman, M.P., 2000. The importance of selenium to human health. Lancet 356, 233–241.
Rocha, J.B.T., Gabriel, D., Zeni, G., Posser, T., Siqueira, L., Nogueira, C.W., Folmer, V., 2005. Ebselen and diphenyl diselenide change biochemical hepatic responses to overdosage with paracetamol. Environ. Toxicol. 19, 255–261.
Rosa, R.M., Roesler, R., Braga, A.L., Saffi, J., Henriques, J.A., 2007. Pharmacology and toxicology of diphenyl diselenide in several biological models. Braz. Med. Biol. Res. 40, 1287–1304.
Rosa, R.M., Flores, D.G., Appelt, H.R., Braga, A.L., Henriques, J.A.P., Roesler, R., 2003. Facilitation of long-term object recognition memory by pretraining administration of diphenyl diselenide in mice. Neurosci. Lett. 341, 217–220.
Savegnago, L., Jesse, C.R., Pinto, L.G., Rocha, J.B.T., Nogueira, C.W., 2007a. Diphenyl diselenide attenuates acute thermal hyperalgesia and persistent inflammatory and neuropathic pain behavior in mice. Brain Res. 1175, 54–59.
Savegnago, L., Pinto, L.G., Jesse, C.R., Alves, D., Rocha, J.B.T., Nogueira, C.W., Zeni, G., 2007b. Antinociceptive properties of diphenyl diselenide: evidences for the mechanism of action. Eur. J. Pharmacol. 555, 129–138.
Savegnago, L., Pinto, L.G., Jesse, C.R., Rocha, J.B.T., Nogueira, C.W., Zeni, G., 2007c. Monoaminergic agents modulate antidepressant-like effect caused by diphenyl diselenide in rats. Prog. Neuro-Psychopharmacol. 31, 1261–1269.
Savegnago, L., Pinto, L.G., Jesse, C.R., Barancelli, D.A., Rocha, J.B.T., Nogueira, C.W., Zeni, G., 2008. Diphenyl diselenide exerts antidepressant-like and anxiolytic-like effects in mice: involvement of L-arginine-nitric oxide-soluble guanylate cyclase pathway in its antidepressant-like action. Pharmacol. Biochem. Behav. 88, 418–426.
Schomburg, L., Schweizer, U., Kohrle, J., 2004. Selenium and selenoproteins in mammals: extraordinary, essential, enigmatic. Cell Mol. Life Sci. 61, 1988–1995.
Stein, G.E., 1996. Pharmacokinetics and pharmacodynamics of newer fluoroquinolones. Clin. Infect. Dis. 23, S19–S24.
Suzuki, K.T., 2005. Metabolomics of arsenic based on speciation studies. Anal. Chim. Acta 540, 71–76.
Suzuki, K.T., Kurasaki, K., Okazaki, N., Ogra, Y., 2005. Selenosugar and trimethylselenonium among urinary Se metabolites: dose- and age-related changes. Toxicol. Appl. Pharm. 206, 1–8.
Ursini, F., Bindoli, A., 1987. The role of selenium peroxidases in the protection against oxidative damage of membranes. Chem. Phys. Lipids 44, 255–276.

4.5 Ação convulsivante do disseleneto de difenila em ratos bebês: Dosagem e correlação com os níveis do composto no plasma, fígado e cérebro

4.5.1 Artigo 5

CONVULSANT ACTION OF DIPHENYL DISELENIDE IN RAT PUPS: MEASUREMENT AND CORRELATION WITH PLASMA, LIVER AND BRAIN LEVELS OF COMPOUND

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TOXICOKINETICS AND METABOLISM

Convulsant action of diphenyl diselenide in rat pups: measurement and correlation with plasma, liver and brain levels of compound

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Abstract Diphenyl diselenide [$(PhSe)_2$], an organoselenium compound, presents toxicological effects in rat pups, manifested by the appearance of seizure episodes. The aim of this study was to carry out the determination and quantification of $(PhSe)_2$ in plasma, liver and brain of rat pups after oral administration (p.o) of this compound (500 mg/kg). The second objective of this study was to correlate the latency to the appearance for the first seizure episode with $(PhSe)_2$ plasma, liver and brain levels. Analysis of $(PhSe)_2$ in plasma, liver and brain samples was performed by gas chromatography/flame ionized detector system (GC/FID). The average levels of $(PhSe)_2$ in plasma, liver and brain of rat pups were 3.67, 5.07 and 1.15 μg/ml, respectively, at 20.58 min post dosing, the latency media for the first seizure episode. $(PhSe)_2$ levels in plasma did not correlate with the latency for the first seizure episode induced by this compound. A significant negative correlation between the latency for the first seizure episode and the levels of $(PhSe)_2$ liver and brain of rat pups was found. It demonstrates that rat pups which had highest levels of $(PhSe)_2$ in liver and brain showed the shortest latency for the first seizure episode.

Keywords Selenium · Diphenyl diselenide · Rat pups · Seizure · Liver · Brain

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Introduction

Diphenyl diselenide $(PhSe)_2$ is a highly lipophylic compound and therefore exhibits a concentration–time profile characterized by an early peak concentration and rapid distribution from blood to the central nervous system, where it exerts its pharmacological and toxicological effects (Maciel et al. 2000; Prigol et al. 2009a). $(PhSe)_2$ has been proven effective against neurotoxicity (Ghisleine et al. 2003), hyperglycemia (Barbosa et al. 2006), lipid peroxidation (Meotti et al. 2004; Luchese et al. 2007; Prigol et al. 2009b), inflammation (Savegnago et al. 2007a), nociception (Savegnago et al. 2007b), anxiety (Savegnago et al. 2007c) and induces a facilitation of formation of long-term object recognition memory (Rosa et al. 2003) and antidepressant-like properties (Ghisleni et al. 2008; Savegnago et al. 2008) in models using rats and mice.

On the other hand, $(PhSe)_2$ has been reported to cause seizure episodes in rat pups with an increase in oxidative stress and inhibition of sulfhydryl enzymes (Prigol et al. 2007, 2008). Additionally, $(PhSe)_2$ induces over stimulation in the glutamate system, by acting on N-methyl-D-aspartate (NMDA) receptors, inhibits [^{3}H]glutamate uptake (Nogueira et al. 2003; Prigol et al. 2008) and reduces the GABAergic transmission by stimulating [^{3}H]GABA uptake in cortex and hippocampal slices from seizing rat pups with decrease in GABA in synaptic cleft systems (Prigol et al. 2009c).

Developmental neuropharmacology has to take into account not only changes in pharmacodynamics but also in pharmacokinetics. Absorption, distribution as well as metabolism and elimination may change during ontogeny (Morselli 1983). Penetration of drugs into the brain is of extraordinary importance. The blood–brain barrier which controls the entry of drugs into the brain is far from being

perfect in the immature central nervous system and it develops during postnatal life in experimental animals (Mareš et al. 2000) as well as in man (Saunders 1986). Some convulsant drugs which do not cross the mature blood–brain barrier might exhibit an action after systemic administration in immature animals (Mareš et al. 2000).

In view of the fact that seizures induced by $(PhSe)_2$ are dependent on animal age (Prigol et al. 2007), the aim of this study was to carry out the determination and quantification of $(PhSe)_2$ in plasma, liver and brain of rat pups after oral administration of this compound. The second objective of this study was to correlate the time to the appearance for the first seizure episode with the levels of $(PhSe)_2$ in plasma, liver and brain.

Materials and methods

Chemicals

$(PhSe)_2$ was prepared in our laboratory according to the literature method (Paulmier 1986). Analysis of the ^1H NMR and ^{13}C NMR spectra showed that the $(PhSe)_2$ obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of compound (99.9%) was determined by GC/HPLC, and it was stable under storage conditions at room temperature, humidity and light for months.

Animals

Pup Wistar rats postnatal day (PND) 12–14 of both sexes were obtained from a local breeding colony. PND 12–14 was chosen based on Mikulecká et al. (2004). The animals were kept in a separate animal room, on a 12 h light/12 dark cycle, in an air-conditioned room (22 ± 2°C). Commercial diet (GUABI, RS, Brazil) and tap water were supplied ad libitum. The dams were allowed to deliver and wean their pups until PND 12–14. Commercial diet (GUABI, RS, Brazil) and tap water were supplied ad libitum. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil.

Preparation of $(PhSe)_2$ solution

The solution was weekly prepared, and the compound was dissolved in canola oil. The concentration of $(PhSe)_2$ was analyzed prior to use. The solutions were stored at 2–8°C and allowed to warm up to room temperature before use. The rat pups were given a single oral administration (p.o) dosage of 500 mg/kg $(PhSe)_2$. The dose of $(PhSe)_2$ used in this study was based on previous reported study dealing with dose–response for the appearance of seizures episode (Prigol et al. 2007). $(PhSe)_2$ solutions were administered to animals in the morning between 8:00 a.m. and 9:30 a.m. at a dosing volume of 10 ml/kg.

Experimental design

The endpoint seizure was chosen to correlate the involvement of $(PhSe)_2$ levels in plasma, liver and brain with seizure episodes in rat pups. To this end, $(PhSe)_2$ at the dose of 500 mg/kg dissolved in canola oil was administered by p.o route to rat pups.

The appearance of seizures was quantified as previously described by Prigol et al. (2007). In short, animals were observed for 1 h in Plexiglas chambers for the appearance of tonic-clonic seizures lasting more than 5 s. The latency for the onset of the first tonic-clonic seizures episode was also recorded. Animals which did not display seizures (1 h) were considered protected.

Twelve animals per group were anesthetized, and blood samples (1 ml) were collected from each animal, by heart puncture, subsequent to the seizure episodes. Blood samples were centrifuged at 3,000 rpm for 5 min. The plasma was separated and stored chilled until assay.

The liver and whole brain of animals were also removed and stored chilled until assay.

Determination and quantification of $(PhSe)_2$ in plasma, liver and brain samples

Determinations of $(PhSe)_2$ concentrations in plasma, liver and brain were made in all samples, collected at the time of euthanasia. The plasma (200 µl), liver (200 mg) or brain (200 mg) was homogenized with ethyl acetate in the ratio of 1:1 (v/v or w/v) to each tube. The extraction was performed by vortex-mixing the tubes for 3 min. After, the samples were centrifuged at 3,000 rpm for 5 min. The supernatants were separated and filtered through a membrane (0.45 µm pore size) Millipore®. The filtrate was transferred to a clean test tube, and a 1 µl aliquot was injected into chromatographic system for analysis. Qualitative and quantitative analysis of $(PhSe)_2$ in plasma samples were conducted by gas chromatography (CG 2010 Shimadzu®) with a flame ionized detector system (FID), according to the methodology described by Prigol et al. (2009a).

The $(PhSe)_2$ concentration in plasma, liver and brain were directly obtained from chromatographic data.

Statistical comparisons

Analysis of variance was performed using one-way ANOVA; latency to seizure and the levels of $(PhSe)_2$ in plasma, liver and brain, followed by the Duncan's multiple

Table 1 The average latency to the appearance of seizure episode and plasma, liver and brain average distribution of $(PhSe)_2$ administered by oral route (dissolved in canola oil) in rat pups

	Latency[a]	Concentration of $(PhSe)_2$[b]		
		Plasma	Liver	Brain
Rat pups	20.58 ± 1.05	3.67 ± 1.06	5.07 ± 0.90	1.15 ± 0.07

Data are reported as means ± S.E.M; $n = 12$

[a] Time (min) to the appearance for the first seizure episode

[b] Concentration (μg/ml) of $(PhSe)_2$ in different tissues

range. Data are expressed as means ± SEM. Values of $P < 0.05$ were considered statistically significant. Pearson's correlation coefficient was used for the estimation of correlation between parameters analyzed.

Results

$(PhSe)_2$-induced seizures in rat pups and its plasma, liver and brain levels

$(PhSe)_2$ induced seizures in 100% of rat pups (As shown in). As showed in Table 1, the average latency for the first seizure episode is 20.58 min. The average levels of $(PhSe)_2$ in plasma, liver and brain of rat pups were 3.67, 5.07 and 1.15 μg/ml, respectively.

Correlation analysis of the latency for the first seizure episode with $(PhSe)_2$ levels

Correlation analysis (Pearson's correlation analysis) revealed that the latency for the first seizure episode induced by $(PhSe)_2$ did not correlate with plasma levels of $(PhSe)_2$ ($r = -0.418$; $P < 0.303$; Fig. 1). Conversely, Pearson's correlation analysis revealed a significant negative correlation between the latency for the first seizure episode induced by $(PhSe)_2$ with the levels of compound in liver ($r = -0.8828$, $P < 0.0001$; Fig. 2) and brain ($r = -0.782$; $P < 0.005$; Fig. 3) of rat pups.

Discussion

The purpose of the current study was to determine and to quantify $(PhSe)_2$ levels in plasma, liver and brain of rat pups and to correlate these results with the latency to the appearance for the first seizure episode induced by $(PhSe)_2$. The results indicated that the average latency for the first seizure episode was 20.58 min and the average concentration of $(PhSe)_2$ in plasma of rat pups was 3.67 μg/ml. $(PhSe)_2$ levels in liver and brain of rat pups were 5.07 and 1.15 μg/ml, respectively, at the time of 20.58 min. Rat pups exhibited a significative negative correlation between the latency for the first seizure episode and the levels of $(PhSe)_2$ in liver and brain.

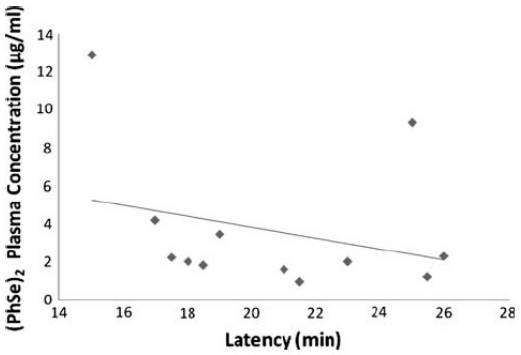

Fig. 1 Correlation between $(PhSe)_2$ plasma concentration (μg/ml) and seizure time (min) in rat pups. Data are individual values for $n = 12$ in each group

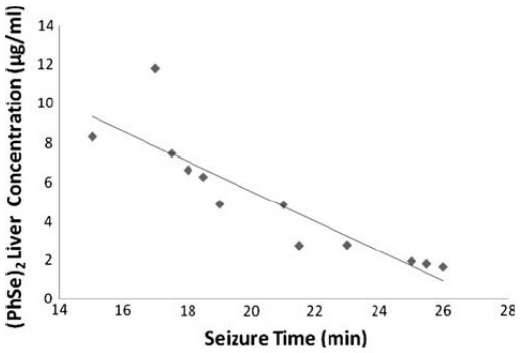

Fig. 2 Correlation between $(PhSe)_2$ liver concentration (μg/ml) and seizure time (min) in rat pups. Data are individual values for $n = 12$ in each group

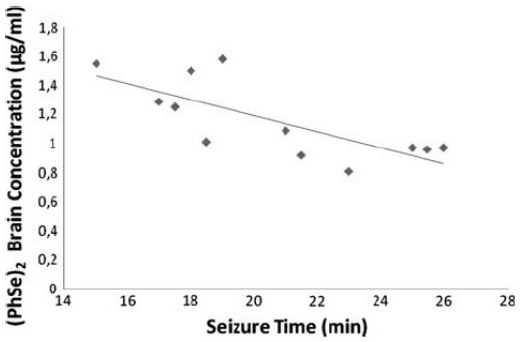

Fig. 3 Correlation between $(PhSe)_2$ brain concentration (μg/ml) and seizure time (min) in rat pups. Data are individual values for $n = 12$ in each group

The $(PhSe)_2$ plasma concentration could be responsible for toxicological effects exhibited by $(PhSe)_2$ in this experimental model. In this way, our research group, using a curve of plasma concentration–time, demonstrated that the maximal concentration of $(PhSe)_2$ 500 mg/kg administrated by oral route in adult rats was 13.13 μg/ml at 30 min after compound administration (Prigol et al. 2009a). The high drug concentration in plasma, demonstrated in our previous study, can be explained by the lipophilic character of $(PhSe)_2$. In the present investigation, we have found that the $(PhSe)_2$ plasma concentration in the pups is about 4.3-fold lower compared to that seen in adult rats (Prigol et al. 2009a). The low concentration found in plasma of pups most likely is due to the fact that rats pup, as young children, have higher water body content compared to adults (Friis-Hansen 1983).

Different to what is observed here, $(PhSe)_2$ administration did not cause seizures in adult rats until a period of 24 h after compound administration by p.o route, although the plasma concentration is 4.3 times higher than the $(PhSe)_2$ concentration detected in rat pups. These data supported the hypothesis that rat pups are more sensitive to the toxic effect produced by $(PhSe)_2$ when compared to adult rats (Prigol et al. 2009a). In fact, several physiological changes with potential impact on drug pharmacokinetics have been observed in children depending on their age (Friis-Hansen 1983). Infants have proportions of body water and adipose tissue different from those in adults, with subsequent effects on the relative distribution volume of lipophilic compounds (Kearns et al. 2003). Albumin and a1-acid glycoprotein are subject to developmental changes in children. In general, drug binding is decreased in children, especially in the newborn, because of quantitative and qualitative changes in albumin (Kearns and Reed 1989). This fact helps to explain the lack of correlation of the latency for the first seizure episode induced by $(PhSe)_2$ with plasma levels of $(PhSe)_2$, since upon entry into blood circulation, most drugs bind to plasma proteins. It is generally accepted that only the free drug molecules can arrive at the site of action that may be enzymes or receptors.

Additionally, there was a significant negative correlation between the latency for the seizure episode and the levels of $(PhSe)_2$ in liver of rat pups. Animals that had the highest levels of compound in the liver showed the shortest latency for the first seizure episode. $(PhSe)_2$ is a highly lipophilic compound, thus it is likely that its metabolism and elimination depends on biotransformation in the liver. The assumption that the entire convulsant effects of $(PhSe)_2$ are due to the parent compound is supported by previous published data. Nogueira et al. (2003) demonstrated that $(PhSe)_2$ when administrated by i.p. route induced seizure and death in mice, conversely, a direct injection of $(PhSe)_2$, at high concentration (1,000 μmol), into the brain of rodents did not cause any overt sign of neurotoxicity, indicating that this compound must be first metabolized to cause seizures. Hepatic biotransformation of drugs is a multifactorial integration of enzymatic actions that alter the initial compound often by adding or exposing a functional group to enable subsequent conjugation and elimination. The appearance and functionality of key enzyme groups, such as cytochrome P450 oxidases, hydrolases, glucuronosyltransferases, sulfotransferases, N-acetyltransferases and glutathione transferases are influenced by gestational age, birth, postnatal age (Blake et al. 2005). We suppose that these developmental differences produce a decrease in drug clearance and an increase in toxicological effect of $(PhSe)_2$, manifested here by seizure episodes in rat pups.

Another finding of this study was the presence of $(PhSe)_2$ in the brain of rat pups. Based on the amount of $(PhSe)_2$ detected in the brain, we can affirm that ~22% of the compound present in plasma reached to the brain and it possibly caused seizure episodes. Accordingly, Sallam et al. (2008) demonstrated that roscovitine, a lipophilic compound analogue of purine, was more toxic and highly distributed over the blood–brain barrier in the 14 days old rat pups and the brain exposure was 30% of that found in plasma, a higher percentage when compared to that of found in the brain of adult rats exposed to the drug. In this view, our previous study reported that adult rats which received $(PhSe)_2$ 500 mg/kg by oral route presented higher plasma concentration when compared to rat pups, but they did not presented seizure episodes. One possibility to explain this difference is that smaller amount of $(PhSe)_2$ crosses the blood–brain barrier and arrives in the brain of adult rats. These hypotheses are supported by Butt et al. (1990) who have shown that blood–brain barrier of the rat fully matures 3–4 weeks postnatal, and that differences observed in brain exposure could be explained by an age-dependent variation in the maturity and function of blood–brain barrier. Moreover, endothelial cells constituting the blood–brain barrier have the character of a lipid membrane, as a result of which lipid soluble non-ionized compounds penetrate easily in the brain (Eyal et al. 2009).

In addition, we revealed a relationship between the latency to the onset of seizure episodes and the levels of $(PhSe)_2$ in brain of rat pups. In fact, a significant negative correlation between these variables was found. These data may explain, at least in part, the neurotoxic effects reported in rat pups treated with $(PhSe)_2$, since the animals that have the highest amount of compound in the brain showed the shortest latency for the first seizure episode. In this view, a study performed by us demonstrated that the appearance of seizures and the latency for the first seizure episode induced by $(PhSe)_2$ in rat pups is dependent on the dose administered (50, 150 or 500 mg/kg; Prigol et al. 2007), indicating that the intensity of the neurotoxic effects is dependent on

the quantity of $(PhSe)_2$ in the brain. Maciel et al. (2003) have reported that acute treatment with $(PhSe)_2$ causes a significant increase in selenium concentration in brain, supporting the hypothesis that the brain is a potential target for the toxicity of highly lipophilic organoselenium compounds.

In brain of rat pups, we previously demonstrated that $(PhSe)_2$ promotes various events that can lead to seizure episodes, among them are the increase in lipid peroxidation, a reduction in δ-ALA-D and Na+, K+ ATPase activities (Prigol et al. 2007) and the interaction with glutamatergic (Prigol et al. 2008) and GABAergic systems (Prigol et al. 2009c). The inhibition in [^{3}H]glutamate uptake and the increase in glutamate in synaptic cleft are mechanisms which contribute to hyperexcitability in rat pups treated with $(PhSe)_2$ (Prigol et al. 2008). $(PhSe)_2$ stimulates [^{3}H]GABA uptake in cortex and hippocampal slices from rat pups, which decreases GABA levels in synaptic cleft. This imbalance between the excitatory and inhibitory neurotransmission is critical to trigger seizure episodes.

In conclusion, our results demonstrated that the $(PhSe)_2$ levels in liver and brain of rat pups showed as significant negative correlation with the latency for the first seizure episode, demonstrating that rat pups which had highest levels of $(PhSe)_2$ in liver and brain showed the shortest latency for the first seizure episode. Differing, the latency for the first seizure episode induced by $(PhSe)_2$ did not correlate with compound plasma levels.

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References

Barbosa NBV, Rocha JBT, Wondracek DC, Perottoni J, Zeni G, Nogueira CW (2006) Diphenyl diselenide reduces temporarily hyperglycemia: possible relationship with oxidative stress. Chem Biol Interact 163:230–238

Blake MJ, Castro L, Leeder JS, Kearns GL (2005) Ontogeny of drugs metabolizing enzymes in the neonate. Sem Fetal Neonatal Med 10:123–138

Butt AM, Jones HC, Abbott NJ (1990) Electrical resistance across the blood–brain barrier in anaesthetized rats: a developmental study. J Physiol 429:47

Eyal S, Hsiao P, Unadkat JD (2009) Drug interactions at the blood–brain barrier: fact or fantasy? Pharmacol Therapeut 123(1):80–104

Friis-Hansen B (1983) Water distribution in the foetus and newborn infant. Acta Paediatr Scand Suppl 305:7–11

Ghisleine G, Porciúncula LO, Cimarosti H, Rocha JBT, Salbego CG, Souza DO (2003) Diphenyl diselenide protects rat hippocampal slices submitted to oxygen–glucose deprivation and diminishes inducible nitric oxide synthase immunocontent. Brain Res 986:196–199

Ghisleni G, Kazauckas V, Both FL, Pagnussat N, Mioranzza S, Rocha JBT, Souza DO (2008) Diphenyl diselenide exerts anxiolytic-like effect in Wistar rats: putative roles of GABAA and 5HT receptors. Prog Neuro-Psychoph 32:1508–1515

Kearns GL, Reed MD (1989) Clinical pharmacokinetics in infants and children. Clin Pharmacokinet 17(1):29–67

Kearns GL, Abdel-Rahman SM, Alander SW, Blowey DL, Leeder JS, Kauffman RE (2003) Developmental pharmacology—drug disposition, action, and therapy in infants and children. N Engl J Med 349:1157–1167

Luchese C, Brandao R, de Oliveira R, Nogueira CW, Santos FW (2007) Efficacy of diphenyl diselenide against cerebral and pulmonary damage induced by cadmium in mice. Toxicol Lett 173(3):181–190

Maciel EN, Bolzan RC, Braga AL, Rocha JBT (2000) Diphenyl diselenide and diphenyl ditelluride differentially affect delta-aminolevulinate dehydratase from liver, kidney, and brain of mice. J Biochem Mol Toxic 14:310–319

Maciel EN, Flores EMM, Rocha JBT, Folmer V (2003) Comparative deposition of diphenyl diselenide in liver, kidney, and brain of mice. B Environ Contam Tox 70:470–476

Mareš P, Chino M, Kubová H, Mathern P, Veliký M (2000) Convulsant action of systemically administered glutamate and bicuculline methiodide in immature rats. Epilepsy Res 42:183–189

Meotti FC, Stangherlin EC, Zeni G, Rocha JBT, Nogueira CW (2004) Protective role of aryl and alkyl diselenides on lipid peroxidation. Environ Res 94:276–282

Mikulecká A, Kubová H, Mares P (2004) Lamotrigine does not impair motor performance and spontaneous behavior in developing rats. Epilep Behav 5:464–471

Morselli PL (1983) Development of physiological variables important for drug kinetics. In: Morselli PL, Pippenger CE, Penry JK (eds) Antiepileptic drug therapy in pediatrics. Raven Press, New York, pp 1–12

Nogueira CW, Meotti FC, Curte E, Pilissao C, Zeni G, Rocha JBT (2003) Investigations into the potential neurotoxicity induced by diselenides in mice and rats. Toxicology 183:29–37

Paulmier C (1986) Selenoorganic functional groups. In: Paulmier C (ed) Selenium reagents and intermediates in organic synthesis, 1st edn. Pergamon Press, Oxford, pp 25–51

Prigol M, Wilhelm EA, Schneider CC, Rocha JBT, Nogueira CW, Zeni G (2007) Involvement of oxidative stress in seizures induced by diphenyl diselenide in rat pups. Brain Res 1147:226–232

Prigol M, Wilhelm EA, Stangherlin EC, Barancelli DA, Nogueira CW, Zeni G (2008) Diphenyl diselenide-induced seizures in rat pups: Possible interaction with glutamatergic system. Neurochem Res 33:996–1004

Prigol M, Schumacher RF, Nogueira CW, Zeni G (2009a) Convulsant effect of diphenyl diselenide in rats and mice and its relationship to plasma levels. Toxicol Lett 189(1):35–39

Prigol M, Luchese C, Nogueira CW (2009b) Antioxidant effect of diphenyl diselenide on oxidative stress caused by acute physical exercise in skeletal muscle and lungs of mice. Cell Biochem Funct 27(4):216–222

Prigol M, Wilhelm EA, Nogueira CW, Zeni G (2009c) Diphenyl diselenide-induced seizures in rat pups: possible interaction with GABAergic system. Neurol Res xx, xx-xx

Rosa RM, Flores DG, Appelt HR, Braga AL, Henriques JAP, Roesler R (2003) Facilitation of long-term object recognition memory by pretraining administration of diphenyl diselenide in mice. Neurosci Lett 341:217–220

Sallam H, Jimenez P, Vita M, Cedazo-Minguez A, Moustapha H (2008) Age dependant pharmacokinetics of roscovitine ant its effect on brain Cdk5 end Erk 1/2 in rat pups haematologica. Hematol J 58(1):32–37

Saunders NR (1986) Development of human blood-CSFbrain barrier. Develop Med Child Neurol 28:261–263

Savegnago L, Jesse CR, Pinto LG, Rocha JBT, Nogueira CW (2007a) Diphenyl diselenide attenuates acute thermal hyperalgesia and persistent inflammatory and neuropathic pain behavior in mice. Brain Res 1175:54–59

Savegnago L, Pinto LG, Jesse CR, Alves D, Rocha JBT, Nogueira CW, Zeni G (2007b) Antinociceptive properties of diphenyl diselenide: evidences for the mechanism of action. Eur J Pharmacol 555:129–138

Savegnago L, Pinto LG, Jesse CR, Rocha JBT, Nogueira CW, Zeni G (2007c) Monoaminergic agents modulate antidepressant-like effect caused by diphenyl diselenide in rats. Prog Neuro-Psychoph 31:1261–1269

Savegnago L, Pinto LG, Jesse CR, Barancelli DA, Rocha JBT, Nogueira CW, Zeni G (2008) Diphenyl diselenide exerts antidepressant-like and anxiolytic-like effects in mice: Involvement of L-arginine-nitric oxide-soluble guanylate cyclase pathway in its antidepressant-like action. Pharmacol Biochem Be 88:418–426

4.6 Determinação das propriedades tipo droga do disseleneto de difenila: Estabilidade, solubilidade, absorção e ligação às proteínas plasmáticas *in vitro*

4.6.1 Manuscrito 1

DETERMINATION OF DRUG-LIKE PROPERTIES OF DIPHENYL DISELENIDE: *IN VITRO* STABILITY, SOLUBILITY, ABSORPTION AND PLASMA PROTEIN BINDING

Em fase de Redação

Determination of drug-like properties of diphenyl diselenide: *In vitro* stability, solubility, absorption and plasma protein binding

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Abstract

In order to increase the success rate in clinical studies, lead compounds in drug discovery research, should not only be potent and selective but also must pocess acceptable pharmacokinetic properties such as absorption and distribution, and a suitable stability. In this study, we carried exploration of drug-like properties of diphenyl diselenide $(PhSe)_2$, a selenoorganic compound, with interesting pharmacological properties. Experimental protocols were established for chemical stability in isotonic phosphate buffer (PBS) pH 7.4 and in simulated gastric and intestinal fluid), biological stability (human serum albumin (HAS) and plasma), solubility in PBS pH 7.4, distribution coefficient (Log D) in octanol/PBS and determination of free $(PhSe)_2$ concentrations in human serum albumin (HAS) and plasma by using liquid chromatography (LC) with UV detection and tandem mass spectrometry (MS). $(PhSe)_2$ was found to be chemically stable and it was not susceptible to degradation in plasma. The aqueous solubility was 0.98 ± 0.072 μM and the distribution coefficient (Log D) in octanol/PBS system was found to be 3.14. The percentage unbound fractions of $(PhSe)_2$ obtained by equilibrium dialysis from HAS and plasma solution incubated with 100 μM of the drug were: 0.69 ± 0.12% and 0.44 ± 0.09 %, respectively. The findings of our study indicated that $(PhSe)_2$ presents a good chemical and biological stability. Though, the compound showed low aqueous solubility, high Log D value and high binding to plasmatic protein. These data permit the knowledge of the pharmacokinetic properties of $(PhSe)_2$ and help to understand its pharmacological and toxicological effects.

Keywords: diphenyl diselenide, selenium, protein binding, stability, solubility.

1. Introduction

Of the many novel compounds, that a drug discovery find that bind to the therapeutic target, only a small fraction has sufficient ADME properties to become a drug product. If the properties are weak, the candidate will have a high risk of failure or be less desirable as a drug product. Thus these studies must be performed in an early stage of discovery so the findings can be incorporated in the development of the product and alterations can be performed in a molecule before it reaches clinical assays (Wang and Urban, 2004). In recent years, many *in vitro* approaches have been developed for evaluation of such properties to speed up the discovery process, reduce failure rate at the final stage, minimize time and cost, and also to avoid complexities associated with animal experiments. These approaches not only show good correlation with *in vivo* findings but also have accelerated the drug-discovery process to a great extent (Kerns, 2000; Di and Kerns 2003; Kaplitaand Liu 2005).

Oral delivery is the most convenient and desirable route of drug administration, which demonstrates for absorption-related studies along with distribution, metabolism, and excretion that are common for all types of formulation irrespective of the route of administration. Stability and dissolution of the drug compound in the gastro-intestinal (GIT) fluid; permeation through gastro-intestinal barrier; binding to plasma proteins; distribution throughout the body are some of the crucial parameters to be evaluated for an oral drug, and an active compound should have all these properties to an optimum level to become a successful drug (Di and Kerns, 2003; Mondal et al., 2009).

Diphenyl diselenide $(PhSe)_2$ is a highly lipophylic compound that, exhibits a concentration-time profile characterized by an early peak concentration, rapid distribution from blood to the central nervous system, where it exerts its pharmacological and

toxicological effects (Maciel et al., 2000; Prigol et al., 2009a, 2010). $(PhSe)_2$ has been proven effective against neurotoxicity (Ghisleine et al., 2003), hyperglycemia (Barbosa et al., 2006), lipid peroxidation (Meotti et al., 2004; Luchese et al., 2007; Prigol et al., 2009b), inflammation (Savegnago et al., 2007a), nociception (Savegnago et al., 2007b), anxiety-like (Savegnago et al., 2007c) and depressant-like (Ghisleine et al., 2008) models using rats and mice. On the other hand, $(PhSe)_2$, when administered at a high dose, has been reported to cause seizure episodes in rats and mice (Prigol et al., 2009a).

The present study investigated the drug-like properties of $(PhSe)_2$ in regards to stability, solubility, absorption, distribution and plasma protein binding (PPB), considering that the study of these stages is crucial to understand the pharmacological or toxicological effect of the compound.

2. Materials and Methods

2.1 Chemicals and reagents

Solvents, for liquid chromatography (LC), and human serum albumin (HAS) were purchased from Sigma (Sigma Aldrich SA). $(PhSe)_2$ was synthesized according to Paulmier (1986). Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that $(PhSe)_2$ obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of compound (99.9%) was determined by liquid chromatography-tandem mass spectrometry (LC-MS). All other chemicals and standard drugs were purchased from Merk. Human plasma was prepared from healthy male volunteers and was kept at 20 ºC until use.

2.2 Chromatographic analyses

Aliquots of samples were analyzed using LC techniques in two different systems. One LC system unit consists of a pump controller and a UV detector. The LC unit consists of a pump controller Merk Hitachi L7110 and a UV detector (Merk Hitachi L7410). $(PhSe)_2$ was quantified at 243 nm using a 12.5cm RP-8 Merck (Licrocart) column (5 µm). The mobile phase was prepared with water: methanol (30:70) both containing 0.1% formic acid, using a flow rate of 1 ml/min and the sample injection volume was 20 µl. Validation of chromatographic method was based on specificity, repeatibility (%RSD for RT and area < 2% with 10 injections of same sample), and linearity using nine calibration standards. The standard curve for $(PhSe)_2$ was linear (R= 0.9908) over the concentration 0.5-1000 µM.

Other LC system (Alliance, Waters 2695 Separation Module) with a photodiode array detector (DAD, Waters 2996) set at 243 nm was used in tandem with a mass spectrometer (Micromass Quattro Micro API). Samples were analyzed using a 150 x 2.1 mm i.d., 5µm Atlantis dC18 column (Waters, Milford, USA) and a pre-column with the same stationary phase was also used all at 35ºC. The analytes were eluted using a gradient mixture of two solvents referred as A (water + 1% formic) and B (methanol+1% formic). A linear gradient was used from 30% to 70% solution B in 30 minutes and the flow rate was 0.2 ml/min. The injection volume was 1 µL. A divert valve was used to discard the eluate from the first 30 second of analysis. The total run time was 30 minutes and the retention time for $(PhSe)_2$ was found to be 23.8 minutes.

Mass Spectrometry. The mass spectrometer was operated using an electrospray ionization source in the negative ion mode (ESI^-) and a triple quadrupole as analiser. Acquisition and data processing were performed using the MassLynx software, from Waters. Analysis were carried out using full scan from and product-ion scan (MS/MS) was performed to detect fragment ions using collision energy. For the monitorization of $(PhSe)_2$

SIR mode (single ion recording) was performed at *m/z* 189, according to data obtained from mass spectra obtained in SCAN mode. Source temperature was 120ºC and desolvation temperature was 350ºC. Cone gas flow and desolvation gas flow were 50 and 750 L/Hr respectively. The capillary voltage was set at 2.5kV and cone voltage 50V.

2.3 Solubility

A 5µl of a 50 mM stock solution of $(PhSe)_2$ in dimethyl sulfoxide (DMSO) was diluted to 1000 µl with phosphate buffer (PBS) pH 7.4 and incubated at 25° C for 2 hours with constant shaking. The undissolved compound was centrifuged to 3,000*x* g for 10 minutes. To 50 µl of the supernatant, 50µl of methanol was added and mixed thoroughly. The concentration of $(PhSe)_2$ in the sample was determined by LC-UV using the calibration curve obtained in the linearity study (range 0.5-1000µM) of the compound prepared in a solution 1:1 (v/v) of PBS and methanol.

2.4 Distribution coefficient (Log D) in octanol/PBS pH 7.4

Octanol and PBS pH 7.4 with ratio 1:1 (v/v) were taken in a flask and shaken mechanically for 2 hours to pre-saturate PBS with octanol and octanol with PBS. These pre-saturated solvents were used for the present study (Hitzel et al., 2000). A 5µl of 50 mM$(PhSe)_2$ in 1000 µl octanol was allowed to undergo partitioning with 1000 µl PBS. After phase mixing by vigorous shaking for 2 hours, phase separation was done by centrifugation at 3,000 *x* g for 10 minutes followed by 1 hour standing without disturbance. The PBS layer was taken out. The concentration of $(PhSe)_2$ in octanol and PBS phases was determined by LC-UV using the calibration curve obtained in the linearity study (range 0.5-1000µM).

Log D (the logarithm of concentration ratio of compound in organic and aqueous phase) was calculated according to Dellis et al (2007). Log D should be within average Log D ± 0.3 log units.

2.5 Chemical stability

Chemical stability was carried out according to Mondal et al (2009). Due to solubility characteristics of $(PhSe)_2$ the chemical stability was carried out using a medium with 50:50 of methanol: PBS pH 7.4. A 5 µl of 50 mM $(PhSe)_2$ solution in DMSO was diluted in the medium above mentioned and was kept at 25°C for 24 hours. Stability was determined based on comparison of the chromatographic peak area of the compound after analysis at 0 hour (freshly prepared and injected) and after 24 hours.

Similar studies were carried out at 37°C replacing PBS with simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) which was prepared following United States Pharmacopoeia without enzyme content. Stability was checked for up to 2 hours for SGF and 4 hours for SIF.

2.6 Biological stability

In a test tube, pooled human plasma (700 µl + 300 µl PBS pH 7.4) or HSA (40 g/lin1000 µl PBS pH 7.4) were mixed with 5 µl of 20 mM $(PhSe)_2$ dissolved in DMSO. The test tube, with a final concentration of 100 µM $(PhSe)_2$, was incubated at 37 ºC and at predetermined time points 0 (freshly prepared and injected) and 1 hour, a 100 µl aliquot was removed, mixed with 200 µl of methanol and centrifuged at 3,000 *x* g for 10 minutes. The supernatant was removed and analyzed by LC-UV. The concentration of $(PhSe)_2$ was determined using the calibration curve obtained in the linearity study (range 0.5-1000µM).

LC-MS analyses were performed to check the possible degradation of $(PhSe)_2$ by plasmatic enzymes.

2.7 Determination of free $(PhSe)_2$ concentrations in HAS and plasma

Determination of free $(PhSe)_2$ concentrations in HAS and plasma study was carried out according to Herforth et al. (2002), by using two chamber equilibrium dialysis cells, each with a capacity of 1.0 ml. The two chambers in each cell are separated by a membrane with a molecular weight cut-off of 12000 Da. Prior to use, the membrane was boiled in distilled water for 2 hours to remove preservatives. Then, it was kept in isotonic phosphate buffer (PBS, pH 7.4). A aliquot of 5 µl of 20 mM $(PhSe)_2$ in DMSO was added to human plasma or HAS solutions. Drug-protein solutions were added to one side chamber (donor) of the dialysis cell (1ml) and the other side (receiver) was filled with 1 ml of PBS. Dialysis was carried out for 8 hours at 37 ºC in a water bath with rotation at 15 rpm. After 8 hours, 100 µl aliquot of PBS solution (receiver dialysis cell) was removed and analyzed by LC-UV. It represents the fraction of unbound drug. The remain bound fraction was obtained by the difference between the initial concentration (100 µM) and unbound fraction of $(PhSe)_2$.

3. Results

Solubility of $(PhSe)_2$ was 0.98 ± 0.072µM in PBS pH 7.4 (Table 1). It showed Log D value of 3.14± 0.09 log units in the octanol /PBS system (Table 1).

It was observed that $(PhSe)_2$ was stable (>88%) in PBS pH 7.4, SGF, and SIF. Stability was 88.5 ± 5.5 % in PBS pH 7.4; 90.33 ± 3.93% in SGF and 96.22 ± 3.30 % in SIF, at the tested conditions (Table 2).

The amount of $(PhSe)_2$ obtained from extracted HAS and serum solution incubated with 100 μM of the drug were : 76 ± 9.71μM at initial time (0 hours) and 65 ± 10,6μM at 1 hour after incubation with HAS; 64 ± 7.6 μM at initial (0 hour) time and 56.5 ± 9.2 μM at 1 hour after incubation with plasma (Table 3). The presence of other possible compounds derived from the molecule of $(PhSe)_2$ was analyzed by LC-MS. Fig. 1 shows the mass chromatograms of the HAS and plasma following different incubation times at 37 °C. Only $(PhSe)_2$ was detected, with a retention time of 23.9min. Mass spectra and retention time of the peak detected are the same of standard solution of $(PhSe)_2$ (Fig. 2), confirming the identification of the compound.

Percentage unbound fraction of $(PhSe)_2$ obtained by equilibrium dialysis from HAS and plasma solution incubated with 100 μM of the drug were: 0.69 ± 0.12% and 0.44 ± 0.09 %, respectively. The remain bound fraction in HAS and plasma, obtained by the difference between the initial concentration (100 μM) and unbound fraction of $(PhSe)_2$ were: 99.31 ± 0.17 and 99.56 ± 0.09, respectively (Table 4).

4. Discussion

The purposes of the current study were to establish *in vitro* drug-like properties of $(PhSe)_2$ such as: (1) solubility in PBS pH7.4, (2) distribution coefficient(Log D) in octanol/PBS pH 7.4; (3) chemical stability (in PBS pH7.4, simulated gastric and intestinal fluid), (4) biological stability in HSA and plasma and (5) determination of free drug concentrations in HAS and plasma.

Determination of compound solubility in PBS pH 7.4 or water has become an essential early measurement in the drug discovery research. Poor aqueous solubility can cause problems in many different *in vitro* testing techniques, resulting in unreliable results and reproducibility problems. Even larger problems may arise when insoluble precipitates

cause false positives in bioassays, potentially wasting time and significant cost (Yan and Caldwell, 2004). The standard way to determine the solubility of a compound is to use the shake-flask solubility method (Chait, 2003). The solubility of $(PhSe)_2$ was found to be 0.98μM in PBS, pH 7.4. This result indicates that the compound is poorly soluble and may create solubility-related problems when formulated as oral dosage form. Accordingly, our previous study demonstrated that $(PhSe)_2$ administered at a high dose (500 mg/kg) by oral route, dissolved in canola oil, produced a lower C max (13.13 and 10.11μg/ml for rats and mice, respectively).These two data together suggest that only a small amount of the compound can be dissolved in gastrointestinal fluid in order to be absorbed into the bloodstream.

Log D value of $(PhSe)_2$ was determined to be 3.14 in Octanol/PBS. Log D (Octanol/PBS) determines lipophilicity of the compounds and thus determines the ability/inability of the drug to cross the gastrointestinal barrier (Di and Kerns, 2003). High Log D (>3) leads to low solubility, erratic/poor absorption, high plasma binding, whereas very low Log D (<0) indicates low permeability and high renal clearance. Compounds with moderate Log D (0-3) have good balance between solubility and permeability and are optimal for oral absorption and cell membrane permeation in cell-based assays (Di and Kerns, 2003; Hitzel et al., 2000; Dellis et al., 2007). $(PhSe)_2$ (Log D = 3.14) presents a high Log D value and, therefore, bears low solubility as observed here. On the other hand, the high Log D of $(PhSe)_2$ in octanol/PBS is indicative of its ability to cross membranes as well as blood-brain barrier. This result helps to explain the early peak concentration of the compound in the plasma (30 minutes) (Prigol et al., 2009) as well as the speed at which the compound reaches the brain and causes seizures in animal models (Prigol et al., 2009, 2010).

Drug chemical stability in the stomach and intestine is critical for promoting drug absorption in the GI tract (Wasan et al., 2009). Stability study in PBS pH 7.4, and SIF revealed that $(PhSe)_2$ is stable at both conditions indicating its acceptability for intended absorption from the high surface area of the intestine.

Drug stability in plasma is a concern in both drug discovery and development areas (Chow et al., 1997; Pop et al., 1999; Shipkova et al., 2000). Except for "pro-drugs", drug candidates undergoing rapid degradation in plasma generally have undesirable pharmacokinetic parameters and pose analytical challenges. For drug development candidates, plasma instability will cause some problems in the validation of the bioanalytical assays, for the unstable pharmaceutical components or their metabolites present in plasma samples (Kerbusch et al., 1998). For these reasons, plasma stability data can be useful in drug discovery programs to avoid the selection of unstable drug candidates. In the he chromatograms obtained in the analysis of $(PhSe)_2$ in HAS and plasma following different incubation times using the mass spectrometry as detector a peak with a with a retention time of 23.9 min was detected and no other compounds, possible metabolites of $(PhSe)_2$ obtained after degradation were detected. This result provides evidence that $(PhSe)_2$ has biological stability, and it is not susceptible to degradation in plasma.

Determination of free drug concentration is another important parameter that needs to be evaluated in the pharmacokinetic studies, as unbound drug plasma concentration decides the pharmacological effects of a compound in regards to tissue distribution, cell entry, receptor interaction and availability for elimination (Yan and Caldwell, 2004; Banker et al, 2003). Previous reports suggest that dialysis for 6 to 8 hours is optimum to reach equilibrium (Banker et al., 2003) and hence 8-hour dialysis was performed in these studies. Since albumin is the most abundant plasma protein and it is well known to bind

and carry out a large number of amphiphilic molecules in plasma, we have performed binding assays using HAS and human plasma proteins. The unbound fraction of $(PhSe)_2$ in HAS and plasma were 0.69 % and 0.44%, respectively, while 99.31% $(PhSe)_2$ was bound to BSA and 99.56% inhuman plasma proteins. These data provide three main evidences: (1) $(PhSe)_2$ unbound fraction in HAS and plasma is much lower, (2) the compound has a large plasma protein binding and (3) most of the compound is bound to albumin.

Plasma protein binding is typically a reversible interaction; however, irreversible or covalent binding can occasionally occur (Nassar et al., 2009). To analyze the interaction between $(PhSe)_2$ and proteins, $(PhSe)_2$ was incubated with HSA and plasma. The mixture was extracted with methanol. The extraction with methanol is able to denature proteins and break weak bonds, but is not capable of breaking covalent bonds. It was observed that when incubated with HSA and plasma, already at baseline, an average value of 77% and 65% of the compound, respectively, appears free of proteins after extraction. These results indicate that most of the compound is covalently bound to proteins and thus remains in the precipitate. $(PhSe)_2$ interacts with sulfhydryl groups to proteins oxidizing them to disulfides (Nogueira et al., 2004), a hypothesis is that it reacts with the sulfhydryl group of cysteine 34, the only reactivethiol group in serum albumin (Cotgreave et al., 1992; Wagner et al., 2004) to form a selenosulfide. Accordingly to a metabolic study with another selenoorganic compound, ebselen, more than 90% of intravenously administered ebselen in mouse plasma is bound by selenium-sulfur bounds to reactive thiols in serum albumin. The compound is transferred from the BSA complex to membrane-associated proteins after reductive cleavage of the Se-S bound effected by endogenous protein thiols or externally added reductants such as, GSH (Wagner et al., 2004).

The findings of the present study indicate that $(PhSe)_2$ presents good chemical and biological stability. However, compound showed low aqueous solubility, high Log D

values and high binding to plasmatic protein. These data are very important since: (a) permit the knowledge of the pharmacokinetic properties of $(PhSe)_2$, (b) explain the low bioavailability of the compound, which was demonstrated in previous studies and (c) suggest future changes in the chemical structure of $(PhSe)_2$ for an improvement in its pharmacokinetic properties.

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5. References

Balimane PV, Han YH, Chong S. Current industrial practices of assessing permeability and p-glycoprotein interaction. AAPS J (2006) 8:E1-13.

Ballimane PV, Chang S. Cell culture-based models for intestinal permeability: A critique. Drug Discov Today (2005)10:335-43.

Banker MJ, Clark TH, Williams JA. Development and validation of a 96-well equilibrium dialysis apparatus for measuring plasma protein binding. J Pharm Sci (2003) 92:967-974.

Barbosa NBV, Rocha JBT, Wondracek DC, Perottoni J, Zeni G, Nogueira CW. Diphenyl diselenide reduces temporarily hyperglycemia: Possible relationship with oxidative stress. Chem. Biol. Interact (2006) 163, 230-238.

Bevan CD, Lloyd RS.A highthroughput screening method for the determination of aqueous drug solubility using laser nephelometry in microtiter plates. Anal Chem (2000) 72:1781-7.

Brunton LL, Lazo JS, Parker KL. Goodman and Gilman′s the pharmacological basis of therapeutics. New York: McGraw-Hill Medical Publishing Division (2006) p. 1.

Chait A. Discovery ADMET profiling: Solubility technique. Biosci Tech (2003) 5:33-38.

Chow D., Bhagwatwar H.P., Phadungpojna S, Andersson B.S. J. Chromatogr. B 704 (1997), p. 277.

Cotgreave IA, Morgenstern R, EngmanL, Ahokas J. Characterization and Quantitation of a Selenol Intermediate in the Reaction of Ebselen with Thiols.Chem.-Biol. Interactions (1992)84, 69.

Dellis D, Giaginis C, Tsantili-Kakoulidou A. Physicochemical profile of nimesulide, Exploring the interplay of lipophilicity, solubility and ionization. J Pharm Biomed Anal (2007) 44:57-62.

Di L, Kerns EH. Profiling drug-like properties in discovery research. Curr Opin Chem Biol (2003) 7:402-8.

Ghisleine G, Porciúncula LO, Cimarosti H, Rocha JBT, Salbego CG, Souza DO. Diphenyl diselenide protects rat hippocampal slices submitted to oxygen–glucose deprivation and diminishes inducible nitric oxide synthase immunocontent. Brain Res. (2003) 986, 196-199.

Ghisleni G, Kazauckas V, Both FL, Pagnussat N, Mioranzza S, Rocha JBT, Souza DO. Diphenyl diselenide exerts anxiolytic-like effect in Wistar rats: Putative roles of GABAA and 5HT receptors. Prog. Neuro-Psychoph (2008) 32, 1508-1515.

Herforth C, Stone JA, Jayewardene AL, Blaschke TF, Fang F, Motoya T, Aweeka FT. Determination of nelfinavir free drug concentrations in plasma by equilibrium dialysis and liquid chromatography/tandem mass spectrometry: important factors for method optimization.Eur J Pharm Sci. (2002) 15(2):185-95.

Hitzel L, Watt AP, Locker KL. An increased throughput method for determination of partition coefficients. Pharm Res (2000) 17:1389-95.

Kansy M, Senner F, Gubernator K. Physicochemical high throughput screening: Parallel artificial membrane permeability assay in the description of passive absorption processes. J Med Chem (1998) 41:1007-10.

Kaplita PV, Liu HH. Automatable formats of higher throughput ADMET profiling for drug discovery support. J Assoc Lab Automation (2005) 10:140-8.

Kellard L, Engelstein M. Automation of cell-based and noncell-based permeability assays. J Assoc Lab Automation (2007) 12:104-9.

Kerbusch T, Huitema ADR, Kettenes-van JJ, Keizer HJ, Ouwerkerk J, de Kraker J, Beijnen JH. J. Chromatogr. B 716 (1998), p. 275.

Kerns EH. High throughput physicochemical profiling for drug discovery. J Pharm Sci (2001) 90:1838-58.

Li AP. Screening for human ADME/Tox drug properties in drug discovery. Drug Discov Today (2001) 6:357-66.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem (1951) 193:265-75.

Luchese C, Brandao R, Oliveira de R, Nogueira CW, Santos FW. Efficacy of diphenyl diselenide against cerebral and pulmonary damage induced by cadmium in mice Toxicol. Lett. (2007) 173 (3), 181-190.

Maciel, E.N., Bolzan, R.C., Braga, A.L., Rocha, J.B.T. Diphenyl diselenide and diphenyl ditelluride differentially affect delta-aminolevulinate dehydratase from liver, kidney, and brain of mice. J. Biochem. Mol. Toxic. (2000) 14: 310-319.

Meotti FC, Stangherlin EC, Zeni G, Rocha JBT, Nogueira CW. Protective role of aryl and alkyl diselenides on lipid peroxidation. Environ. Res. (2004) 94, 276–282.

Mondal SK., Mazumder UK, Mondal NB, Banerjee S. Optimization of rat microsomal stability assay using HPLC. J Biol Sci (2008) 8: 1110-1114.

Paulmier C. Selenoorganic functional groups. In: Paulmier, C. (Ed.), Selenium Reagents and Intermediates in Organic Synthesis, 1st ed. Pergamon Press, Oxford, England, pp. (1986) 25-51.

Pop E, Rachwal S, Vlasak J, Zharikova A, Prokai L. J. Pharm. Sci. (1999) 88, p. 1156.

Prigol M, Schumacher RF, Nogueira CW, Zeni G. Convulsant effect of diphenyl diselenide in rats and mice and its relationship to plasma levels. Toxicol Lett (2009a)189(1): 35-39.

Prigol M, Luchese C, Nogueira CW. Antioxidant effect of diphenyl diselenide on oxidative stress caused by acute physical exercise in skeletal muscle and lungs of mice. Cell Biochem. Funct. (2009b) 27 (4), 216-222.

Prigol M, Pinton S, Schumacher RF, Nogueira CW, Zeni G. Convulsant action of diphenyl diselenide in rat pups: measurement and correlation with plasma, liver and brain levels of compound. Arch Toxicol (2010) 84: 373–378.

Savegnago L, Jesse CR, Pinto LG, Rocha JBT, Nogueira CW. Diphenyl diselenide attenuates acute thermal hyperalgesia and persistent inflammatory and neuropathic pain behavior in mice. Brain Res. (2007a) 1175, 54-59.

Savegnago L, Pinto LG, Jesse CR, Alves D, Rocha JBT, Nogueira CW, Zen, G. Antinociceptive properties of diphenyl diselenide: Evidences for the mechanism of action. Eur. J. Pharmacol. (2007b) 555, 129-138.

Savegnago L, Pinto LG, Jesse CR, Barancelli DA, Rocha JBT, Nogueira CW, Zeni G. Diphenyl diselenide exerts antidepressant-like and anxiolytic-like effects in mice: Involvement of L-arginine-nitric oxide-soluble guanylate cyclase pathway in its antidepressant-like action. Pharmacol. Biochem. Be. (2008) 88, 418-426.

Shipkova M, Schutz E, Armstrong VW, Niedmann PD, Oellerich M, Wieland E. Clin. Chem. (2000) 46, p. 365.

Sugano K, Hamada H, Machida M, Ushio H. High Throughput prediction of oral absorption: Improvement of composition of the lipid solution used in Parallel artificial membrane permeability assay. J Biomol Screen (2001) 6:189-96.

Wagner G, Schuch G, Akerboom TP, Sies H. Transport of ebselen in plasma and its transfer to binding sites in the hepatocyte.BiochemPharmacol. (1994) 15;48(6):1137-44.

Wang J, Urban L. The impact of early ADME profiling on drug discovery and development strategy. Drug Discov World (2004) 5:73-86.

Wasana EK., Bartlett K, Gershkovich P, Sivak O, Banno B, Wong Z, Gagnon J, Gates B, Leon CG, Wasan KM. Development and characterization of oral lipid-based Amphotericin B formulations with enhanced drug solubility, stability and antifungal activity in rats infected with Aspergillusfumigatus or Candida albican. International Journal of Pharmaceutics (2009) 372:76-84.

Yan Z, Caldwell GW. Methods in pharmacology and toxicology: Optimization in Drug Discovery: In vitro Methods. New Jersey: Humana Press, p. 1, 2004.

Tables

Table 1: Solubility and distribution coefficient (Log D) in octanol/PBS pH 7.4 of $(PhSe)_2$.

Solubility (μM)[a]	**Log D Octanol/PBS (log Unit)[b]**
0.98 ± 0.072	3.13 ± 0.091

[a,b]Results are average of two experiments ± SD.
[b]Individual distribution coefficient (Log D) values were within ± 0.3 log unit of average Log D $(PhSe)_2$.

Table 2: Chemical Stability of $(PhSe)_2$ in PBS pH 7.4, simulated gastric fluid and simulated intestinal fluid

Medium	**$(PhSe)_2$chemical stability(%)[a]**
PBS pH 7.4	88.11 ± 5.5
Simulated Gastric Fluid	90.33 ± 3.93
Simulated Intestinal Fluid	96.22 ± 3.30

Results are average of three experiments ± SD.
[a]Chemical Stability was reported as percentage of compound that remains after incubation time predetermined for each medium.

Table 3: Amount of $(PhSe)_2$ obtained from extrated HAS and plasma solution incubated with 100 μM of the drug.

	Concentration (μM)	
Medium	**0 hour[a]**	**1 hour**
HSA	76 ± 9.71	65 ± 10.6
Plasma	64 ± 7.6	56.5 ± 12.2

Results are average of two or three experiments ± SD.
[a]Time point 0 hour denotes to freshly prepared and injected solution

Table 4: Percentage unbound fraction of $(PhSe)_2$ obtained by equilibrium dialysis from HAS and plasma solution incubated with 100 µM of the drug.

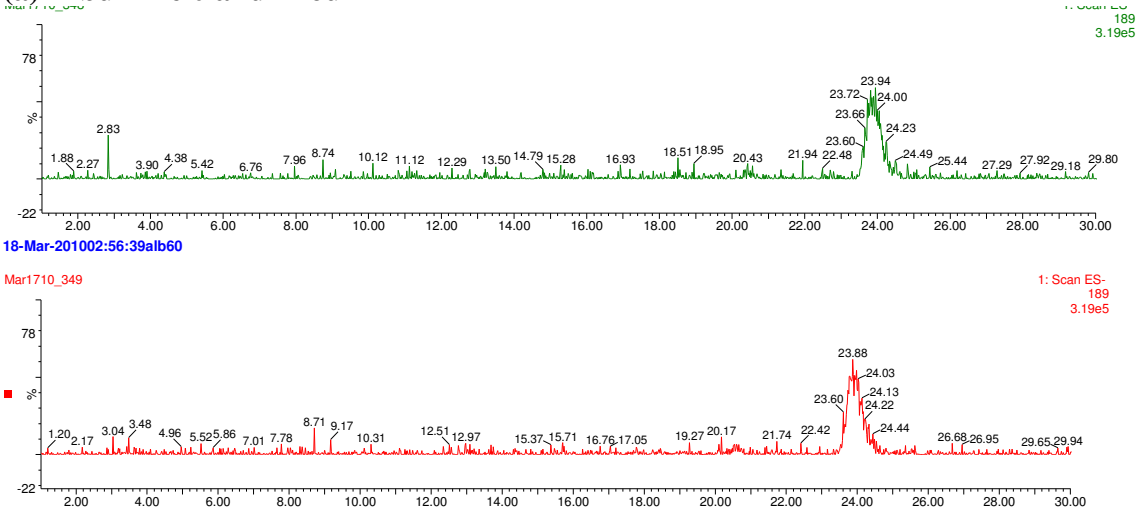
	Unbound fraction (%)[a,b]	**Remain bound fraction (%)[a,c]**
HSA	0.69 ± 0.12	99.31 ± 0.17
Plasma	0.44 ± 0.09	99.56 ± 0.09

Results are average of two or three experiments ± SD.
[a]Unbound fraction and remain bound fraction were reported as percentage of compound that remains after incubation time predetermined for each medium.
[b]Unbound fraction were obtained by equilibrium dialysis after 8 hours of incubation.
[r]Remain bound fraction was obtained by the difference between the initial concentration (100 µM) and unbound fraction of $(PhSe)_2$

Figures:

Figure 1

(a) Albumine 0 and 1hour



(b)Plasma 0 and 1 hour

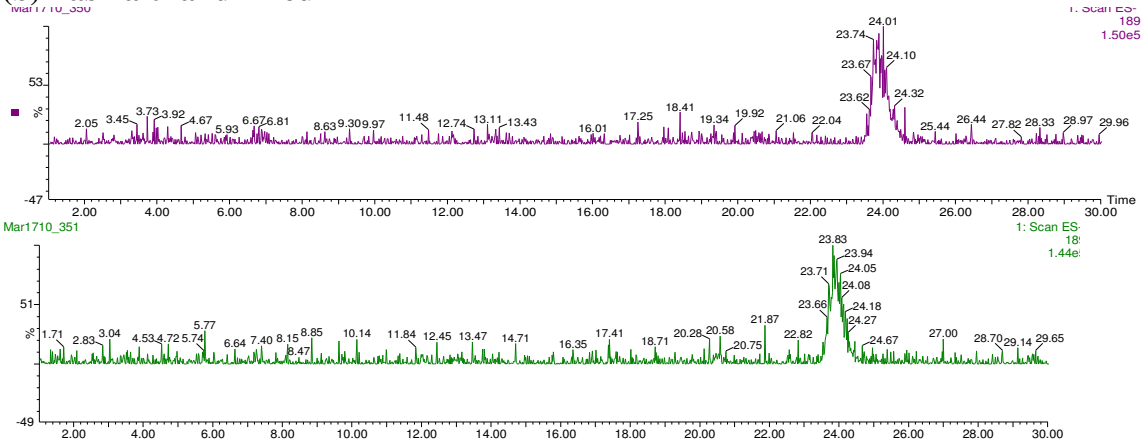


Figure 1: Chromatogram of LC-MS analysis of (a) HAS sample (0 and 1 hour) and (b) plasma sample (0 and 1 hour).

Figure 2

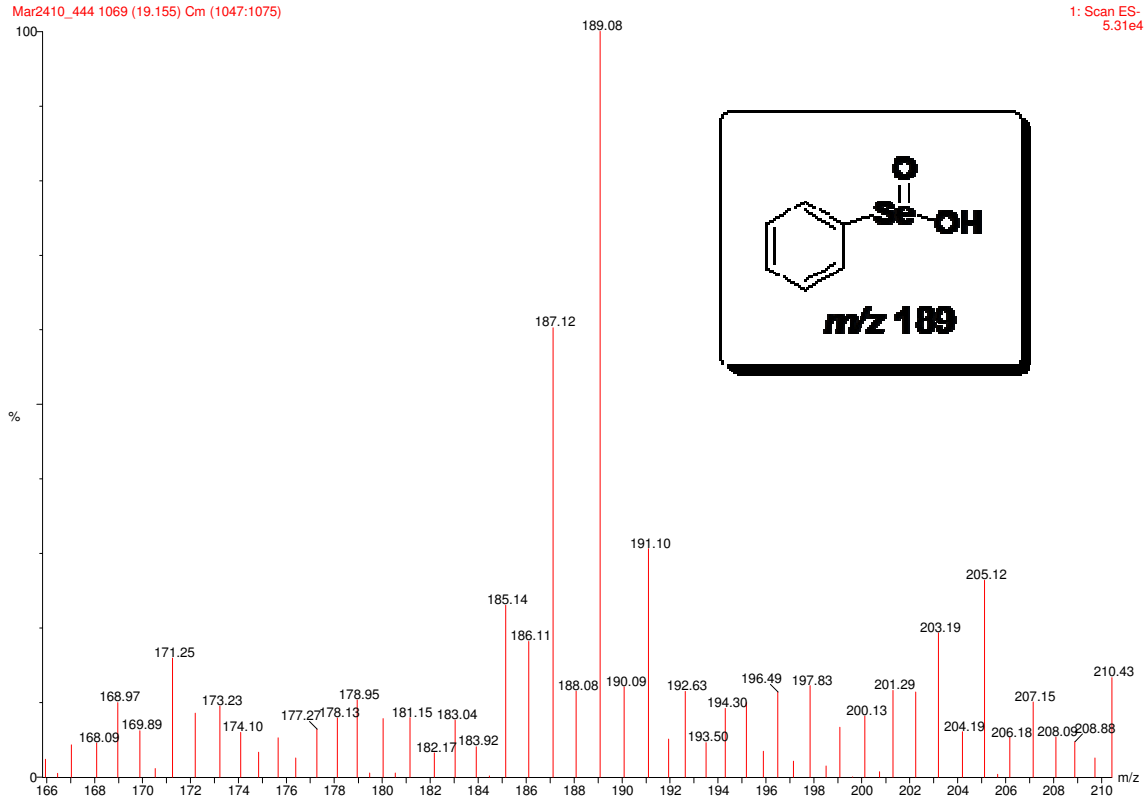


Figure 2: MS ion spectra obtained of $(PhSe)_2$ (*m/z* 189) eluted at 23.9 min. The ion structure is presented.

4.7 Estudo do metabolismo do disseleneto de difenila *in vitro:* Identificação de intermediários reativos por análise da formação de adutos com a glutationa

4.7.1 Manuscrito 2

METABOLIC STUDY OF DIPHENYL DISELENIDE IN VITRO: IDENTIFICATION OF REACTIVE INTERMEDIATES THROUGH ANALYSIS OF GLUTATHIONE ADDUCTS

Em fase de Redação

Metabolic study of diphenyl diselenide *in vitro*: Identification of reactive intermediates through analysis of glutathione adducts

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Abstract

In spite of extensive literature describing pharmacological and toxicological properties of diphenyl diselenide $(PhSe)_2$, little is known about its metabolism. The present study investigated, for the first time, $(PhSe)_2$ metabolic pathways *in vitro*: (1) 100 µM $(PhSe)_2$ was incubated with different liver fractions (Total Homogenate, S9, Soluble and Microsomal Faction). Samples were qualitatively analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Reduced glutathione (GSH)-selenol adduct was detected in four different liver fractions, with a ion scan spectrum of *m/z* 462; (2) Microsomal fraction was incubated with 100 µM $(PhSe)_2$, supernatant and precipitate were quantitatively analyzed using liquid chromatography (LC) and inductively coupled plasma (ICP) respectively.45 µM $(PhSe)_2$ appears in supernatant at baseline and 18 µM after 1 hour of incubation, while 57 ppb of selenium remaining in the precipitate after 1 hour of incubation. The addition of GSH maintains constant $(PhSe)_2$ levels in supernatant and significantly reduces the amount of selenium in the precipitateafter 1 hour of incubation. In microsomal incubation of $(PhSe)_2$ with N-acetylcysteine (NAC), a NAC-selenol adduct was detected by LC-MS with a ion scan spectrum of *m/z* 318. NAC significantly reduces the amount of selenium in the precipitate. The inhibition of microsomal P450 activity by carbon monoxide did not alter the amount of $(PhSe)_2$ in supernatant and selenium levels in precipitate. These data indicate that $(PhSe)_2$ is not metabolized by phase I reactions and it reacts chemically with GSH and NAC forming adducts, these decreases covalent binding of the compound with proteins; (3) Potential of $(PhSe)_2$ to inhibit the cytochrome P450 enzymes was assessed $(PhSe)_2$ already at 1 µM reduced microsomal activity. The calculated IC_{50} value for microsomal activity inhibition by $(PhSe)_2$ was 78 µM.

Keywords: diphenyl diselenide, metabolism, rat liver microsome, selenium.

1 Introduction

In last years, a large number of novel pharmaceutical agents derived from selenium are under research (Mugesh et al., 2001; Nogueira et al., 2004; Rosa et al., 2007). Among them are the organoselenium compounds, with emphasis to diphenyl diselenide $(PhSe)_2$, widely studied in several animal models (Nogueira et al., 2004; Rosa et al., 2007; Savegnago et al., 2007; Ghisleni et al., 2008; Prigol et al., 2009a).

$(PhSe)_2$ is a highly lipophylic compound that possesses double face due to its contrasting behaviour, which depends on the dose used (Nogueira et al., 2004). $(PhSe)_2$ has antioxidant activity, confirmed in several *in vitro* and *in vivo* models (Meotti et al., 2004; Luchese et al., 2007; Prigol et al., 2009a), and thus has a protective effect against hepatic (Borges et al., 2005), and gastric (Savegnago et al., 2005) injuries, in addition to its neuroprotective (Ghisleine et al., 2003), anti-inflammatory (Savegnago et al., 2007) and antidepressant-like (Ghisleni et al., 2008) activities.

On the other hand, $(PhSe)_2$ has been reported to cause neurotoxicity in rodents (Nogueira et al., 2003a; Prigol et al., 2007, 2008) and inhibition of sulfhydryl enzymes (Barbosa et al., 1998; Borges et al., 2005; Nogueira et al., 2003b; Prigol et al., 2007). In addition, a different toxicity across species induced by $(PhSe)_2$ has been documented, since the chemical failed to produce any adverse effects in rats, despite a much higher dose. In contrast, a similar dose of the compound induces seizures and death in mice (Nogueira et al., 2003a). The reasons for differential toxicity between mice and rats are presently unknown.

A recent pharmacokinetic study of $(PhSe)_2$ in rats and mice indicated that the maximum concentration for $(PhSe)_2$ in plasma of rats and mice occurs at 30 min after oral administration of compound dissolved in canola oil. Animals exhibited a concentration–time profile characterized by an early peak concentration followed by a continuous decay

in plasma concentration, which remained at detectable levels for up to 8 h after dosing. Furthermore, the same study demonstrated that the use of different administration routes (p.o., i.p. and s.c.) and vehicle solutions (canola oil or DMSO) in rats and mice indicates that the appearance of seizures and $(PhSe)_2$ plasmatic levels are dependent of administration routes (i.p. >p.o.>s.c.), vehicle solutions (DMSO> canola oil) and animal species (mice > rat) (Prigol et al., 2009b). Similar study done in rat pups showed that plasma concentration of $(PhSe)_2$ in the rat pups is about 4.3-fold lower compared to that of seen in adult rats. However $(PhSe)_2$ has shown evidence of toxicity in rat pups, even though in lower concentrations in plasma, demonstrating that rat pups is more sensible to toxic effect produced by $(PhSe)_2$ when compared to adult rats (Prigol et al., 2010).

In spite of extensive literature reporting pharmacological properties of $(PhSe)_2$, little is known about its metabolism. The aim of our study was to identify possible metabolic pathways of $(PhSe)_2$ by (1) incubation of the compound with different liver fractions (Total Homogenate, S9, Soluble and Microsomal Faction). Based on these results, the next objective were (2) to carry out $(PhSe)_2$ incubations with microsomal fraction in different conditions and (3) assessed the potential of $(PhSe)_2$ to inhibit the cytochrome P450 enzymes.

2. Materials and Methods

2.1 Chemicals and reagents

Solvents for high performance liquid chromatography (LC) and liquid chromatography-tandem mass spectrometry (LC-MS), nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, reduced glutathione (GSH) and N-acetylcysteine (NAC) were purchased from Sigma (St. Louis, MO, USA). $(PhSe)_2$ was synthesized according to Paulmier (1986). Analysis of the ^1H

NMR and ^{13}C NMR spectra showed that $(PhSe)_2$ obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of compound (99.9%) was determined by liquid chromatography-tandem mass spectrometry (LC-MS). All other chemicals and standard drugs were purchased from Merk.

2.2 Liver Fractions preparation

Ethical authorization was obtained from Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil for use of animals for the present study.

The liver fractions were prepared accordingly to Constantino et al. (1999), with same modifications. Rats were euthanized and liver was rapidly removed, immersed in ice-cold phosphate buffer (PBS) pH 7.4, minced and washed with the same solution. The mince was homogenized with Teflon/glass homogenizer, immersed in ice, using 3 ml of washing solution per g of liver.

Total Homogenate: The homogenate was centrifuged at 1,000 *x* g for 10 minutes at 4 ºC. The pellet was discarded and supernatant was used as liver total homogenate. This preparation was divided in two parts. One was characterized and stored at -80C to use in incubation studies. The other was used to prepare other liver fractions.

S9 fraction: Liver homogenate was centrifuged at 9,000 *x* g for 10 minutes at 4 ºC. The pellet was discarded and supernatant was used as S9 fraction. This preparation was divided in two parts. One was characterized and stored at -80C to use in incubation studies. The other was used to prepare other liver fractions.

Microsomal and soluble fractions: S9 fraction was centrifuged at 100,000 *x* g for 1 hour at 4 ºC. The supernatant was used as soluble fraction and stored at -80C. The pellet

was suspended in PBS (same to initial volume) and was used as microsomal fraction and stored at -80C.

Protein concentration was determined by the method of Lowry (Lowry et al., 1951) using bovine serum albumin as a standard.

2.3 *In vitro* assays

2.3.1 $(PhSe)_2$ incubations with different liver fractions

Incubations were performed at 37ºC in PBS pH 7.4 using total volume of 1 ml with 0.5 mg protein/ml of the liver fraction under study, 6.25 mmol/ml glucose-6-phosphate, 1.25 mmol/ml NADP, 5mmol/ml $MgCl_2$ and 2.5 U/ml of glucose-6-phosphate dehydrogenase. Reactions are started by the addition 20 µl of $(PhSe)_2$ dissolved in methanol. Final substrate concentration was 100 µM. Aliquots (100 µl) were withdrawn at timed intervals (0 and 1 hour), and quenched by the sequencial addition of 400 µl methanol. After centrifugation to 3,000*x* g for 10 minutes, the supernatant was analyzed by LC-MS to verify the formation of possible metabolites and characterize them.

2.3.2 $(PhSe)_2$ incubations with microsomal fraction

Incubations were performed at 37ºC in PBS pH 7.4 using total volume of 1 ml with 0.5 mg protein/ml of microsomal fraction, 6.25 mmol/ml glucose-6-phosphate, 1.25 mmol/ml NADP, 5mmol/ml $MgCl_2$ and 2.5 U/ml of glucose-6-phosphate dehydrogenase. Reactions are started by the addition 20 µl of $(PhSe)_2$ dissolved in methanol. Final substrate concentration was 100 µM. Aliquots (100 µl) were withdrawn at timed intervals (0 and 1 hour), and quenched by the sequential addition of 400 µl methanol. After centrifugation to 3,000*x* g for 10 minutes, the supernatant was analyzed by LC-UV to assess the concentration of the $(PhSe)_2$ and LC-MS to verify the formation of possible metabolites and characterize them. The protein precipitate was analyzed by inductively

coupled plasma (ICP) to verify Se concentration, it is an indicative of $(PhSe)_2$ covalent binding in microsomal proteins.

Experiments using GSH or NAC were conducted to verify the formation of adducts, since GSH and NAC are nucleophilic agents that react with electrophilic compounds. GSH or NAC both at 1mM were added to the incubation medium.

Experiment using carbon monoxide was conducted in order to cause inhibition of enzyme activity of the P450 complex, which is oxygen-dependent. Carbon monoxide as bubbled into the microsomal solution for 5 min prior to the addition of the substrate.

2.3.3 Assessment of $(PhSe)_2$ potential to inhibit the Cytochrome P450 enzymes.

$(PhSe)_2$ potential to inhibit the cytochrome P450 enzymes was assessed through the initial rate of formation of *N*, ethilbenzamide, a metabolite of *N, N* diethilbenzamide, formed from a reaction with cytochrome P450.

Incubations were performed at 37ºC in PBS pH 7.4 using total volume of 1 ml with 0.5 mg microsomal fraction, 6.25 mmol/ml glucose-6-phosphate, 1.25 mmol/ml NADP, 5mmol/ml $MgCl_2$ and 2.5 U/ml of glucose-6-phosphate dehydrogenase. Reactions are started by the addition 20 µl of $(PhSe)_2$ dissolved in methanol, at concentrations ranging from 1 to 1000 µM. The incubation mixture was pre incubated for 10 minutes prior to the initiation of metabolic reaction with N, N diethilbenzamide (final concentration of 100 µM). Aliquots (100 µl) were withdrawn at timed intervals (0, 10 and 20 minutes), and quenched by the sequential addition of 400 µl methanol. After centrifugation to 3,000*x* g for 10 minutes, the supernatant was analyzed by LC to assess the concentration of *N*, ethilbenzamide.

Potential of $(PhSe)_2$ to inhibit cytochrome P450 was assessed by calculating the initial rate of formation of the substrate *N*, ethilbenzamide in the first 20 minutes of incubation of the N, N diethilbenzamide in the presence and absence of $(PhSe)_2$.

2.4 Analysis Methods

2.4.1 Chromatographic analyses

Aliquots of samples were analyzed using LC techniques in two different systems, to assess the concentration of the samples. One LC system unit consists of a pump controller and a UV detector. The LC unit consists of a pump controller Merk Hitachi L7110 and a UV detector (Merk Hitachi L7410). $(PhSe)_2$ was quantified at 243 nm using a 12.5cm RP-8 Merck (Licrocart) column (5 µm). The mobile phase was prepared with water: methanol (30:70) both containing 0.1% formic acid, using a flow rate of 1 ml/min and the sample injection volume was 20 µl. Validation of chromatographic method was based on specificity, repeatibility (%RSD for RT and area < 2% with 10 injections of same sample), and linearity using nine calibration standards. The standard curve for $(PhSe)_2$ was linear (R= 0.9908) over the concentration 0.5-1000 µM.

Other LC system (Alliance, Waters 2695 Separation Module) with a photodiode array detector (DAD, Waters 2996) set at 243 nm was used in tandem with a mass spectrometer (Micromass Quattro Micro API). Samples were analyzed using a 150 x 2.1 mm i.d., 5µm Atlantis dC18 column (Waters, Milford, USA) and a pre-column with the same stationary phase was also used all at 35ºC. The analytes were eluted using a gradient mixture of two solvents referred as A (water + 1% formic) and B (methanol+1% formic). A linear gradient was used from 30% to 70% solution B in 30 minutes and the flow rate was 0.2ml/min. The injection volume was 1 µL. A divert valve was used to discard the eluate from the first 30 second of analysis. The total run time was 30 minutes.

Mass Spectrometry. The mass spectrometer was operated using an electrospray ionization source in the negative ion mode (ESI^-) and a triple quadrupole as analiser. Acquisition and data processing were performed using the MassLynx software, from Waters. Analysis were carried out using full scan from and product-ion scan (MS/MS) was performed to detect fragment ions using collision energy. For the monitorization of $(PhSe)_2$ and GSH-selenol and NAC-selenol adducts, SIR mode (single ion recording) was performed at *m/z* 189, *m/z* 462 and *m/z* 318, respectively, according to data obtained from mass spectra obtained in SCAN mode. Source temperature was 120ºC and desolvation temperature was 350ºC. Cone gas flow and desolvation gas flow were 50 and 750 L/Hr respectively. The capillary voltage was set at 2.5kV and cone voltage 50V.

2.4.2 Inductively coupled plasma (ICP) analyses

A microsomal precipitate was treated at 60 ºC for 1 hour with concentrated nitric acid (100 µl) and H_2O_2 (10 µl). Samples were diluted to 5 ml with water and analyzed in ICP. Selenium was determined with ICP (Shimadzu Corp).

Selenium concentration in microsomal precipitate is expressed as ppb (parts per billion) in the sample.

2.5 Statistical analysis

The results are presented as means ± S.D. Statistical analyses were performed using a one-way analysis of variance (ANOVA), followed by the Duncan's range test when appropriate. The IC_{50} (inhibitory concentration) was calculated by using the two data points traversing the 50% inhibition lines in terms of the activity. The *p* values less than 0.05 were considered as indicative of significance.

3 Results

3.1 $(PhSe)_2$ incubations with different liver fractions

$(PhSe)_2$ was incubated with four different liver fractions: Total homogenate, S9, soluble and microsomal fraction. The presence of other possible metabolites derived from the molecule of $(PhSe)_2$ was analyzed by LC-MS. Fig. 1, 2 and 3 show the mass chromatograms of total homogenate, S9, soluble and microsomal fraction following different incubation times (0 and 1 hour) at 37 °C. $(PhSe)_2$ was found, with a retention time of 23.9 min and ion scan *m/z* 189, the same of standard solution of $(PhSe)_2$ (Fig. 4). GSH-selenol adduct was detected in four different liver fractions, already at baseline, with a retention time of 7 minutes (Fig. 1-3) and ion scan spectrum of *m/z* 462. Collision-induced dissociation of GSH-selenol adduct gave rise to a major fragment ion *m/z* 304 with resulted from selenol loss (Fig. 5).

3.2 $(PhSe)_2$ incubations with microsomal fraction

In microsomal incubation with $(PhSe)_2$ the amount of compound which remains free in the supernatant was analyzed by LC (Table 1) and the amount of compound that remains bound to protein in the precipitate was measured in the form of selenium by ICP (Table 2).

It was observed that when microsomal fraction was incubated with 100 µM $(PhSe)_2$, already at baseline, an amount of 45 µM $(PhSe)_2$ appears in the supernatant, regardless of the different conditions tested. After 1 hour, only 18 µM $(PhSe)_2$ appears in the supernatant (Table 1). The quantity of 57 ppb of selenium remained in the precipitate after 1 hour of incubation (Table 2).

The addition of GSH in incubation medium maintained constant the levels of $(PhSe)_2$ in the supernatant, even after 1 hour of incubation of 46 µM $(PhSe)_2$ (Table 1). The addition of GSH significantly reduced the amount of selenium in the precipitate (35 ppb) after 1 hour, when compared to all other groups (Table 2).

In microsomal incubation of $(PhSe)_2$ with NAC, a NAC-selenol adduct was detected, already at baseline, with a retention time of 13 minutes (Fig. 6) and ion scan *m/z* 318. Collision-induced dissociation of NAC-selenol adduct gave rise to two major fragment ions *m/z* 189 and 157 with resulted from selenol-thiol and selenol loss (Fig. 7). The amount $(PhSe)_2$ in supernatant after 1 hour of incubation (26 µM) is significantly less than the amount at 0 hour. The addition of NAC significantly reduced the amount of selenium in the precipitate (39 ppb), when compared to $(PhSe)_2$ group (Table 2).

The inhibition of microsomal P450 activity by preincubating the microsomal fraction with carbon monoxide did not alter the amount of $(PhSe)_2$ that appears in supernatant (52 µM at 0 hour and 20 µM at 1 hour) when compared to $(PhSe)_2$ group. Levels of selenium in the precipitate after 1 hour of incubation were 46 ppb. These values are significantly similar when compared to the values of microsomal fraction incubated in the presence of oxygen.

3.3 Potential of $(PhSe)_2$ to inhibit cytochrome P450 enzymes

The microsomal activity of cytochrome P450 enzymes responsible by formation of *N*, ethilbenzamide, a metabolite of *N, N* diethilbenzamide, was significantly reduced by $(PhSe)_2$ already at the lowest concentration tested (1 µM). The calculated IC_{50} value for microsomal activity inhibition by $(PhSe)_2$ was 78 µM (Table 3).

4 Discussion

$(PhSe)_2$ is a highly lipophilic compound, thus it is likely that its metabolism and elimination depends on metabolism in the liver. The aim of this study was to characterize the metabolism of the compound in rat liver *in vitro*. Metabolism occurs through reactions representing two distinct phases. Phase I reactions are most commonly described as "functionalization" reactions and include oxidations, reductions and hydrolyses, these

introduce a new polar functional group and are majority catalyzed by cytochrome P450 enzymes. Phase II reactions are most commonly describes as conjugation reactions, and include glucuronidation, sulfonation, acetylation, methylation and GSH conjugation. These cause a drastic increase in the polarity, and thus excretion of a drug (Nassar et al., 2009). In this way, we carried out metabolic study of $(PhSe)_2$ using four different liver homogenate fractions: total homogenate, S9, soluble and microsomal fraction. The results of four different fractions analyzed by LC-MS demonstrated the same profile described by appearance of initial compound $(PhSe)_2$ and a selenol-GSH adduct, characterized by LC-MS-MS.

There are many evidences that toxicology of $(PhSe)_2$ involves the oxidation of SH groups from proteins and of low-molecular weight thiol containing molecules such as GSH (Barbosa, 1998; Bruning et al., 2009). In this way, many works of our research group suggested the formation of the complex selenol-SR as product of oxidation of thiol groups (Nogueira 2004; Bruning et al., 2009), but it was never structurally characterized. This study showed, for the first time, the existence and characterization of adduct selenol-GSH.

An important observation of this study is that when $(PhSe)_2$ was incubated with different liver fractions, already at initial time occurred the formation of selenol-GSH adduct. These data indicates that $(PhSe)_2$ does not react enzymatically with GSH, but chemically, forming adducts. We propose that GSH, an endogenous nucleophile, reacts with $(PhSe)_2$ via a thiol nucleophilic attack in the selenium atom of $(PhSe)_2$, capturing the chemically reactive intermediate selenol, this electrophilic species is then free to covalently bind to proteins; thus preventing it from reacting with thiols of endogenous proteins.

To prove the formation of adducts between selenol and SH groups, we carried out microsomal incubations using NAC, an exogenous thiol compound. Already at initial time occurs the formation of selenol-NAC adduct, proving that complex formation occurred by

a chemical mechanism of nucleophilic attack in the selenium atom of $(PhSe)_2$. This study showed, for the first time, the existence and characterization of complex selenol-NAC.

Based on data from this study, we propose that the formation of adduct with GSH could be the first step in metabolism of the compound, involving the formation of mercapturic acid derivatives which are except in the urine. Mercapturic acids (MAs) are excretion products formed during the metabolism of electrophilic chemicals. MAs are formed from GSH S-conjugate via the MA pathway. When GSH S-conjugates metabolize to the corresponding MAs, γ-glutamyl and glycinyl residues are removed from de GSH S-conjugate by γ-glutamyl-transpeptidase and dipeptidases, with subsequent acetylation to MAs (*N*-acetyl-*L*-cysteine S-conjugates) by cysteine S-conjugate N-acetyltransferase (De Rooij et al., 1998). Future studies, using *in vivo* experimental models, will be needed to identify whether the liver selenol-GSH adducts identified by us in this work are excreted in urine as the corresponding MAs.

Many xenobiotics are converted to highly reactive intermediates in living systems where they subsequently interact with cell constituents. This has been widely implicated as a critical event in target organ toxicity induced by therapeutic and environmental chemicals (Hinson et al., 1994). The mechanism by which chemicals cause toxicity is for the most part unknown, but protein covalent binding by xenobiotic metabolites has long been associated with target organ toxicity (Cohen et al., 1997).

To analyze the interaction between $(PhSe)_2$ and microsomal fraction, microsomal fraction was incubated with 100 μM $(PhSe)_2$. It was observed that already at baseline, an amount of 45 μM $(PhSe)_2$ appeared in the supernatant, after 1 hour only 18 μM $(PhSe)_2$ was found in the supernatant, as well 57 ppb of selenium remaining in the precipitate after 1 hour of incubation. These results indicate that most of the compound, upon contact with liver microsomal fraction, rapidly bound to proteins and that the amount of compound that

bound to proteins increased with the increase of time. We believe that $(PhSe)_2$ reacted with essential thiol groups of proteins via a thiol nucleophilic attack in the selenium atom of $(PhSe)_2$, capturing the chemically reactive intermediate selenol, and oxidizing endogenous proteins. In this way, several works of our research group demonstrated that $(PhSe)_2$ causes reduction on the activity of δ-ALA-D and Na^+, K^+ ATPase, sulfhydryl containing enzymes, and relate, at least in part, this event to the toxicological effect of the compound (Nogueira et al., 2004, Borges et al., 2005; Prigol et al., 2007, 2008). Besides, studies with ebselen, an organoselenium compound, demonstrated that intracellular effect of covalent binding of the compound to essential thiol groups in protein causes inhibition in enzymes such as glutathione S-transfereses (Nikawa et al., 1994), monooxygenases (Ziegler et al., 1992) and the inhibition of IP_3-induced calcium release (Dimmeler et al., 1991).

To understand the role of GSH on $(PhSe)_2$ metabolism, further experimental protocols were carried out in the presence of GSH. The addition of GSH did not alter the initial amount of $(PhSe)_2$ in supernatant (45μM), however after 1 hour of incubation the levels of compound remained equal to the initial levels (46%). Furthermore, levels of selenium in the precipitate after 1 hour of incubation were 35 ppb, much lower than those observed in the incubation without GSH. These data indicate that GSH acts has a protective effect in tissues, since it forms an adduct with selenol, consequently preventing the oxidation of endogenous proteins. In this manner, several researchers have demonstrated the relationship between $(PhSe)_2$ administration and GSH levels. Maciel et al. (2000) have shown that GSH and other thiols (such as DTT) could be oxidized by $(PhSe)_2$ and that acute and two week exposure to $(PhSe)_2$ caused a decrease in the hepatic and renal content of non-protein-SH in mice. Adams et al. (1989) demonstrated that administration of $(PhSe)_2$ in mice decreased hepatic GSH content by 50% after 1 h exposure.

The hepatic cytochrome P450 monooxygenase catalyzes the oxidation of a large number of endogenous compounds and the majority of ingested environmental chemicals, leading to their elimination and often to their metabolic activation to toxic products. This enzyme system therefore provides our primary defense against xenobiotics and is a major determinant in the therapeutic efficacy of pharmacological agents (Raucy and Allen., 2001). The hepatic cytochrome P450 (CYP) monooxygenase catalyze is strictly on molecular oxygen and is inhibited by carbon monoxide. To evaluate the importance of hepatic P450s in $(PhSe)_2$ biotransformation we have conditionally inhibited microsomal P450 activity by preincubating the microsomes with carbon monoxide. We observed that on average 52 µM $(PhSe)_2$ appeared free after extraction, after 1 hour only 20 µM $(PhSe)_2$ was detected free. Levels of selenium in the precipitate after 1 hour of incubation were 46 ppb. These values are significantly similar when compared to the values of microsomes incubated in the presence of oxygen. These *in vitro* data together with those shown above indicate that $(PhSe)_2$ biotransformation does not depend on the cytochrome P450 monooxygenase enzymes.

Some studies conducted in our research group demonstrated that administration of the $(PhSe)_2$ in different experimental models tends to be more toxic for mice than for rats (Nogueira et al., 2004; Prigol et al., 2009b). Moreover, it was shown that the compound caused more toxicity for rat pups than for adult rats (Prigol et al., 2007; 2008; 2009b; 2010). There were speculations that these differences were related to a possible biotransformation of the compound, and a possible metabolite would be responsible for the toxic effects of the compound in animals. Considering results of present study, we can infer that $(PhSe)_2$ causes toxicity by reacting with endogenous thiol groups in protein to form protein seleno dissulfides. We can also infer that differences in toxicity related to age or species could be associated to conjugation of the compound with GSH, since the

formation of this adduct protects against protein oxidation. In this manner, Degan and Neumann (1978) report that GSH content may be critical in inhibition of the cellular macromolecule bindings to foreign compounds like aflatoxin B1, elimination of which rely mainly on GSH-conjugation. Additionally, Prescott (1983) related species differences in susceptibility to the acetaminophen hepatotoxicity, in his study, mice and hamsters are more sensitive, while rats are more resistant. Other studies revealed that he hepatic GSH levels are equal in resistant and susceptible species, while the rate of GSH conjugation to acetaminophen varies greatly in different animal species (Allamed et al., 1987; Smolarek et al., 1990).

Oxidative metabolism mediated by the superfamily of hepatic enzymes known as the cytochrome P450 represents a significant clearance pathway for many drugs prescribed today, and the inhibition of the metabolism of one drug by another drug taken concurrently by the patient has now been shown to be a very important cause of adverse drug reactions (Wienkers and Heath., 2005). At the center of P450 catalysis is the iron protoporphyrin IX (heme) with a thiolate of conserved cysteine residue as the fifth ligand (Nassar et al., 2009). Given that, the reactive groups present within enzyme's active site are nucleophiles, irreversible inhibition via covalent binding to electrophilic species can occur. Thus, we investigated whether $(PhSe)_2$ could cause an inhibitory effect on cytochrome P450s, since it readily reacts with thiol groups. Findings reported in this study clearly demonstrated that $(PhSe)_2$ inhibited P450 mediated metabolism of *N, N,*dietil benzamide. Our data indicate that $(PhSe)_2$ can interfere with the metabolism of other drugs that need to be metabolized by P450, when administered concomitantly with them. This interaction can reduce the formation of metabolites of drugs and change their effect.

Partial inactivation of cytochrome P-450 was also observed by organochalcogen compound ebselen (Kuhn-Velten and Sies., 1989). It appears to be associated with a Se-N

bond cleavage in the ebselen molecule due to interaction with SH groups of microsomal proteins *in vitro* (Kamigata et al., 1986). Ebselen could interact either directly (i.e. by formation of a Se-S bond) or indirectly (i.e. by formation of a $Se\text{-}Fe^{3+}$ complex and consecutive protonation of the S- anion) with the thiolate group in rat liver microsomal cytochrome P-450 (Kuhn-Velten and Sies., 1989). Furthermore*, in vivo*, ebselen is obviously largely bound to plasma proteins and may react with intracellular thiols, notably GSH, before reaching the cytochrome P-450-containing membranes of the endoplasmic reticulum *in vivo.* This may contribute to the low toxicity of this substance (Wendel et al., 1986).

The main results of our in vitro study indicate that: (1) $(PhSe)_2$ was not metabolized by phase I reactions catalyzed by cytochrome P450 enzymes; (2) $(PhSe)_2$ reacted chemically with GSH and NAC forming adducts; (3) $(PhSe)_2$ reacted with SH groups from proteins, and the presence of GSH and NAC decreased this covalent binding and (4) $(PhSe)_2$ reduced microsomal activity of cytochrome P450 enzymes.

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5 References

Adams Jr, W J, Kocsis JJ, Snyder R. Acute toxicity and urinary excretion of Diphenyl diselenide. Toxicology Letters, (1989) 48: 301-310.

Allameh A, Saxena M, Mehta LM, Raj HG. Comparison of pulmonary and hepatic glutathione depletion by diethyl maleate. Med. Sci. Res., (1987) 15: 957–958.

Barbosa, N.B.V., Rocha, J.B.T., Zeni, G., Emanuelli, T., Beque, M.C., Braga, A.L. Effect of organic forms of selenium on delta-aminolevulinate dehydratase from liver, kidney, and brain of adult rats. Toxicol. Appl. Pharm., (1998) 149: 243-253.

Borges LP, Nogueira CW, Panatieri RB et al. Acute liver damage induced by 2-nitropropane in rats: Effect of diphenyl diselenide on antioxidant defenses. Chem. Biol. Interactions, (2005) 160: 99-107.

Borges, V.C., Rocha, J.B.T., Nogueira, C.W. Effect of diphenyl diselenide, diphenyl ditelluride and ebselen on cerebral Na+, K+-ATPase activity in rats. Toxicology, (2005) 215: 191-197.

Brüning, CA., Prigol, M., Barancelli, DA., Nogueira, CW., Zeni, G. Disubstituted diaryl diselenides inhibit δ-ALA-D and Na+, K+-ATPase activities in rat brain homogenates in vitro. Molecular and Cellular Biochemistry., (2009) 332: 17-24.

Cohen SD, Pumford NR, Khairallah EA, Boekelheide K, Pohl LR, Amouzadeh HR, Hinson JA. Selective protein covalent binding and target organ toxicity. Toxicology And Applied Pharmacology, (1997) 143(1), 1-12.

Constantino, L., Paixão P., Moreira R., Iley, J., Do Rosário V.E. Metabolism of primaquine by liver homogenate fractions. Evidence for MAO and Cyt P450 involvement in the oxidative deamination of primaquine to Carboxyprimaquine. Exp. Toxic Pathol., (1999) 51: 299-303.

De Rooij BM, Commandeur JN, Vermeulen PE. Mercapturic acid as biomarkers of exposure to electrophilic chemicals: aplications to environmental and industrial chemicals. Biomarkers, (1998) 3: 239-303,

Degan GH, Neumann, HG. The major metabolite of aflatoxin B1 in the rat is a glutathione conjugate. Chemico Biol. Interaction, (1978) 22: 239-255.

Dimmeler S, Briine B and Ullrich V. Ebselen prevents inositol (1,4,5)-trisphosphate binding to its receptor. Biochem Pharmaco (1991) l42: 1151-1153.

Ghisleine, G., Porciúncula, L.O., Cimarosti, H., Rocha, J.B.T., Salbego, C.G., Souza, D.O. Diphenyl diselenide protects rat hippocampal slices submitted to oxygen–glucose deprivation and diminishes inducible nitric oxide synthase immunocontent. Brain Res. (2003) 986: 196-199.

Ghisleni, G., Kazauckas, V., Both, FL., Pagnussat, N, Mioranzza, S, Rocha, JBT, Souza, DO. Diphenyl diselenide exerts anxiolytic-like effect in Wistar rats: Putative roles of GABAA and 5HT receptors. Prog. Neuro-Psychoph. (2008) 32: 1508-1515.

Hinson JA, Pumford NR, Nelson SD. The role of metabolic activation in drug toxicology. Drug Metabolism Rev. (1994) 26, 395-412.

Kamigata N, Takata M, Matsuyama H and Kobayashi M. Novel ring opening reaction of 2-aryl-1,2-benzisoselenazol- 3(2H)-one withthiols. Heterocycles (1986) 24: 3027- 3030.

Kuhn-Velten N and Sies H, Optical spectral studies of ebselen interaction with cytochrome P450 of rat liver microsomes. Biochem Pharmacol (1989) 38: 619-625.

Lowry, O H., Rosebrough, N.J., Farr, A L., Randall, R. Protein measurement with the Folin phenol reagent. J. Biol. Chem, (1951) 193: 265-275.

Luchese, C., Brandao, R., Oliveira de, R., Nogueira, C.W., Santos, F.W. Efficacy of diphenyl diselenide against cerebral and pulmonary damage induced by cadmium in mice Toxicol. Lett. (2007) 173 (3): 181-190.

Maciel, E.N., Bolzan, R.C., Braga, A.L., Rocha, J.B.T. Diphenyl diselenide and diphenyl ditelluride differentially affect delta-aminolevulinate dehydratase from liver, kidney, and brain of mice. J. Biochem. Mol. Toxic. (2000) 14: 310-319.

Meotti, F.C., Stangherlin, E.C., Zeni, G., Rocha, J.B.T., Nogueira, C.W. Protective role of aryl and alkyl diselenides on lipid peroxidation. Environ. Res. (2004) 94: 276–282.

Mugesh, G., Du Mont, WW., Sies, H. Chemistry of biologically important synthetic organoselenium compounds. Chem. Rev. (2001) 101: 2125-2179.

Nassar AF, Hollenberg P F, Scatina JA. Drug Metabolism Handbook: Concepts and Aplications. A John Wiley & Sons, INC., Publication, 2009.

Nikawa T, Schuch G, Wagner G and Sies H, Interaction of ebselen with glutathione S-transferase and papain in vitro. Biochem Pharmacol , (1994) 47: 1007-1012.

Nogueira CW, Meotti FC, Curte E, Pilissao C, Zeni G, Rocha JBT. Investigations into the potential neurotoxicity induced by diselenides in mice and rats. Toxicology (2003a) 183: 29-37.

Nogueira CW, Borges VC, Zeni G, Rocha JBT. Organochalcogens effects on delta-aminolevulinate dehydratase activity from human erythrocytic cells in vitro. Toxicology (2003b)191: 169-178.

Nogueira CW, Zeni G, Rocha JBT. Organoselenium and organotellurium compounds: toxicology and pharmacology. Chem. Rev. (2004) 104: 6255-6285.

Prescott LF, Critchley J. The Treatment of Acetaminophen Poisoning. Annual Review of Pharmacology and Toxicology. (1983) 23: 87-101.

Prigol M, Wilhelm EA, Schneider CC, Rocha JBT, Nogueira CW, Zeni G. Involvement of oxidative stress in seizures induced by diphenyl diselenide in rat pups. Brain Res. (2007) 1147: 226-232.

Prigol M, Wilhelm EA, Stangherlin EC, Barancelli DA, Nogueira CW, Zeni G. Diphenyl diselenide-induced seizures in rat pups: Possible interaction with glutamatergic system. Neurochem. Res. (2008) 33: 996-1004.

Prigol M, Luchese C, Nogueira CW. Antioxidant effect of diphenyl diselenide on oxidative stress caused by acute physical exercise in skeletal muscle and lungs of mice. Cell Biochem. Funct. (2009a) 27 (4), 216-222.

Prigol M, Schumacher RF, Nogueira CW, Zeni G. Convulsant effect of diphenyl diselenide in rats and mice and its relationship to plasma levels. Toxicol Lett (2009b) 189(1): 35-39.

Prigol M, Pinton S, Schumacher RF, Nogueira CW, Zeni G. Convulsant action of diphenyl diselenide in rat pups: measurement and correlation with plasma, liver and brain levels of compound. Arch Toxicol (2010) 84: 373–378.

Raucy JL, Allen SW. Pharmacogen. J. (2001) 1: 178–186

Rosa RM, Roesler R, Braga AL, Saffi J, Henriques JA. Pharmacology and toxicology of diphenyl diselenide in several biological models. Braz. Med. Biol. Res. (2007) 40: 1287-1304.

Savegnago L, Trevisan M, Alves D et al. Antisecretory and antiulcer effects of diphenyl diselenide. Environ. Toxicol. Pharmacol. (2006) 21: 86-92.

Savegnago L, Pinto LG, Jesse CR, Alves D, Rocha JBT, Nogueira CW, Zeni G. Antinociceptive properties of diphenyl diselenide: Evidences for the mechanism of action. Eur. J. Pharmacol. (2007) 555: 129-138.

Smolarek TA, Higging CV, Amacher T. Metabolism and cytotoxicity of acetaminophen in hepatocyte cultures from rat, rabbit, dog and monkey. Drug Metab. Dis., (1990) 18: 859–863.

Wendel A, Otter R, Tiegs G. Inhibition by ebselenof microsomal NADPH-cytochrome P450-reductase in vitro but not in vivo.BiochemPharmacol (1986) 35: 2995- 2997.

Wienkers LC, Heath TG. Predicting in vivo drug interactions from in vivo drud discovery data. Biological Chemistry, (2005) 257:3073-3083.

Ziegler DM, Graf P, Poulsen LL, Stahl W and Sies H, NADPH-dependent oxidation of reduced ebselen, 2- selenylbenzanilide, and of Z-(methylseleno)benzanilidecatalyzed by pig liver flavin-containing monooxygenase, Chem Res Toxic., (1992) 5: 163-166.

Tables

Table 1: $(PhSe)_2$ concentration in supernatant after incubation with liver microsomal fraction. The sample preparation and data processing are described in Section 2.3.2

	Incubation Time		
	0 hour	**0.5 hour**	**1 hour**
$(PhSe)_2$ 100 µM	44.1 ± 8.4 µM	26.2 ± 13.4 µM	18.1 ± 10.07 µM[a, b]
$(PhSe)_2$ 100 µM + GSH 1mM	45.5 ± 0.8 µM	45.1 ± 0.10 µM	46.3 ± 4.50 µM
$(PhSe)_2$ 100 µM + NAC 1mM	35.8 ± 4.1 µM	32.2 ± 1.5 µM	26.5 ± 0.30 µM[a,c]
$(PhSe)_2$ 100 µM at CO_2	52.0 ± 10.8 µM	35.2 ± 12.4 µM	20.6 ± 6.60 µM[a,d]

Results are average of three experiments ± SD.
([a]) Denotes $p < 0.05$ as compared to the $(PhSe)_2$ 100 µM + GSH 1mM (1 hour) group.
([b]) Denotes $p < 0.05$ as compared to the $(PhSe)_2$ 100 µM (0 hour) group.
([c]) Denotes $p < 0.05$ as compared to the $(PhSe)_2$ 100 µM + NAC 1mM (0 hour) group.
([d]) Denotes $p < 0.05$ as compared to the $(PhSe)_2$ 100 µM + at CO_2 (0 hour) group.

Table 2: Concentration of selenium in microsomal fraction precipitate.

Incubation Medium	**Selenium (ppb)**[a]
$(PhSe)_2$ 100 uM	52.76 ± 10.79[a]
$(PhSe)_2$ 100 uM + GSH 1mM	35.50 ± 0.84[b]
$(PhSe)_2$ 100 uM + NAC 1mM	39.45 ± 0.35 [b]
$(PhSe)_2$ 100 uM at CO_2	46.00 ± 3.9 [a]

Results are average of three experiments ± SD.
Selenium concentration in microsomal precipitate is expressed as ppb (parts per billion) in the sample.
([a]) Denotes $p < 0.05$ as compared to the $(PhSe)_2$ 100 µM + GSH 1mM and (PhSe)2 100 µM + NAC 1mM groups.
([b]) Denotes $p < 0.05$ as compared to other groups.

Table 3: $(PhSe)_2$ potential to inhibit cytochrome P450 enzymes, assessment through the initial rate of formation of *N*, ethilbenzamide, a metabolite of *N, N* diethilbenzamide.

$(PhSe)_2$ (uM)	**Microsomal Activity (%)**[a]
0	100 ± 0.00
1	93.0 ± 4.4
10	64.6 ± 6.42
100	46.1 ± 1.73
1000	23.1 ± 8.88

Results are average of three experiments ± SD.

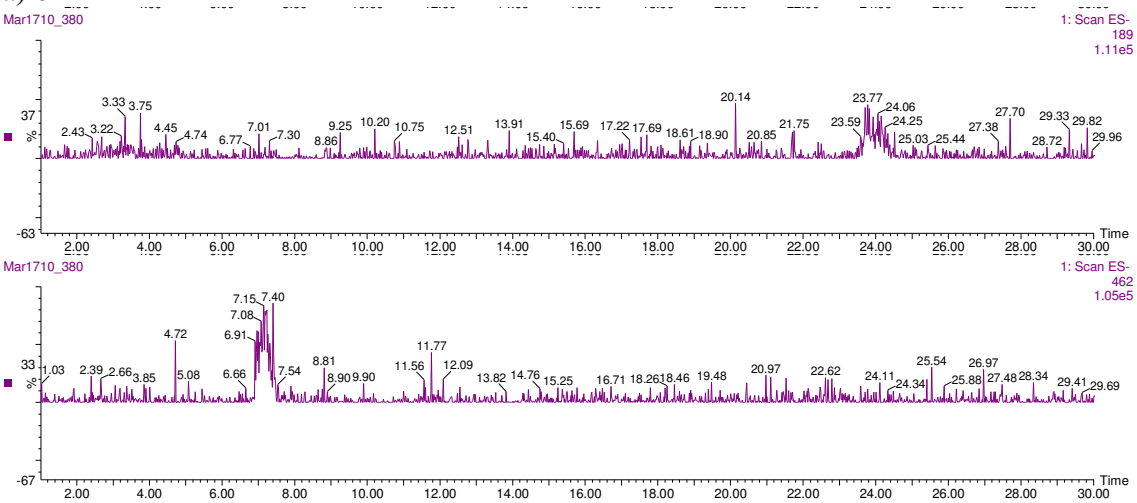
[a] Microsomal activity was reported as percentage of microsomal activity after preincubation with different $(PhSe)_2$ concentrations. Potential of $(PhSe)_2$ to inhibit cytochrome P450 was assessment by calculating the initial rate of formation of the substrate N, ethilbenzamide in the first 20 minutes of incubation of the N, N diethilbenzamide in the presence and absence of $(PhSe)_2$.

The incubation medium without pre incubation with $(PhSe)_2$ was considered as 100% microssomal activity. All results are different all results from each other.

Figures:

Figure 1

a) 0



b)

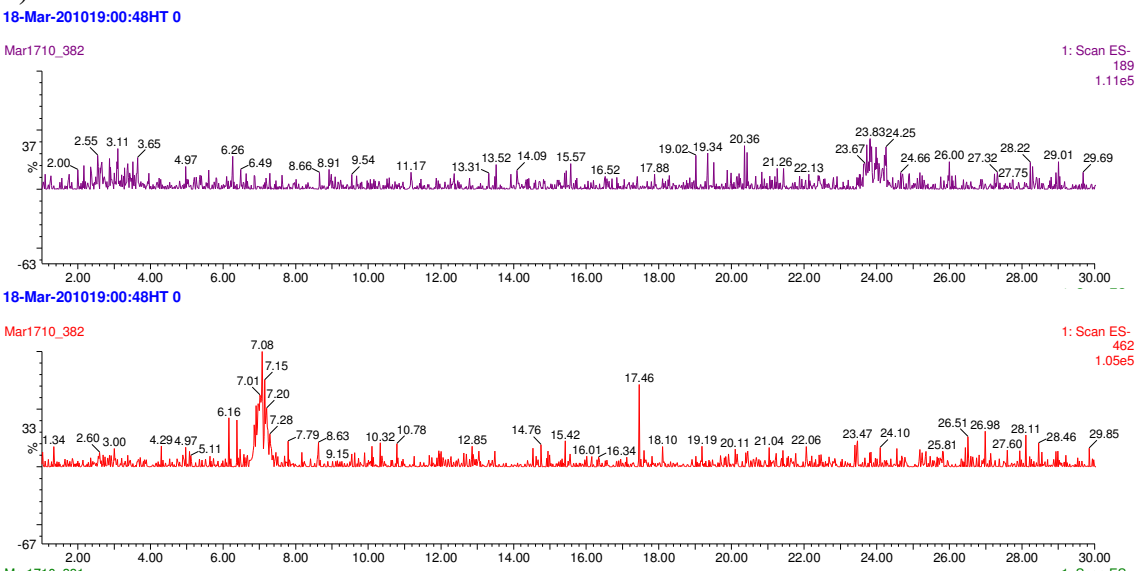
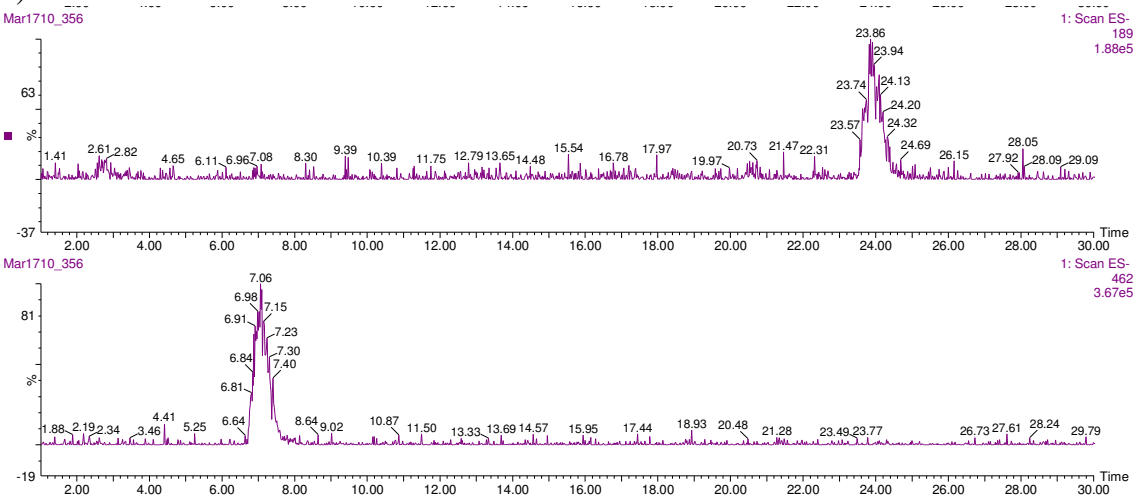


Figure 1: Chromatogram of LC-MS analysis of Liver Total Homogenate incubated with $(PhSe)_2$ 100 uM (a) 0 hour (b) 1 hour.

Figure 2

a)



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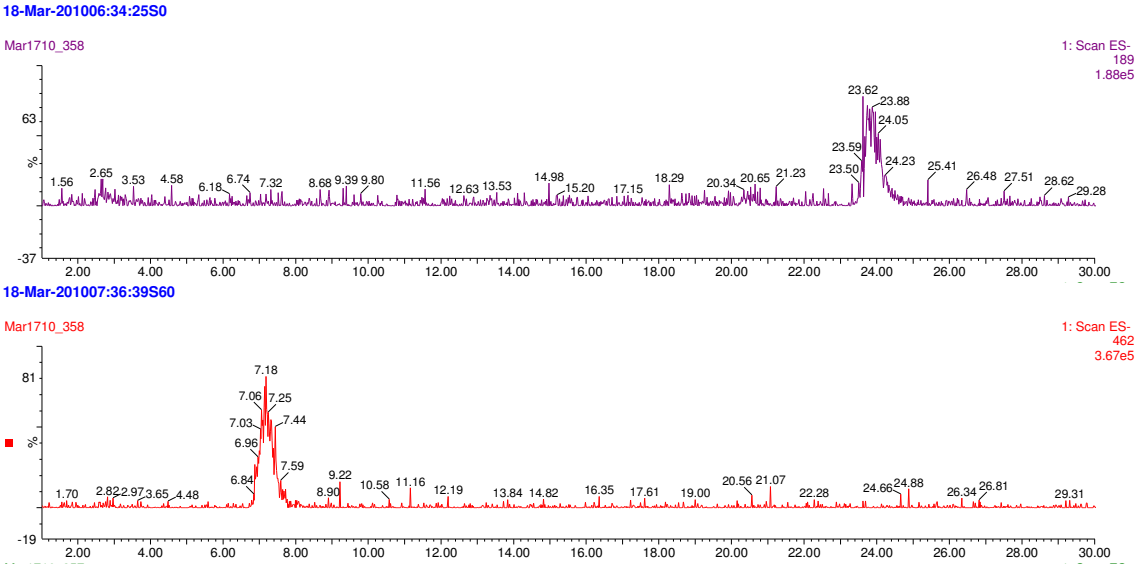
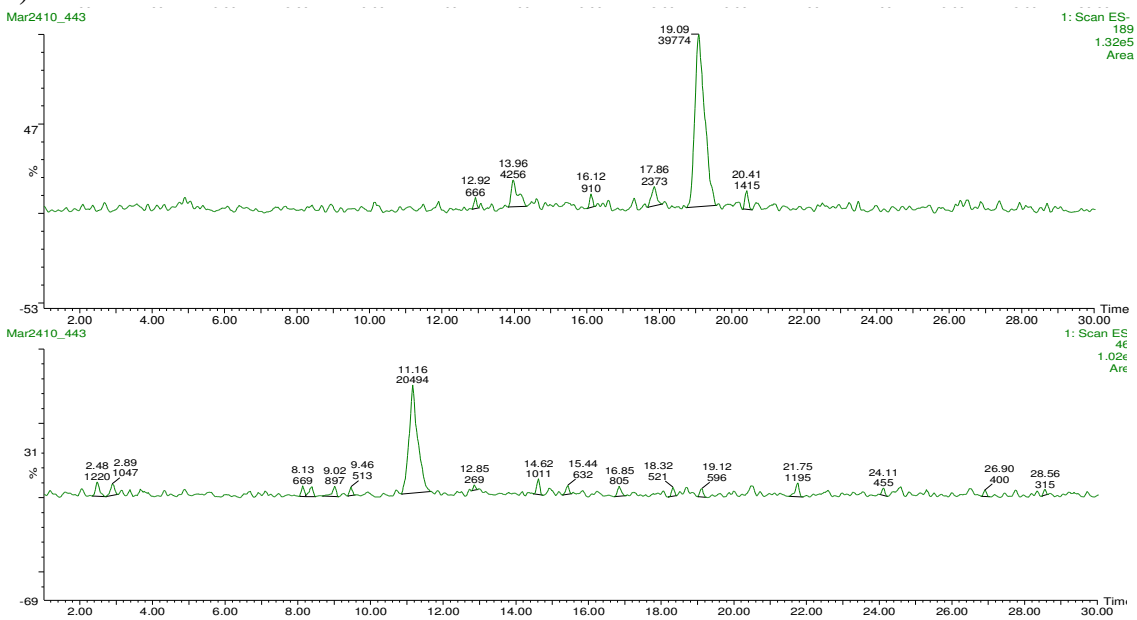


Figure 2: Chromatogram of LC-MS analysis of Liver Soluble Fraction incubated with $(PhSe)_2$ 100 uM (a) 0 hour (b) 1 hour.

Figure 3

a)



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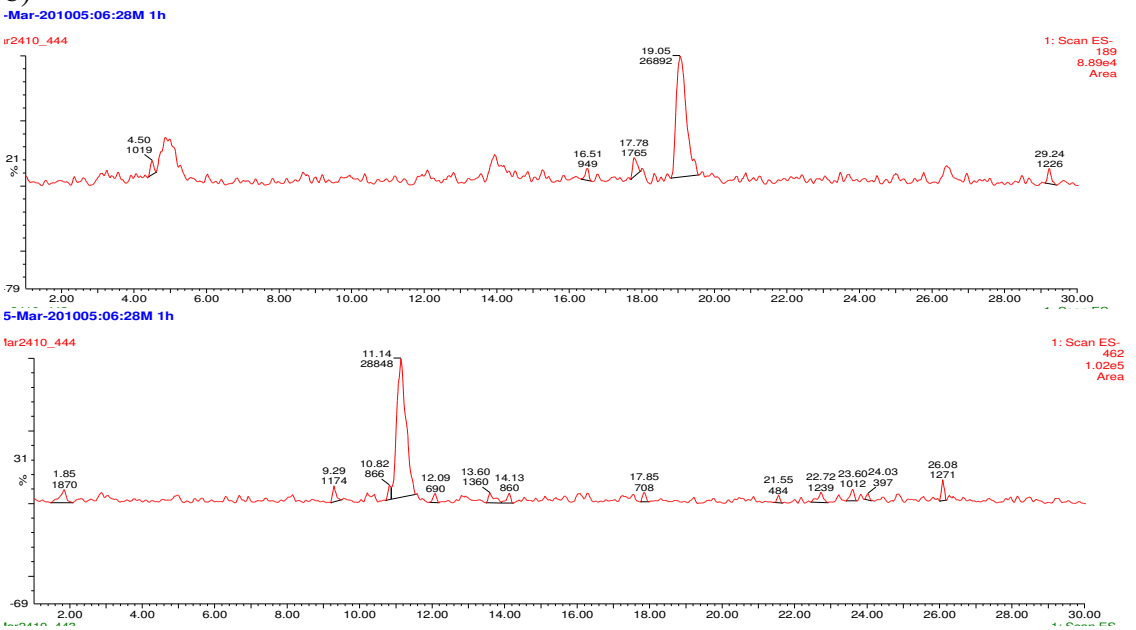

Figure 3: Chromatogram of LC-MS analysis of Liver Microsomal Fraction incubated with $(PhSe)_2$ 100 uM (a) 0 hour (b) 1 hour.

Figure 4

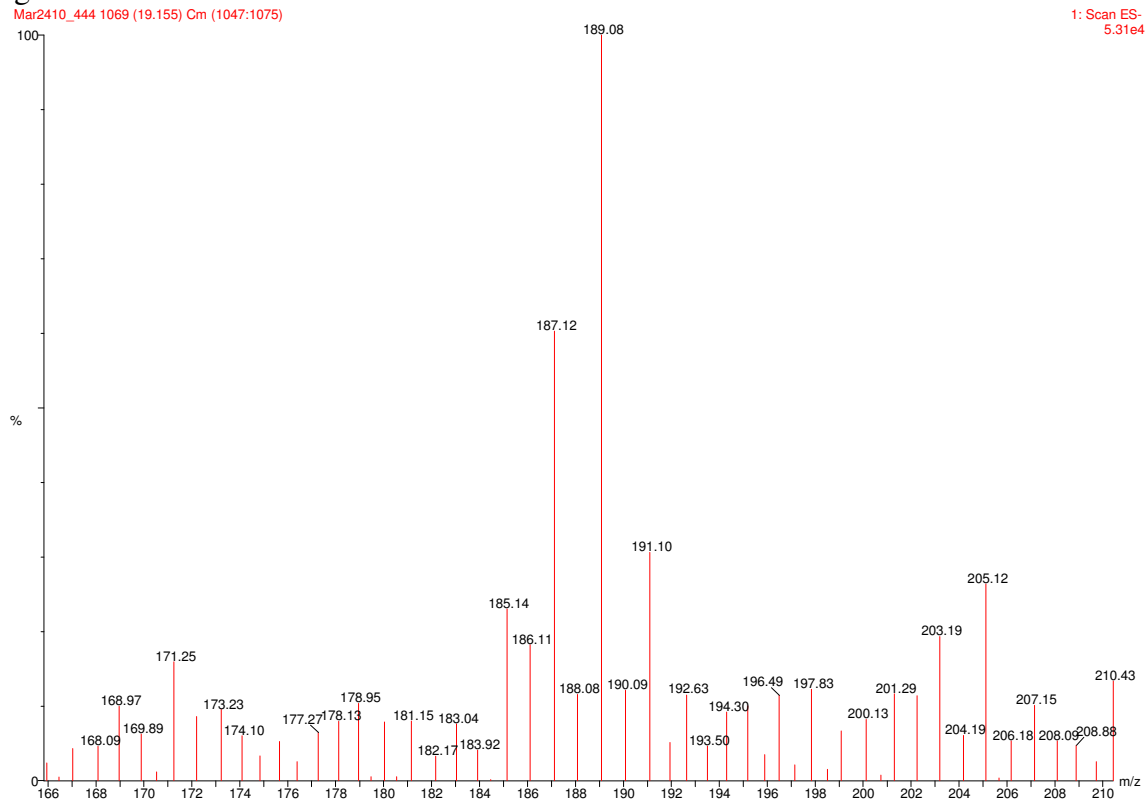

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Figure 4: MS ion spectra obtained of $(PhSe)_2$ (*m/z* 189) eluted at 23.9 min. The ion product fragment ion and proposed structure are presented.

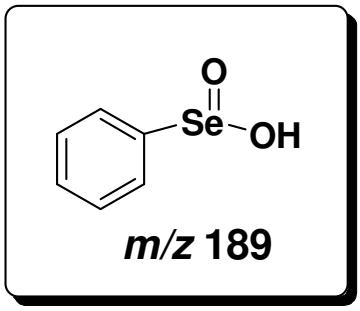


Figure 5

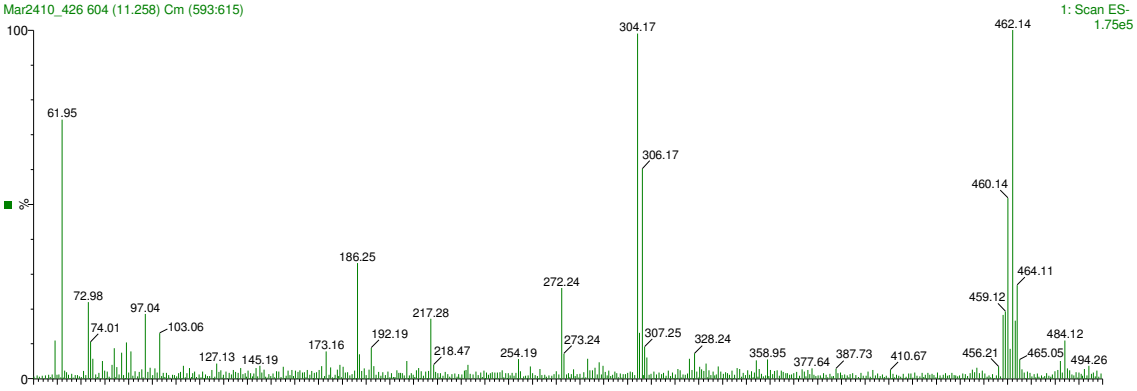


Figure 5: MS ion spectra obtained of GSH-selenol adduct (*m/z* 462) eluted at 7 min. The ion structure, product fragment ions and proposed structure are presented.

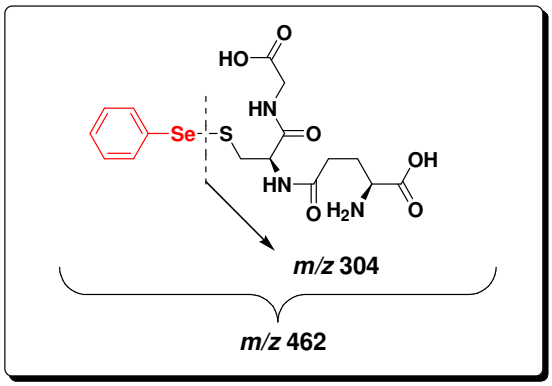
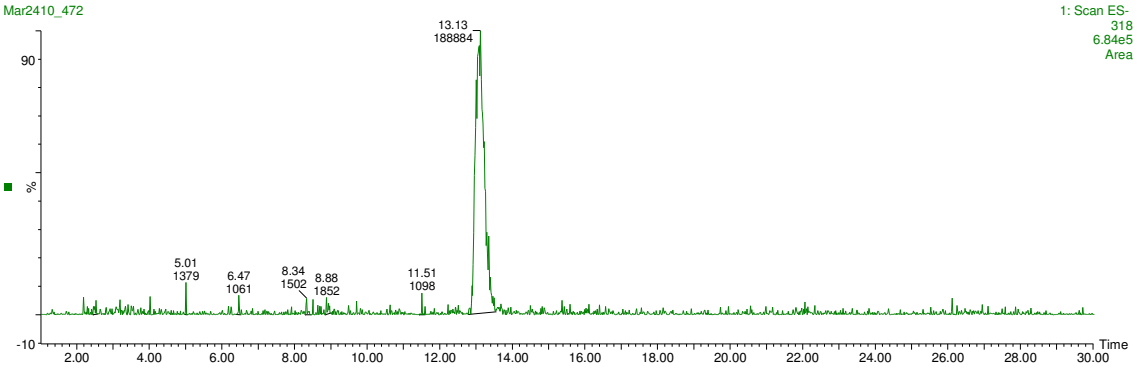


Figure 6

(a)



(b)

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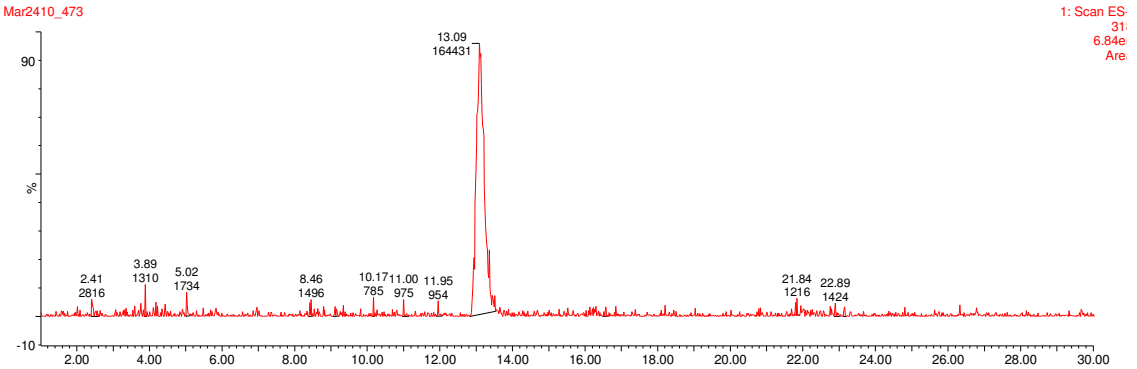


Figure 6: Chromatogram of LC-MS analysis of Liver Microsomal Fraction incubated with $(PhSe)_2$ 100 uM + NAC 1mM (a) 0 hour (b) 1 hour.

Figure 7

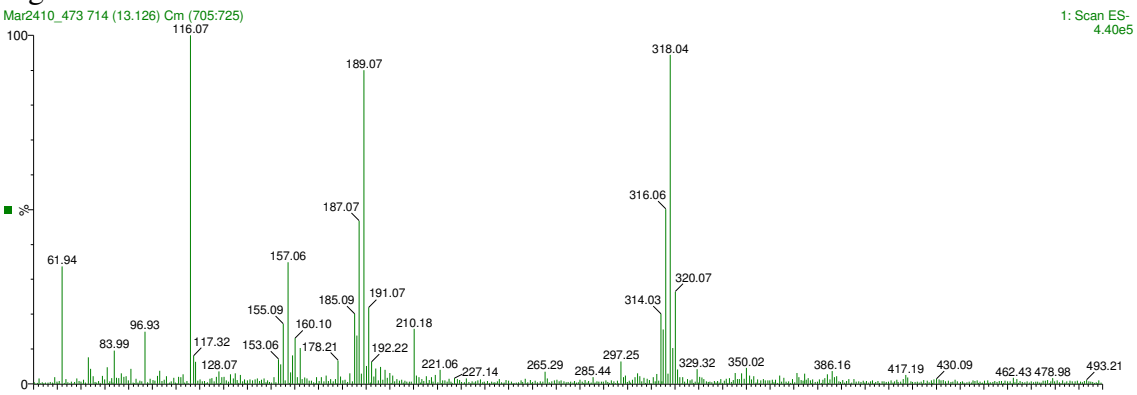
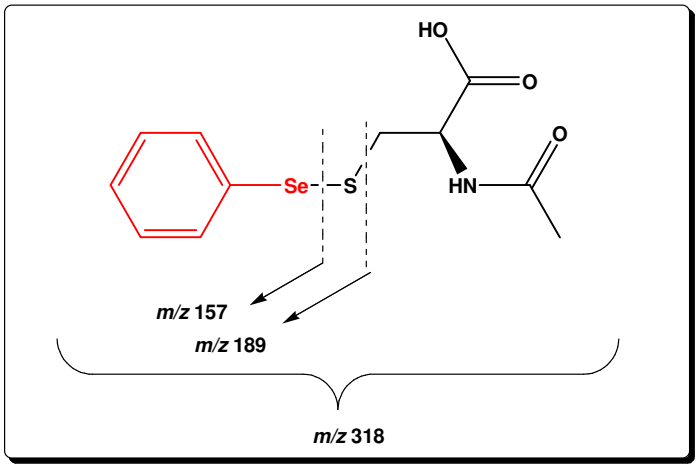


Figure 7: MS ion spectra obtained of NAC-selenol adduct (*m/z* 318) eluted at 13 min. The ion structure, product fragment ions and proposed structures are presented.



5. DISCUSSÃO

5.1 Convulsões Induzidas pelo Disseleneto de Difenila e Mecanismos Neuroquímicos Envolvidos nas Convusões Induzidas pelo Composto

Muitos estudos tem sido realizados com o objetivo de elucidar as propriedades farmacológicas do $(PhSe)_2$. Tem sido documentado que este composto de Se possui propriedade antioxidante (Rossato et al., 2002; Meotti et al., 2004; Prigol et al., 2009), anti-úlcera (Savegnago et al., 2006), neuroproteção (Ghisleni et al., 2003), hepatoproteção (Borges et al., 2005), anti-inflamatória e antinociceptiva (Savegnago et al., 2007a,b), do tipo ansiolítica (Ghisleni et al., 2008) e anti-hiperglicêmica (Barbosa et al., 2006). Porém, destacam-se também vários efeitos toxicológicos do composto, principalmente relacionados ao SNC (Nogueira et al., 2001, 2002, 2003a). No entanto, os estudos realizados, até o momento, com o $(PhSe)_2$ utilizaram somente modelos experimentais com animais adultos.

Nas últimas décadas tem aumentado o número de pesquisas demonstrando que há uma grande diferença na suscetibilidade aos efeitos tóxicos de drogas, e que esta está relacionada a diferentes estágios da vida (bebês, crianças, adultos e velhos) (Alcorn and McNamara., 2003). Os bebês, em particular, apresentam muitas mudanças fisiológicas e bioquímicas relacionadas ao desenvolvimento, que afetam a disposição e conseqüente efeito das drogas. Neste contexto, consideramos importante a investigação dos efeitos toxicológicos do $(PhSe)_2$ em ratos bebês, para melhor entender o comportamento deste composto na fase do desenvolvimento.

No **Artigo 1** demonstrou-se, pela primeira vez, que o $(PhSe)_2$, administrado pela via oral, causa toxicidade em ratos bebês, evidenciada pelo aparecimento de convulsões. Nogueira et al. (2003a) demonstraram que o $(PhSe)_2$ causava convulsões em camundongos adultos, porém este efeito não era observado em ratos adultos. A respeito desta diferença entre adultos e bebês, Mares et al. (2004) demonstraram que a penetração de drogas no cérebro é de extraordinária importância para a obtenção dos efeitos farmacológicos ou toxicológicos. Neste sentido, a barreira hemato-encefálica, que controla a entrada de drogas no cérebro não está perfeitamente formada no período pós natal, tanto de animais

experimentais quanto no homem (Mares et al., 2000; Morgane et a., 2002). E muitas drogas com efeito convulsivante, que não atravessam a barreira hemato-encefálica madura, podem exibir efeitos tóxicos após a administração sistêmica em ratos bebês (Mares et al., 2004).

Do mesmo modo, verificou-se que as convulsões causadas pela administração do $(PhSe)_2$ são dependentes da dose, sendo que a dose do composto a qual induz convulsão é cerca de 10 vezes superior a dose que possui propriedades farmacológicas (Nogueira et al., 2004; Savegnago et al., 2007a,b). Porém, sabe-se que em virtude de nos primeiros anos de vida o cérebro estar em desenvolvimento, convulsões nesta faixa etária podem desencadear sérios prejuízos neurológicos que podem se refletir na idade adulta (Vingerhoets, 2006).

As análises bioquímicas realizadas no cérebro dos animais, após o episódio convulsivo, demonstraram um aumento nos níveis de peroxidação lipídica e um aumento da atividade da enzima catalase, sugerindo que o estresse oxidativo está associado às convulsões induzidas pelo $(PhSe)_2$, podendo ocasionar um dano neuronal. Além disso, observou-se uma significante diferença nos níveis de TBARS e na atividade da δ-ALA-D e Na^+, K^+-ATPase entre os ratos que apresentaram e os que não apresentaram convulsão, na dose de 150 mg/kg, o que sugere que o episódio convulsivo pode estar diretamente relacionado com o estresse oxidativo e a injúria cerebral no modelo convulsivo induzido por $(PhSe)_2$**.** Na dose de 50 mg/kg de $(PhSe)_2$, os níveis de TBARS não foram alterados em ambos os grupos, com ou sem convulsão, o que sugere que o estresse oxidativo está em parte relacionado com a dose do composto administrada. As espécies reativas podem ser nocivas para as células, devido à oxidação de constituintes essenciais como lipídeos, proteínas e DNA, que podem ser medidos por identificação de seus produtos formados durante as reações, como o MDA (Reiter, 1995). Tem sido descrito que os radicais livres estão envolvidos na gênese das convulsões, um exemplo disso é que a administração intracortical de ferro induz descargas epileptiformes e concomitante aumento de radicais livres no cérebro e, ambos os fenômenos são inibidos por antioxidantes (Jyoti et al., 2009).

Outro resultado importante demonstrado neste trabalho foi a redução da atividade das enzimas δ-ALA-D e Na^+, K^+-ATPase, que são enzimas sulfidrílicas e sensíveis a agentes oxidantes (Nogueira et al., 2003b; Borges et al., 2005). De fato, estudos demonstraram que a toxicidade dos compostos orgânicos de selênio está diretamente relacionada com sua capacidade de oxidar grupos SH (Spalholz et al., 2001).

A importância destes achados está no fato de que a inibição da δ-ALA-D pode levar ao acúmulo do seu substrato, o δ-ALA (Emanuelli et al., 2001), o qual pode se auto-oxidar,

dando origem às espécies reativas de oxigênio. Estas, por sua vez, são nocivas aos sistemas biológicos, uma vez que podem oxidar biomoléculas importantes, provocando injúria tecidual e morte celular (Emanuelli et al. 2003). Existem evidências que sugerem o envolvimento do δ-ALA, substrato da δ-ALA-D, em manifestações neurológicas como as convulsões (Kappas et al., 1995; Emanuelli et al., 2003).

Do mesmo modo, tem sido descrito que os radicais livres podem estar relacionados com a inativação da Na^+-K^+-ATPase (Dawson & Dawson, 1996). Como a Na^+-K^+-ATPase é uma proteína integral de membrana, ela é um alvo fácil ao ataque dos radicais livres na membrana lipídica, principalmente no cérebro, uma estrutura rica em ácidos graxos poliinsaturados, os quais são altamente suscetíveis à lipoperoxidação (LPO). O prejuízo na atividade desta enzima ocasiona um aumento da excitabilidade neuronal (Grisar et al., 1983; Furian et al., 2007). Sabe-se que a Na^+-K^+-ATPase desempenha papel primordial na manutenção do gradiente iônico, uma vez que sua inibição aumenta a excitabilidade neuronal e facilita o aparecimento da convulsão (Vasilets and Schwarz, 1993; Fighera et al., 2006; Royes et al., 2007; Furian et al., 2007).

Além desses achados, também se observou que a administração oral do $(PhSe)_2$ em ratos bebês inibiu a atividade da enzima δ-ALA-D hepática. De fato, compostos que são absorvidos pelo trato gastrointestinal sofrem um extenso metabolismo hepático (Timbrell, 2000). Associado a isso está a capacidade dos compostos de selênio em oxidar grupos SH (Nogueira et al., 2004), tornando a enzima δ-ALA-D hepática alvo da ação destes compostos (Barbosa et al., 1998; Farina et al., 2003).

Neste contexto pode-se inferir que o $(PhSe)_2$ demonstrou efeitos neurotóxicos quando administrado pela via oral em ratos bebês. Esses efeitos foram caracterizados pelo aparecimento de convulsões, as quais podem estar, pelo menos em parte, associadas ao estresse oxidativo.

Estudos têm demonstrado que compostos organocalcogênios, dentre eles o $(PhSe)_2$, atuam sobre o sistema glutamatérgico (Nogueira et al., 2004). O **artigo 2**, no qual o objetivo foi investigar a interação entre o sistema glutamatérgico e o aparecimento de convulsões induzidas pelo (PhSe) em ratos bebês, demonstrou que a administração concomitante de glutamato e $(PhSe)_2$ aumenta a latência para o primeiro episódio convulsivo induzido pelo $(PhSe)_2$. Este resultado sugere que o efeito observado nos animais seja apenas do glutamato, já que os dois compostos, como são administrados em grande quantidade (glutamato 4g/kg e $(PhSe)_2$ 500 mg/kg), devem competir pela

metabolização hepática, fazendo com que uma menor dose de $(PhSe)_2$ chegue ao cérebro. Alguns dados obtidos neste trabalho sustentam esta hipótese, tais como: marcadores de estresse oxidativo, como o TBARS e enzimas antioxidantes, como a catalase, estão alterados nos grupos tratados com $(PhSe)_2$, enquanto estes mesmos parâmetros estão inalterados nos animais tratados somente com o glutamato. Estes resultados corroboram com os apresentados no **artigo 1**, evideciando que as convulsões induzidas pelo $(PhSe)_2$ em ratos bebês estão associadas ao estresse oxidativo.

Além disso, demonstrou-se que o $(PhSe)_2$ modula receptores glutamatérgicos do tipo NMDA, uma vez que a pré-administração de MK-801 aumentou a latência para o primeiro episódio convulsivo induzido por $(PhSe)_2$, na dose de 500 mg/kg (**Artigo 2**) e aboliu completamente as convulsões induzidas por $(PhSe)_2$, na dose de 50 mg/kg (**Anexo 1**). Em contrapartida, o $(PhSe)_2$ não causa convulsões por agir em receptores glutamatérgicos do tipo AMPA/cainato, uma vez que a pré-administração de DNQX não alterou a latência para o primeiro episódio convulsivo induzido por $(PhSe)_2$ nas doses de 50 e 500 mg/kg (**Artigo 2**) e (**Anexo 1**). Por fim, demonstrou-se que o $(PhSe)_2$ diminui a captação de glutamato em sinaptossomas de ratos bebês. A captação de glutamato é um dos mecanismos pelo qual o glutamato é removido da fenda sináptica, e esta inibição contribui para um aumento nas concentrações do glutamato extracelular. Em conjunto, os resultados do **artigo 2** demonstram que o $(PhSe)_2$ produz convulsões em ratos bebês atuando, pelo menos em parte, nos receptores glutamatérgicos ionotrópicos do tipo NMDA e inibindo a captação de glutamato, causando uma superestimulação do sistema glutamatérgico.

Sabe-se que não apenas a superestimulação do sistema glutamatérgico pode causar convulsões, mas também a inibição da resposta GABAérgica é um importante mecanismo envolvido na gênese das convulsões. Com isso, no **artigo 3** verificou-se os efeitos sobre o sistema GABAérgico do aparecimento de convulsões induzidas pelo $(PhSe)_2$ nos ratos bebês.

Agonistas de receptor $GABA_{A,}$ diazepam, fenobarbital e GABA protegeram completamente das convulsões induzidas por $(PhSe)_2$ na dose de 50 mg/kg, e aumentaram a latência para o início do primeiro episódio convulsivo induzido por $(PhSe)_2$ na dose de 500 mg/kg. Além disso, o antagonista de receptor $GABA_{A,}$ picrotoxina, diminuiu a latência para o primeiro episódio convulsivo induzido por $(PhSe)_2$ nas doses de 50 e 500 mg/kg. Estes resultados demonstram o envolvimento do receptor $GABA_{A,}$ direta ou indiretamente, nas convulsões induzidas pelo $(PhSe)_2$ em ratos bebês.

Adicionalmente, o pré-tratamento com AOAA, inibidor da GABA transaminase, enzima que degrada o GABA na fenda sináptica foi capaz de abolir as convulsões induzidas por $(PhSe)_2$, na dose de 50 mg/kg, e aumentou a latência para o início do primeiro episódio convulsivo induzido por $(PhSe)_2$, na dose de 500 mg/kg. Estes resultados demonstram o envolvimento da enzima GABA transaminase, direta ou indiretamente, nas convulsões induzidas pelo $(PhSe)_2$ em ratos bebês.

Por último, o pré-tratamento com DABA, um inibidor da captação do GABA protegeu completamente as convulsões induzidas por $(PhSe)_2$, na dose de 50 mg/kg e aumentou a latência para o início do primeiro episódio convulsivo induzido por $(PhSe)_2$, na dose de 500 mg/kg. Além de disso, o $(PhSe)_2$ aumentou a captação de GABA em fatias de córtex e hipocampo dos ratos bebês, que apresentaram convulsões. Este resultado demonstra um efeito direto do $(PhSe)_2$ sobre o sistema GABAérgico. De fato, a diminuição na neurotransmissão GABAérgica no SNC contribui para a hiperexcitabilidade, que é responsável pelo estatus epilético (Olsen et al., 1999).

Juntos os **artigos 2** e **3** evidenciaram que um aumento na transmissão excitatória glutamatérgica associado a uma diminuição na transmissão GABAérgica inibitória é um importante mecanismo envolvido na gênese das convulsões (Mares et al., 2000).

Os **artigos 1**, **2** e **3** demostraram que o $(PhSe)_2$ apresenta efeito convulsivante em ratos bebês e alguns dos mecanismos envolvidos neste efeito foram elucidados e estão sumarizados na **figura 11**.

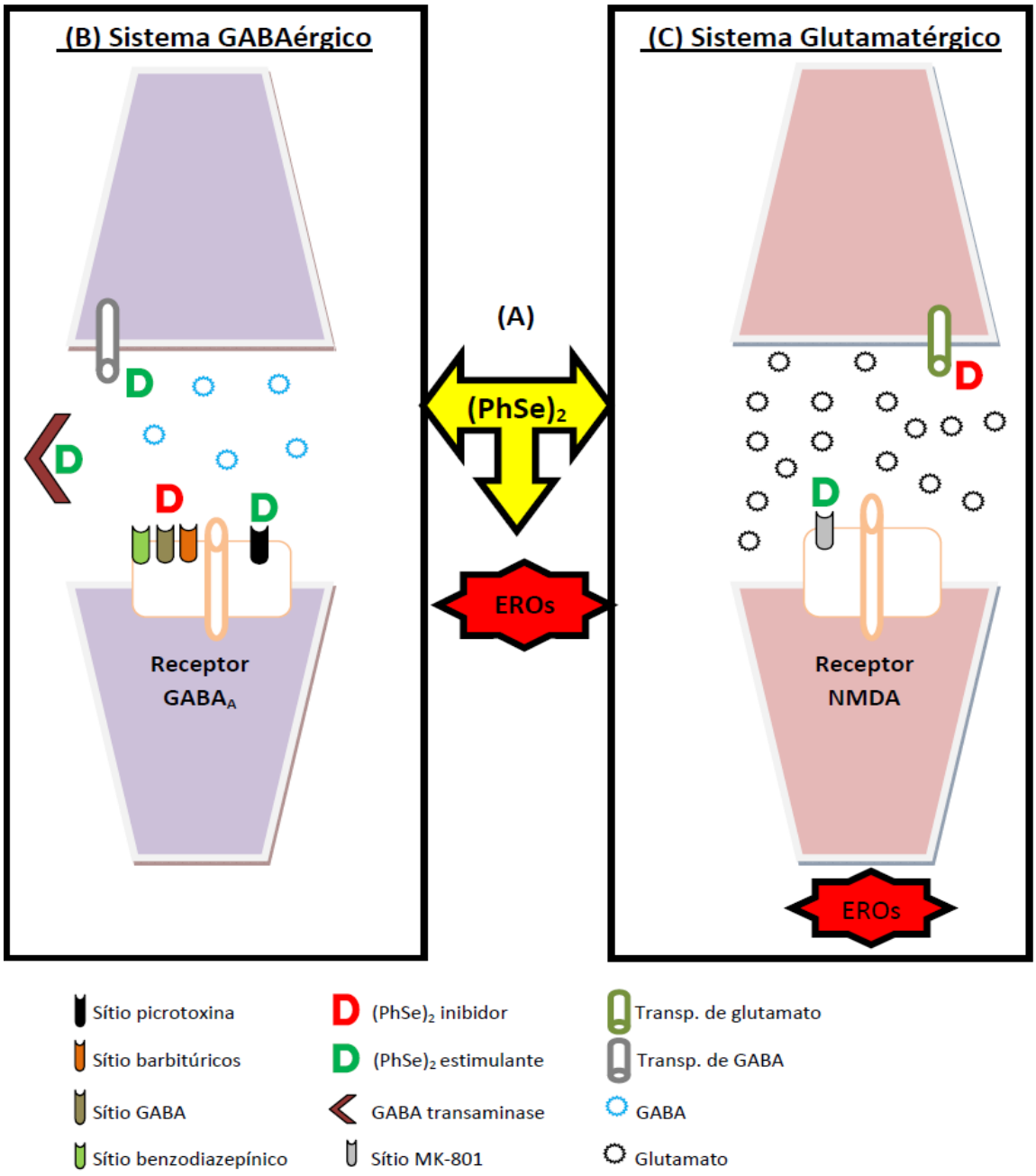


Figura 11: Mecanismos de ação do $(PhSe)_2$ envolvidos no efeito convulsivante do composto. O $(PhSe)_2$ provoca convulsões através: (A) da produção direta de espécies reativas de oxigênio (EROs); (B) da inibição da resposta GABAérgica e (C) da superesimulação da resposta excitatória glutamatérgica.

5.2 Estudo da Absorção, Distribuição e Biotransformação do Disseleneto de Difenila

O mais elementar princípio que rege a farmacologia é que a intensidade dos efeitos terapêuticos e tóxicos de uma droga e dos seus metabólitos no organismo, e estes efeitos são determinados pelas suas características farmacocinéticas e farmacodinâmicas (Martín-Liménz et al., 2008)

Em relação ao $(PhSe)_2$, estudos utilizando diferentes modelos experimentais demonstraram a existência de diferentes propriedades farmacológicas e toxicológicas conhecidas até o momento. No entanto, pouco se conhece sobre a cinética e o metabolismo deste composto.

No **artigo 4**, realizou-se pela primeira vez a determinação e a quantificação do $(PhSe)_2$ no plasma de ratos e camundongos. Os achados demonstraram que os níveis máximos do composto no plasma ocorrem 30 minutos após sua administração por via oral, e são semelhantes tanto para ratos quanto para camundongos. Estes dados correspondem a dados prévios de curva de dose-resposta realizados em modelos experimentais usando ratos e camundongos, na qual o $(PhSe)_2$ teve atuação máxima como antinociceptivo, tipo-antidepressivo e antiinflamatório, 30 minutos após sua administração (Savegnago et al., 2007a,b; 2008).

As concentrações de $(PhSe)_2$ encontradas na circulação são baixas (12 μg/mL), se relacionadas a dose administrada (500 mg/kg). Uma explicação para isso é o alto grau de lipofilicidade do composto. Sabe-se que a lipossolubilidade é um dos fatores envolvidos na absorção dos medicamentos. A eficácia das drogas lipofílicas é reduzida devido à sua pequena solubilidade em água o que faz com que a sua absorção seja menor. A lipofilicidade explicaria também a rápida absorção e disponibilidade na corrente sanguínea e a rápida queda nos níveis do composto, já que se observou que o $(PhSe)_2$ apresenta-se circulante até 8 horas após sua administração.

Outros importantes achados deste estudo envolvem mudanças nas vias de administração (oral, intraperitoneal e subcutânea), no solvente utilizado para diluir o $(PhSe)_2$ (óleo de canola ou DMSO) e na espécie de animal utilizada (ratos ou camundongos). Estas variações nos permitiram chegar a várias conclusões, relacionadas

aos diferentes efeitos farmacológicos e toxicológicos do composto, nos diferentes modelos testados.

O efeito tóxico do $(PhSe)_2$, caracterizado pelo aparecimento de convulsões, demonstrou ser dependente: da espécie testada, sendo os camundongos mais sensíveis que os ratos; da via de administração, sendo a via intraperitoneal a menos segura, seguida pela via oral e pela via subcutânea; e do solvente utilizado, sendo o óleo mais inerte que o DMSO. A respeito disso, diferentes estudos utilizando doses de $(PhSe)_2$ e vias de administração similares, porém utilizando diferentes solventes exibiram diferentes resultados. O $(PhSe)_2$ se mostrou tóxico quando dissolvido em DMSO, causando convulsão em camundongos (Nogueira et al., 2003a), porém apresentou atividade ansiolítica quando diluído em óleo de canola (Ghisleni et al., 2008).

Juntos os **artigos 1** e **4** destacaram o fato de que as convulsões induzidas por $(PhSe)_2$ são dependentes da idade, já que o $(PhSe)_2$ na dose de 500 mg/kg, administrado pela via oral, dissolvido em óleo de canola, provoca convulsão em 100% dos ratos bebês e não causa nenhum sinal de convulsão em ratos adultos. Sendo assim, no **artigo 5**, buscou-se determinar e quantificar os níveis do $(PhSe)_2$ no plasma, fígado e cérebro de ratos bebês, após o primeiro episódio convulsivo.

O **artigo 5** demonstrou que os níveis plasmáticos de $(PhSe)_2$ nos ratos bebês após o episódio convulsivo era de 3.67 μg/ml, valor 4 vezes menor que o encontrado no **artigo 4** para ratos adultos. Mesmo apresentando níveis plasmáticos menores de composto, os ratos bebês apresentaram convulsão, o que nos leva a entender que os ratos bebês são mais sensíveis aos efeitos tóxicos do $(PhSe)_2$ do que os adultos.

Observou-se ainda uma correlação negativa entre os níveis hepáticos do composto e a latência para o primeiro episódio convulsivo. Isto possivelmente está relacionado com as características químicas do composto (altamente lipofílico) associado às diferenças fisiológicas nos animais (relacionadas à idade). Estas diferenças podem produzir uma diminuição na taxa de eliminação do composto, aumentando assim seu efeito tóxico, manifestado aqui pelas convulsões.

O estudo revela ainda uma correlação negativa entre os níveis cerebrais do $(PhSe)_2$ e a latência para o primeiro episódio convulsivo, demonstrando que os animais que apresentam uma maior quantidade de composto no cérebro, convulsionam antes. Os dados encontrados no **artigo 5** explicam, pelo menos em parte, os efeitos neurotóxicos apresentados pelos ratos bebês tratados com $(PhSe)_2$ (**Artigos 1**, **2** e **3**). E corroboram com os dados encontrados no **artigo 1** que demonstraram que a latência para o primeiro

episódio convulsivo é dependente da dose administrada (50, 150 ou 500 mg/kg). De fato, quanto maior a dose administrada, maior o potencial do composto em chegar ao cérebro e causar convulsão nos animais.

Até o momento (**Artigos 4** e **5**) conseguiu-se desvendar parte do comportamento cinético do $(PhSe)_2$ no modelos *in vivo* realizados. Contudo, devido à necessidade de obter mais informações sobre o composto, que viessem a complementar os dados obtidos, realizou-se modelos cinéticos *in vitro*, nos quais foram investigados parâmetros relacionados ao comportamento da droga no organismo (**Manuscritos 1** e **2**).

Estima-se que grande parte dos compostos sintetizados, mesmo após passar por uma modelagem molecular, deixam de se tornar drogas bem sucedidas, principalmente devido a sua pobre farmacocinética ou toxicidade (Wang e Urban, 2004). Assim, a avaliação inicial das propriedades farmacocinéticas torna-se obrigatória para aumentar a taxa de sucesso no estudo de drogas. Nos últimos anos muitas abordagens *in vitro* têm sido desenvolvidas para a avaliação das propriedades farmacocinéticas de drogas, com o objetivo de acelerar o processo de descoberta, minimizar tempo e custo, e reduzir a taxa de insucesso nos testes *in vivo* em animais (Kerns, 2000; Di e Kerns 2003; Kaplitaand and Liu 2005).

O **manuscrito 1** mostrou que o $(PhSe)_2$ apresenta uma baixa solubilidade em PBS pH 7.4 (0.98 μM). Os compostos com baixa solubilidade em água apresentam baixa biodisponibilidade, uma vez que não se dissolvem adequadamente no fluído gastrointestinal, possuindo assim uma baixa taxa de absorção para a circulação sanguínea. Segundo Gursoy and Beita (2004), a taxa de absorção gastrointestinal é controlada pela dissolução do composto no fluído gastrointestinal. Este dado explica os resultados obtidos nos **artigos 4** e **5**, nos quais a administração oral de uma alta dose de $(PhSe)_2$ (500 mg/kg) produziu uma baixa Cmáx (13.13, 10.11 e 3.67 μg/ml para ratos, camundongos e ratos bebês, respectivamente). Provavelmente apenas uma pequena quantidade do composto administrado pela via oral foi absorvida para a circulação sanguínea.

Observou-se ainda que o logarítimo do coeficiente de partição octanol/água (o qual prediz a habilidade do composto em atravessar as membranas) do $(PhSe)_2$ foi bastante elevado (3.14). Este valor indica que o composto apresenta uma rápida absorção pelo trato intestinal e explica os resultados obtidos no **artigo 4**, no qual o composto atinge uma concentração plasmática máxima 30 minutos após a administração pela via oral em ratos e camundongos adultos. Este resultado indica ainda que o $(PhSe)_2$, devido a sua lipofilicidade, atravessa a barreira hemato-encefálica, corroborando com o **artigo 5**, no

qual 1.15 μg/ml de $(PhSe)_2$ é encontrado no cérebro de ratos bebês 20 minutos após a administração do composto pela via oral. Essa rápida distribuição deve ser responsável pelos efeitos tóxicos do composto demonstrados nos **artigos 1**, **2**, **3**, **4** e **5**.

O **manuscrito 1** evidenciou ainda que o $(PhSe)_2$ apresenta uma forte ligação às proteínas plasmáticas (□99%), principalmente à albumina. A determinação da percentagem de droga que está livre ou ligada às proteínas é um parâmetro muito importante na farmacocinética de uma droga, uma vez que somente a droga livre consegue passar pelas membranas e exercer seu efeito nos tecidos alvos (Yan and Caldwell, 2004; Banker et al, 2003). Contudo, nossos dados indicaram que a ligação do $(PhSe)_2$ ocorre principalmente de forma covalente. Uma hipótese é que o $(PhSe)_2$ reaja com o grupo sulfidrila da cisteína 34, o único tiól reativo da albunina, formando um selenosulfeto. Os **artigos 1** e **2** já demonstravam que o $(PhSe)_2$ interage com grupos sulfidrila das enzimas como a δ-ALA-D e a Na^+, K^+-ATPase, oxidando-os, o que é responsável pela perda da função da enzima.

Em relação à distribuição do $(PhSe)_2$, acredita-se que mesmo ligado, ele seja transferido da albumina para os tecidos através de tióis de proteínas associadas a membrana ou por tióis endógenos como o GSH, que sejam capazes de clivar a ligação Se-S, deixando-o livre. Esta hipótese é baseada no estudo realizado por Wagner et al. (1994) para o composto orgânico de selênio ebselen.

Por fim o **manuscrito 1** evidenciou que o $(PhSe)_2$ apresenta estabilidade química frente aos fluídos gástrico e intestinal simulados, bem como apresenta estabilidade quando em contato com o plasma. Estes dados são importantes, uma vez que complementam os resultados obtidos nos **artigos 4** e **5**, enfatizando que o $(PhSe)_2$ quando administrado aos animais não sofre degradação estomacal, intestinal ou mesmo pelas enzimas plasmáticas. Muitas drogas são descartadas dos estudos farmacológicos por apresentarem degradação frente a diferenças de pH ou por enzimas estearases plasmáticas.

Como já citado anteriormente, o $(PhSe)_2$ é um composto altamente lipofílico, portanto, ele necessita ser biotransformado no organismo em compostos mais polares para serem posteriormente excretados. No **manuscrito 2** realizou-se um estudo *in vitro* para identificar as vias metabólicas responsáveis pela biotransformação do $(PhSe)_2$ no organismo.

O **manuscrito 2** revelou que o $(PhSe)_2$ incubado com diferentes frações hepáticas (homogenato total, S9, fração solúvel e microssomas) sofre conjugação com o GSH endógeno gerando um complexo selenol-SG, o qual foi, pela primeira vez, caracterizado

por espectro de massas. A formação do conjugado ocorre no tempo inicial de incubação, indicando que não se trata de uma reação enzimática de conjugação com o GSH, mas sim uma reação química, através da formação de aduto.

O GSH, um tiol endógeno, reage com o $(PhSe)_2$, via ataque nucleofílico do grupo tiól no átomo de selênio do $(PhSe)_2$, capturando quimicamente o intermediário reativo selenol, e gerando o produto final PhSe-SG. A adição de NAC, um tiól exógeno, ao meio de incubação, também foi capaz de formar aduto com o $(PhSe)_2$, o qual foi caracterizado pelo espectro de massas.

A formação de adutos com espécies nucleofílicas como GSH e NAC demonstram que o $(PhSe)_2$ apresenta um intermediário reativo, o selenol, que tem o potencial de formar ligações covalentes com um grande número de constituíntes celulares tais como proteínas e enzimas, causando prejuízo as mesmas. A respeito disso o **artigo 1** demontrou que a administração oral de $(PhSe)_2$ em ratos bebês causou inibição nas enzimas sulfidrílicas δ-ALA-D e Na^+, K^+-ATPase bem como levou ao aparecimento de convulsões nos animais. Adicionalmente, o **manuscrito 1** demonstrou uma extensa ligação do $(PhSe)_2$ à albumina plasmática, a qual ocorre provavelmente por ligação covalente do composto ao grupo sulfidrila presente na albumina.

Os **artigos 1**, **2**, **3**, **4** e **5** sugerem que o $(PhSe)_2$ seja metabolizado no fígado pelo complexo de enzimas do sistema P450, uma vez que há diferenças entre espécies (ratos e camundongos) e idade (ratos bebês e adultos) no efeito tóxico do composto. Além disso, Nogueira et al. (2003) demonstraram que a administração de $(PhSe)_2$ pela via intracerebroventricular não produz convulsões nos animais.

No **manuscrito 2**, a incubação do $(PhSe)_2$ com a fração microsomal (fração rica em enzimas do sistema P450) não produziu nenhum metabólito diferente, apenas o conjugado PhSe-SG. A inibição das enzimas microssomais com a utilização de CO não alterou a taxa de aparecimento do conjugado PhSe-SG, confirmando que ele ocorre de forma independente das enzimas do sistema P450. A partir destes dados sugere-se duas hipóteses: (1) não ocorre a formação de metabólito hepático; (b) o(s) possível (is) metabólito(s) do $(PhSe)_2$ poderia(m) estar sendo produzido(s) em quantidade reduzida, estando abaixo do limite de detecção do aparelho.

Em relação à excreção do $(PhSe)_2$, não tem-se dados concretos, mas sugere-se que a formação do aduto com a glutationa pode ser o primeiro passo para a excreção do composto, a qual envolve a formação de derivados do ácido mercaptúrico, forma mais polar e excretada na urina. Segundo De Rooij et al. (1998) e Scholz et al. (2005), grande

parte das drogas eletrofílicas são excretadas pela urina na forma de produtos do ácido mercaptúrico.

O **manuscrito 2** mostrou ainda que o $(PhSe)_2$ é capaz de inibir o metabolismo da *N,N* dietilbenzamida, droga que é metabolizada pelo citocromo P450 em *N* etilbenzamida. Este resultado indica que o $(PhSe)_2$ pode interferir no metabolismo de drogas que necessitam ser metabolizadas pelo citocromo P450, reduzindo a formação de metabólitos e mudando o efeito das mesmas. Supõe-se que o $(PhSe)_2$ possa interagir com grupamentos tiólicos do sítio ativo das proteínas microssomais, causando oxidação e perda da função dos mesmos. Kuhn-Velten and Sies. (1989) demonstraram que o ebselen, causa inativação do citocromo P450 por interagir diretamente com tióis do sítio ativo dos microssomas.

Os **artigos 4** e **5** e os **manuscritos 1** e **2** demonstraram a absorção, distribuição e metabolismo do $(PhSe)_2$ através de estudos *in vitro* e *in vivo*. Algumas destas etapas foram elucidadas e estão sumarizadas na **figura 12**.

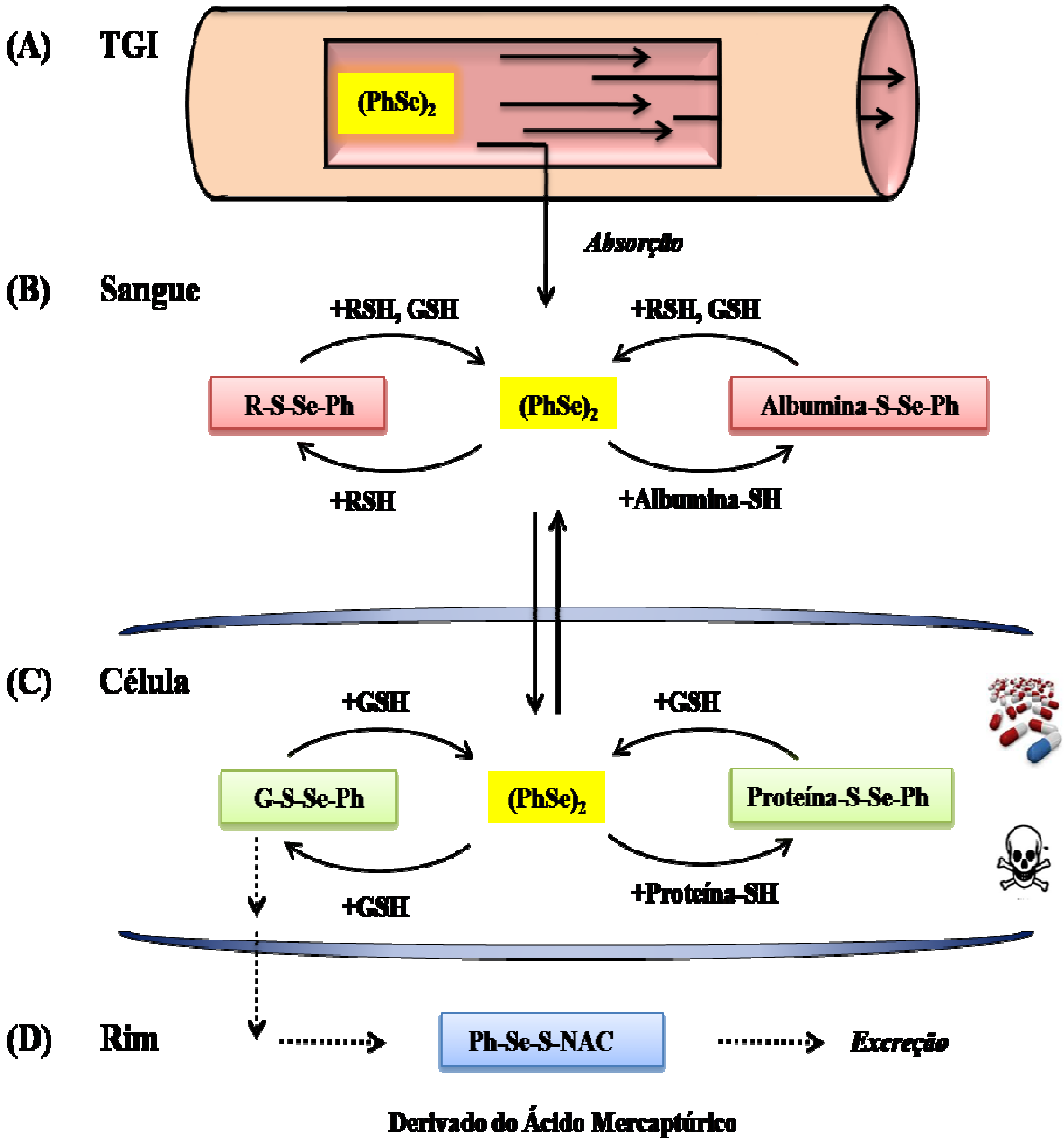


Figura 12: Esquema demonstrando: (A) a absorção do $(PhSe)_2$ pelo trato gastrointestinal (TGI); (B) o transporte do composto na circulação sanguínea através da ligação à albumina; (C) a entrada do $(PhSe)_2$ nas células e a oxidação de proteínas, levando aos efeitos farmacológicos e/ou toxicológicos do composto e (D) um possível mecanismo de excreção do $(PhSe)_2$, na urina, através da formação de derivados do ácido mercaptúrico.

6. CONCLUSÕES

De acordo com os resultados obtidos podemos concluir que:

- A**rtigo 1:** A administração de $(PhSe)_{2,}$ pela via oral, causou neurotoxicidade em ratos bebês, evidenciada pelo aparecimento de convulsões. As convulsões causadas pela administração do $(PhSe)_2$ foram dependentes da dose. O estresse oxidativo está relacionado, pelo menos em parte, com as convulsões induzidas pelo $(PhSe)_{2;}$

- A**rtigo 2**: Um dos mecanismos de ação envolvidos no efeito convulsivante do $(PhSe)_2$ envolve a interação com o sistema glutamatérgico, por estimular os receptores glutamatérgicos ionotrópicos do tipo NMDA e por inibir a captação de glutamato, causando uma superestimulação do sistema glutamatérgico;

- A**rtigo 3**: O $(PhSe)_2$ interaje com o sistema GABAérgico, antagonizando os receptores GABAégicos do tipo $GABA_A$, estimulando a enzima GABA transaminase e estimulando a captação de GABA, diminuindo assim a neurotransmissão inibitória GABAérgica;

- A**rtigo 4**: A concentração máxima de $(PhSe)_2$ no plasma de ratos e camundongos adultos ocorreu 30 minutos após a administração do composto, e decaiu continuamente, permanecendo detectável até 8 horas após sua administração. O uso de diferentes vias de administração e veículos em ratos e camundongos indicou que o aparecimento das convulsões e os níveis plasmáticos de $(PhSe)_2$ são dependentes da via de administração (i.p > p.o > s.c), do veículo (DMSO > óleo de canola) e da espécie animal (camundongo > rato);

- **Artigo 5**: Os níveis de $(PhSe)_2$ no fígado e no cérebro de ratos bebês após o primeiro episódio convulsivo, apresentam uma correlação negativa com a latência para o primeiro episódio convulsivo. Este resultado ratifica a estreita relação entre as convulsões causadas pelo composto e a quantidade dele que é biotransformada e chega ao cérebro. Os ratos bebê convulsionam, mesmo apresentando menores valores plasmáticos de composto que os adultos, o que nos leva a entender que estes são mais sensíveis aos feitos tóxicos do $(PhSe)_2$.

- M**anuscrito 1**: O $(PhSe)_2$ apresentou estabilidade química e biológica. No entanto, o composto apresentou uma baixa solubilidade em água, um alto coeficiente de partição octanol-água e uma extensa ligação às proteínas plasmáticas. Estes dados são muito importantes uma vez que nos permitem entender a baixa biodisponibilidade do composto demonstrada em estudos prévios;

- M**anuscrito 2**: O $(PhSe)_2$ não é biotransformado por reações de fase I catalizadas pelo citocromo P450. Ele reage quimicamente com GSH e NAC, formando aductos ou ainda reage com grupos SH de proteínas. A presença de GSH ou NAC no meio de incubação diminuiu a ligação do $(PhSe)_2$ às proteínas. O $(PhSe)_2$ reduziu a atividade das enzimas do citocromo P450.

7. REFERÊNCIAS BIBLIOGRÁFICAS

ADAMS JR W, KOCSIS J, SNYDER R. Acute toxicity and urinary excretion of diphenyldiselenide. **Toxicology Letters** 48:301-310, 1989.

ALAKUIJALA A, PALGI M, WEGELIUS K, SCHMIDT M, ENZ R, PAULIN L, SAARMA M, PASTERNACK M. GABA receptor rho subunit expression in the developing rat brain. **Dev. Brain Res.** 154:15-23, 2005.

ALAVIJEH MS, PALMER AM. The pivotal role of drug metabolism and pharmacokinetics in the discovery and development of new medicines. **I. Drugs** 7:755–763, 2004.

ALBERTS B, BRAY D, LEWIS J, RAFF M, ROBERTS K, WATSON JD. Molecular Biology of the cell. 3ª ed, New York & London: Garland Publishing, 1994.

ALCORN J, MCNAMARA PJ. The ontogeny of hepatic and renal systemic clearance pathways in infants: A review (Part I), **Clin. Pharmacokinet.** 41:959-998, 2002.

ALCORN J, MCNAMARA PJ. Pharmacokinetics in the newborn. **Adv. Drug Rev.** 55:667-686, 2003.

ALMEIDA A, HEALES SJR, BOLAÑOS JP. Glutamate neurotoxicity is associated with nitric oxide-mediated mitochondrial dysfunction and glutathione depletion. **Brain Research** 790:209-216, 1998.

AVDEEF A. Physicochemical profiling (solubility, permeability and charge state). **Curr. Top Med. Chem.** 1:277–351, 2001.

BANKER MJ, CLARK TH, WILLIAMS JA. Development and validation of a 96-well equilibrium dialysis apparatus for measuring plasma protein binding. **J. Pharm. Sci.** 92:967-974, 2003.

BARBOSA NBV, ROCHA JBT, ZENI G, EMANUELLI T, BEQUE MC, BRAGA AL. Effect of organic forms of selenium on δ-Aminolevulinate dehydratase from liver, kidney and brain of adult rats. **Toxicol. Appl. Pharmacol.** 149:243-253, 1998.

BARBOSA NBV, ROCHA JBT, WONDRACEK DC, PEROTONI J, ZENI,G, NOGUEIRA CW. Diphenyl diselenide reduces temporarily hyperglycemia: possible relationship with oxidative stress. **Chem. Biol. Interact.** 163:230-238, 2006.

BASHKATOVA V, NARKEVICH V, VITSKOVA G, VANIN A. The influence of anticonvulsivant drugs on nitric oxide levels and lipid peroxidation in the rat brain during penthylenetetrazole-induced epileptiform model seizures. **Prog. Neuro pychopharmacol. Biol. Psychiatry** 27:487-492, 2003.

BEDWAL RS, NAIR N, SHARMA MP, MATHUR RS. Selenium-its biological perspectives. **Med Hypotheses** 41(2):150-159, 1993.

BEHNE D, KYRIAKOPOULOS A. Identification of type I iodothyronine 5'-deiodinase as a selenoenzyme. **Biochem. Biophys. Res. Co.** 173:1143-1149, 1990.

BERGSTROM CA. In silico predictions of drug solubility and permeability: two rate-limiting barriers to oral drug absorption. **Basic Clin. Pharmacol. Toxicol.** 96:156-161, 2005.

BESUNDER JB, REED MD, BLUMER JL. Principles of drug biodisposition in the neonate. A critical evaluation of the pharmacokinetic–pharmacodynamic interface (Part I). **Clin. Pharmacokinet.** 14:189–216, 1988.

BLAKE MJ, CASTRO L, LEEDER JS, KEARNS GL. Ontogeny of drug metabolizing enzymes in the neonate. **Seminars in Fetal and Neonatal Medicine** 10:123-138, 2005.

BORGES LP, BORGES VC, MORO AV, ROCHA JBT, NOGUEIRA CW, ZENI G. Protective effect of diphenyl diselenide on acute liver damage induced by 2-nitropropane in rats. **Toxicology** 210:1-8, 2005.

BORGES VC, ROCHA JBT, NOGUEIRA CW. Effect of diphenyl diselenide, diphenyl ditelluride and ebselen on cerebral Na^+, K^+-ATPase activity in rats. **Toxicology** 215:191-197, 2005.

BORMANN J. The 'ABC' of GABA receptors. **Trends Pharmacol. Sci.** 21:16-19, 2000.

BRAGA AL, SILVEIRA CC, ZENI G, SEVERO WA, STEFANI HA. Synthesis of selenocetais from enol ethers. **J. Chem. Res.** 15:206-207, 1996.

BRITO VB, FOLMER V, PUNTEL GO, FACHINETTO R, SOARES JCM, ZENI G, NOGUEIRA CW, ROCHA JBT. Diphenyl diselenide and 2,3-dimercaptopropanol increase the PTZ-induced chemical seizure and mortality in mice. **Brain Res. Bull.** 68:414-418, 2006.

CALDWELL J. Conjugation reactions in foreign compound metabolism: definition, conseqüences and species variations. **Drug Metab. Rev.** 13:745-777, 1982.

CAVAZZUTI GB. Epidemiology of different types of epilespsy in school age childen Dev Med Child Neurol of Modena, Italy. **Epilepsia** 21:57-62, 1980.

CENTURIÃO FB, CORTE CL, PAIXÃO MW, BRAGA AL, ZENI G, EMANUELLI T. Effect of ebselen and organochalcogenides on excitotoxicity induced by glutamate in isolated chick retina. Brain Res. 1039:146–152, 2005.

CHAN PH, FISHMAN RA, LEE JL, CANDELISE L. Effects of excitatory Neurotransmitter aminoacids on swelling of rat brain cortical slices. **Journal Neurochemistry** 33:1309-1315, 1979.

CHAPMAN AG. Glutamate and epilepsy. **Am. Soc. Nutr. Sci.** 1043S-1045S, 2000.

CHAUDHRY FA, REIMER RJ, BELLOCCHIO EE. The vesicular GABA transporter, VGAT, localizes to synaptic vesicles in sets of glycinergic as well as GABAergic neurons. **J Neurosci.** 18:9733-9750, 1998.

CHERUBINI E, CONTI F. Generating diversity at GABAergic synapses. **Trends Neurosci.** 24:155-162, 2001.

CHOONARA I, CONROY S. Unlicensed and off-label drug use in children: implications for safety. **Drug Saf.** 25:1-5, 2002.

COMASSETO JV. Vinylic selenides. **J. Organomet Chem.** 253:131-181, 1983.

COMASSETO JV, LO W, PETRAGNANI N, STEFANI HA. Vinylic selenides and tellurides - Preparation, reactivity and synthetic applications. **Synthesis** 373, 1997.

COMMISSION ON CLASSIFICATION AND TERMINOLOGY OF THE INTERMATIONAL LEAGUE AGAINS EPILEPSY. Proposal for revised classification of epilepsies and epileptic syndromes. **Epilepsia** 22:489-501, 1981.

CONSIGLIERI VO, STORPIRTIS S. Bioequivalência de medicamentos: objetivos, parâmetros farmacocinéticos, delineamento experimental e critérios de avaliaçäo. **Rev. Bras. Ciênc. Farm.** 36(1):13-21, 2000.

COTMAN CW, KAHLE JS, MILLER SE, ULAS J, BRIDGES RJ. Excitatory aminoacid neurotransmisson. **Psycopharmacology** 7:75-85, 1995.

COWAN LD. The epidemiology of the epilepsies in children. **Ment. Retard. Dev Disabil. Res. Rev.** 8:171-181, 2002.

DANBOLT NC. Glutamate uptake. **Progress in Neurobiology** 65:1-105, 2001.

DAWSON VL, DAWSON TM. Free radicals and neuronal cell death. **Cell Death and Differentiation** 3:71-78, 1996.

DEVAUD LL. Ethanol dependence has limited effects on GABA or glutamate transporters in rat brain. **Alcohol. Clin Exp Res** 25:606–611, 2001.

DI L, KERNS EH. Profiling drug-like properties in discovery research. **Curr. Opin. Chem. Biol.** 7:402-408, 2003.

DIAZ JP, NAVARRO M, LOPEZ H, LOPEZ MC. Determination of selenium levels in dairy products and drinks by hydride generation atomic absorption spectrometry: Correlation with daily dietary intake. **Food Addit. Contam.** 14:109-114, 1997.

DICHTER MA., WILCOX KS. Excitatory synaptic transmission. Epilepsy: A comprehensive Textbook. J. Engel, Jr. and T.A. Pedley. Eds. Lippincott-Raven Publishers, Philadelphia, 1997.

DIMMELER S, BRIINE B, ULLRICH V. Ebselen prevents inositol (1,4,5)-trisphosphate binding to its receptor. **Biochem. Pharmacol.** 42:1151-1153, 1991.

DOOSE H, SITEPU L. Epidemiology of epilepsy in childrem. In: Dodson & Pellock, Pediatric and Terapy (eds). Demos Publications, 2 ed, 1993.

DRÖGE W. Free radicals in the physiological control of cells function. **Phisyology Review** 82:47-95, 2002.

EDMONDS B, GIBB AJ, COLQUHOUN D. Mechanisms of activation of glutamate receptors and the time course of excitatory synaptic currents. **Annu. Rev. Physiol.** 57:495-519, 1995.

ELGER CE, HELMSTAEDTER C, KURTHEN M. Chronic epilepsy and cognition. **Lancet** (Neurol) 3:663-672, 2004.

EMANUELLI T, PAGEL FW, ALVES LB, REGNER A, SOUZA DO. Inhibition of adenylate cyclase activity by 5-aminolevulinic acid in rat and human brain. **Neurochem. Int.** 35:213-218, 2001.

EMANUELLI T, PAGEL FW, PORCIÚNCULA LO, SOUZA DO. Effects of 5-aminolevulinic acid in the glutamatergic neurotansmission. **Neurochem. Int.** 42:115-121, 2003.

ENGELBORGHS RS, D'HOOGE RPP, DE DEYN PP. Pathopysiology of epilepsy. **Acta Neurol. Belg.** 100:201-213, 2000.

ERAKOVIC V, ZUPAN G, VARLJEN J, JA J, SIMONIC A. Altered activities of rat brain metabolic enzymes by pentylenetetrazol kindling and pentylenetetrazol-induced seizures. **Epilepsy Research** 43:165-173, 2001.

FARINA M, BRANDÃO R, LARA FS, SOARES FA, SOUZA DO, ROCHA JBT. Profile of nonprotein thiols, lipid peroxidation and delta-aminolevulinate dehydratase

activity in mouse kidney and liver in response to acute exposure to mercuric chloride and sodium selenite. **Toxicology** 184:179-187, 2003.

FERNANDES JG & SANDER JW. Epidemiologia e história das epilepsias. In: Costa JCC. (eds). Fundamentos Neurobiológicos das Epilepsias: Aspectos Clínicos e Cirúrgicos. Lemos Editorial, São Paulo 1:3-20, 1998.

FIGHERA MR, ROYES LFF, FURIAN AF, OLIVEIRA MS, FIORENZA NG, FRUSSA-FILHO R, PETRY JC COELHO, RC, MELLO CF. GM1 ganglioside prevent seizures, Na+, k+ ATPase activity inhibition and oxidative stress induced by glutaric acid and pentylenetetrazole. **Neurobiology of Disease** 22:611-623, 2006.

FISCHER H, DEREU N. Mechanism of the catalytic reduction of hydroperoxides by ebselen: a selenium- 77 NMR studv. **Bull Sot Chim Bek** 96:757-768, 1987.

FRAHM C, DRAGUHN A. GAD and GABA transporter (GAT-1) mRNA expression in the developing rat hippocampus. **Dev. Brain Res.** 132:1-13, 2001.

FURIAN AF, FIGHERA MR, OLIVEIRA MS, FERREIRA APO, FIORENZA NG, MYSKIW JC, PETRY JC, COELHO RC, MELLO CF, ROYES LFF. Methylene blue prevents methylmalonate-induced seizures and oxidative damage in rat striatum. **Neurochemistry Internactional** 50:164-171, 2007.

GADEA A, LOPEZ-COLOME AM. Glial transporters for glutamate, glycine, and GABA: II. GABA transporters. **J. Neurosci. Res.** 63:461–468, 2001.

GALÉ K. GABA and epilepsy: basic consepts from preclinical research. **Epilepsia.** 33(5):S3-S12, 1992.

GHISLENI G, PORCIÚNCULA LO, CIMAROSTI H, ROCHA JBT, SALBEGO CG, SOUZA DO. Diphenyl diselenide protects rat hippocampal slices submitted to oxygen–glucose deprivation and diminishes inducible nitric oxide synthase immunocontent. **Brain Research** 986:196-199, 2003.

GOODMAN & GILMAN: As Bases Farmacológicas da Terapêutica, 11ª Ed. edição, J.G. Hardman, L.E. Limbird, P.B. Molinoff, R.W. Ruddon, A. Goodman Gilman, McGraw-Hill, 2007.

GOZLAN H, BEM-ARI Y. NMDA receptor redox sites: are they targets for selective neuronal protection? **TiPS** 16:368-375, 1995.

GRISAR T, FRANCK GV. Glial Contribution to Seizure - K+ Activation of (Na+,K+)-ATPase in Bulk Isolated Glial-Cells and Synaptosomes of Epileptogenic Cortex. Brain Research 261(1):75-84, 1983.

GRUNEWALD R. Childhood seizures and their consequences for the hippocampus. **Brain.** 125:1935-1936, 2002.

HALLIWELL B, GUTTERIDGE JMC. Free radicals in Biology and Medicine, 2ª ed, New York: Clerendon Press, 1989.

HALLIWELL B, GUTTERIDGE JMC. Free radicals in biology and medicine. 4th ed. Oxford/UK: Clarendon Press/Oxford Science Publications, 2007.

HOLMES GL. Effects of seizures on brain development: lessons from the laboratory. **Pediatr Neurol**. 33:1-11, 2005.

HOLMGREN A. Thioredoxin. Annu. Rev. Biochem. 54:237-271, 1985.

HUANG Y, ZHANG Z. Binding study of drug with bovine serum albumin using a combined technique of microdialysis with flowinjection chemiluminescent detection. **J. Pharm. Biomed. Anal.** 35:1293-1299, 2004.

INEU RP, PEREIRA ME, ASCHNER M, NOGUEIRA CW, ZENI G, ROCHA JBT. Diphenyl diselenide reverses gastric lesions in rats: Involvement of oxidative stress. **Food. Chem. Toxicol.** 46:3023-3029, 2008.

IZQUIERDO I, BEVILACQUA L, ROSSATO JI, BONINI JS, MEDINA JM, CAMMAROTA M. Different molecular cascades in different sites of the brain control memory consolidation. **Trends Neurosci.** 29:496-515, 2006.

JACQUES-SILVA MC, NOGUEIRA CW, BROCH LC, FLORES EM, ROCHA JBT. Diphenyl diselenide and ascorbic changes deposition of selenium and ascorbic in liver and brain of mice. **Pharmacol. Toxicol.** 88:119-125, 2001.

JYOTI A, SETHI P, SHARMA D. Curcumin protects against electrobehavioral progression of seizures in the iron-induced experimental model of epileptogenesis. **Epilepsy & Behavior** 14(2):300-308, 2009.

KADE IJ, PAIXÃO MW, RODRIGUES OED, IBUKUNB EO, BRAGA AL, ZENI G, NOGUEIRA CW. Rocha J.B.T. Studies on the antioxidant effect and interaction of diphenyl diselenide and dicholesteroyl diselenide with hepatic δ-aminolevulinic acid dehydratase and isoforms of lactate dehydrogenase. **Toxicology in Vitro** 23(1):14-20, 2009.

KAMIGATA N, TAKATA M. MATSUYAMA H AND KOBAYASHI M. Novel ring opening reaction of Z-aryl-1,2- benziselenazol-3(2H)-one with thiols. **Heterocycles** 24:3027-3030, 1986.

KANDA T, ENGMAN L, COTGREAVE IA, POWIS G. Novel water-soluble diorganyl tellurides with thiol peroxidase and antioxidant activity. **J. Org. Chem.** 64:8161-8169, 1999.

KANEKO K, ITOH K, BERLINER LJ, MIYASAKA K, FUJII H. Consequences of nitric oxide generation in epileptic-seizure rodent models as studied by in vivo EPR. **Magn. Redon. Med.** 48:1051-1056, 2002.

KAPLITA PV, LIU HH. Automatable formats of higher throughput ADMET profiling for drug discovery support. **J. Assoc. Lab. Automation** 10:140-8, 2005.

KAPPAS A, SASSA S, GALBRAITH RA, NORDMANN Y. The porphyrias. In: Scriver, C.R., Beaudet, A.l., Sly, W.S., Valle, D. (Eds.), The Metabolic Bases of Inherited, 7th Edition, McGraw Hill, New York, pp. 2103-2160, 1995.

KERNS EH. High throughput physicochemical profiling for drug discovery. **J Pharm Sci** 90:1838-58, 2001.

KHAZIPOV R, KHALILOV I, TYZIO R, MOROZOVA E, BEN-ARI Y, HOLMES GL. Developmental changes in GABAergic actions and seizure susceptibily in the rat hippocampus. **Eur. J. Neurosci**. 19:590-600, 2004

KLAYMAN DL, GÜNTHER WH. (eds.). Organic selenium compounds: their chemistry and biology. New York: John Wiley and sons, 68-157, 1973.

KLEINROK Z, TURSKI WA, CZUCZWAR SJ. Excitatory amino acid antagonists and the anticonvulsive activity of conventional antiepileptic drugs. **Pol. J. Pharmacol.** 47(3):247-52, 1995.

KOBAYASHI Y, OGRA Y, ISHIWATA K, TAKAYAMA H, AIMI N, SUZUKI KT. Selenosugars are key and urinary metabolites for selenium excretion within the required to low-toxic range. **Proc. Natl. Acad. Sci. U. S. A.** 99:15932–15936, 2002.

KORPI ER, GRÜNDER G, LÜDDENS H. Drug interactions at $GABA_A$ receptors. **Progr. Neurobiol.** 67:113-159, 2002.

KOSTYNIAK PJ. Preliminary toxixity studies of bis, beta-(*n,n*-dimorpholino) ethylselenide (MOSE). a New Radioimaging Agent. **Drug Chem Toxicol.** 7:41-55. 1984.

KRATOCHWIL NA, HUBER W, MULLER F, KANSY M, GERBER PR. Predicting plasma protein binding of drugs—revisited. **Curr. Opin. Drug. Discov. Dev.** 7:507-512, 2004.

KUHN-VELTEN N, SIES H. Optical spectral studies of ebselen interaction with cytochrome P450 of rat liver microsomes. **Biochem. Pharmacol.** 38: 619-625, 1989.

KVAMME E. Synthesis of glutamate and its regulation. **Prog. Brain Res**.16:73-85, 1998.

LENOX WG. Historical perspective –review and preview – childhood convulsions and epilepsy. In: Epilepsy and Related Disorders (ed). Little Brown and Company, Boston, Toronto; 1 and 2:11-436, 1981.

LEVSEN K, SCHIEBEL HM, BEHNKE B, DÖTZER R, DREHER W, ELEND M, THIELE H. Structure elucidation of phase II metabolites by tandem mass spectrometry: an overview. **Journal of Chromatography A** 1067:55-72, 2005.

LIANG LP, HO YS, PANTEL M. Mitochondrial superoxide production in kainate-induced hippocampal damage. **Neuroscience** 101:563-570, 2000.

LIN J, SAHAKIAN DC, DE MORAIS SM, XU JJ, POLZER RJ, WINTER SM. The role of absorption, distribution, metabolism, excretion and toxicity in drug discovery. Curr. Top. Med. Chem. 3:1125-1154, 2003.

LIPTON SA, ROSENBERG PA. Excitatory amino acids as a final commom pathway for neurologic desorders. **New Eng. J. Med.** 330:613-622, 1994.

LÖSCHER W. New visions in the pharmacology of anticonvulsion. **Eur. J. Pharmacol.**, 342, 1-13, 1997.

LUCHESE C, BRANDAO R, OLIVEIRA R, NOGUEIRA CW, SANTOS FW. Efficacy of diphenyl diselenide against cerebral and pulmonary damage induced by cadmium in mice. **Toxicol. Lett.** 173(3):181-190, 2007.

MACIEL EN, FLORES EM, ROCHA JBT Comparative deposition of diphenyl diselenide in liver, kidney and brain of mice. **Bull. Environ. Contam. Toxicol.** 70:470-476, 2003.

MADL JE. & BURGESSER K. Adenosine triphosphate depletion reverses sodium-dependent, neuronal uptake of glutamate in rat hippocampal slices. **The Journal of Neuroscience** 13:4429-4444, 1993.

MANNHOLD R. The impact of lipophilicity in drug research: a case report on β-blockers. **Mini Rev. Med. Chem**. 5:197-205, 2005.

MARES P, CHINO M, KUBOVÁ H, MATHERN P. Convulsant action of systemically administered glutamate and bicuculline methiodide in immature rats. **Epilepsy Research** 42:183-189, 2000.

MARES P, FOLBERGROVA J, KUBOVA H. Excitatory aminoacids and epileptic seizures in immature brain. **Physiological Research** 53(1):S115-S124, 2004.

MARSHALL FH, JONES KA, KAUPMANN K, BETTLER B. $GABA_B$ receptors – the first 7TM heterodimers. **Trends Pharmacol. Sci.** 20:396-399, 1999.

MARTIN DC, PLAGENHOEF M, ABRAHAM J, DENNISON RL, ARONSTAM RS. Volatile anesthetics and glutamate activation of N-methyl-D-aspartate receptors. **Biochem. Pharmacol.** 49:809-817, 1995.

MARTÍN-JIMÉNEZ T, LINDEBLAD M, KAPETANOVIC IM, CHENB Y, LYUBIMOV A. Comparing pharmacokinetic and pharmacodynamic profiles in female rats orally exposed to lovastatin by gavage versus diet. **Chem. Biol. Interact.** 171:142-151, 2008.

MCCARVER DG, HINES RN. The ontogeny of human drug metabolizing enzymes: phase II conjugation enzymes and mechanisms. **J. Pharmacol. Exp. Ther.** 300:361-366, 2002.

McDONALD JW & SCHOEPP DD. Aminooxyacetic acid produces excitotoxic brain injury in neonatal rats. **Brain Research** 624:239-244, 1993.

MCNAMARA JO. Emerging insights into the genesis of epilepsy. **Nature** 399:A15-22,1999.

MELDRUM BS. Excitotoxicity and epileptic brain damage. **Epilepsy Res.** 10:55-61, 1991.

MELDRUM BS. Neurotransmission In Epilepsy. **Epilepsia** 36:S30-S35, 1995.

MELDRUM BS, AKBAR MT, CHAPMAN AG. Glutamate receptors and transporters in genetic an acquired modles of epilepsy. **Epilepsy Res.** 36:189-204, 1999.

MEOTTI FC, BORGES VC, ZENI G, ROCHA JBT, NOGUEIRA C W. Potential renal and hepatic toxicity of diphenil diselenide, diphenil ditelluride and Ebselen for rats and mice. **Toxicol. Lett.** 143:9-16, 2003.

MEOTTI FC, STANGHERLIN EC, ZENI G, ROCHA JBT, NOGUEIRA CW. Protective role of aryl and alkyl diselenides on lipid peroxidation. **Environm. Res.** 94:276-282, 2004.

MODY I, MACDONALD JF. NMDA receptor-dependent excitotoxicity: the role of intracellular Ca^{2+} release. **Trends Pharmacol. Sci.** 16:356-359, 1995.

MORGANE PJ, MOKLER DJ, GALLER JR. Effects of prenatal protein malnutrition on the hippocampal formation. **Neurosci. Biobehav. Rev.** 26:471-483, 2002.

MÜLLER A, GABRIEL H, SIES H. A novel biologically active selenoorganic compound. IV. Protective glutathione-dependent effect of PZ 51 (ebselen) against ADP-Fe induced lipid peroxidation in isolated hepatocytes. **Biochem. Pharmacol.** 34:1185-l 189, 1985.

MÜLLER A, GABRIEL H, SIES H, TERLINDEN R, FISCHER H, ROMER A. A novel biologically active selenoorganic comoound-VII. Biotransformation of ebselen in perfused rat liver. **Biochem. Pharmacol.** 37:1103-1109, 1988.

NASSAR AF, HOLLENBERG PF, SCATINA JA. Drug Metabolism Handbook: Concepts and Aplications. A John Wiley & Sons, INC., Publication, 2009.

NAVARRO-ALARCÓN M, LÓPEZ-MARTINEZ MC. Essentiality of selenium in the human body: relationship with different deseases. **Sci. Tot. Environ.** 249:347-371, 2000.

NICOLETTI F, BRUNO V, COPANI A, CASABONA G, KNOPFEL F. Metabotropic glutamate receptors: a new target for the therapy of neurodegenerative disorders? **Trends Neurosci.** 19:267-272, 1996.

NOGUEIRA CW, ROTTA LN, PERRY ML, SOUZA DO, ROCHA JBT. Diphenyl diselenide and diphenyl ditelluride affect the rats glutamatergic system in vitro and in vivo. **Brain Res.** 906:157-163, 2001.

NOGUEIRA CW, ROTTA LN, ZENI G, SOUZA DO, ROCHA JBT. Exposure to Ebselen changes glutamate uptake and release by rat brain synaptosomes. **Neurochem. Res.** 27:283-288, 2002..

NOGUEIRA CW, MEOTTI FC, CURTE E, PILISSÃO C, ZENI G, ROCHA JBT. Investigations into the potential neurotoxicity induced by diselenides in mice and rats. Toxicology 183:29-37, 2003a.

NOGUEIRA CW, BORGES VC, ZENI G, ROCHA JBT. Organochalcogens effects on d-aminolevulinic acid dehydratase activity from human erythrocytic cells in vitro. **Toxicology** 191:169-178, 2003b.

NOGUEIRA CW, ZENI G, ROCHA JBT. Organoselenium and organotellurium compounds: toxicology and pharmacology. **Chem. Rev.** 104:6255–6286, 2004.

OBRENOVITCH TP, URENJAK J, ZILKHA E, JAY TM. Excitotoxicity in neurological disorders-the glutamate paradox. **Int. J.Develop. Neurosc.** 18:281-287, 2000.

OLIVEIRA MS, FURIAN AF, ROYES LFF, FIGHERA MR, CARVALHO MJ, GINDRI FN, MELLO CF. Ascorbate Modulates Pentylenetetrazol-Induced Convulsions Biphasically. **Neuroscience** 128:721-728, 2004.

OLSEN RW, DELOREY TM, GORDEY M, KANG MH. GABA receptor function and epilepsy. **Adv. Neurol.** 79:499-510, 1999.

OZAWA S, KAMIYA H, TSUZUKI K. Glutamate receptors in the mammalian central nervous system. **Prog. Neurobiol.** 54:581-618, 1998.

PAINTER EP. The chemistry and toxicity of selenium compounds, with special reference to the selenium problem. **Chem. Rev.** 28:179-213, 1941.

PARDRIDGE WM. The blood-brain barrier: bottleneck in brain drug development. **NeuroRx** 2:3-14, 2005.

PARNHAM MJ, GRAF E. Pharmacology of synthetic organic selenium compounds. Prog. Drug Res. 36:9-47, 1991.

PATEL, M. Mitochondrial dysfunction and oxidative stress: cause and consequence of epileptic seizures. **Free Radical Biology & Medicine** 29:1951-1962, 2004.

PATSOUKIS N, ZERVOUDAKIS G, PANAGOPOULOS NT, GEORGIU CD, ANGELATOU F, MATSOKIS NA. Thiol redox state (TRS) and oxidative stress in the mouse hippocampus after pentylenetetrazol-induced epileptic seizure. **Neurosci. Lett.** 357:83-86, 2004.

PAULMIER C. Selenoorganic functional groups. In: Paulmier, C. (Ed.), Selenium Reagents and Intermediates in Organic Synthesis, First ed. Pergamon Press, Oxford, England, 25-51, 1986.

PORCIÚNCULA LO, ROCHA JBT, BOECK CR, VENDITE D, SOUZA DO. Ebselen prevents excitotoxicity provoked by glutamate in rat cerebellar granule neurons. **Neurosci. Lett.** 299:217–220, 200.

PRICE DL. New order from neurological disorders. Nature 399:A3-A5, 1999.

PRIGOL M, LUCHESE C, NOGUEIRA CW. Antioxidant effect of diphenyl diselenide on oxidative stress caused by acute physical exercise in skeletal muscle and lungs of mice. **Cell Biochemistry and Function** 27:216-222, 2009.

RAOL YH, LYNCH DR, BROOKS-KAYAL A. Role of exitatory aminoacids in developmental epilepsies. Ment. Retard. Dev. Disabil. **Res. Rev.** 7:254-260, 2001.

REITER RJ. Oxidative Processes and Antioxidative Defense-Mechanisms in the Aging. **Brain Faseb Journal** 9(7):526-533, 1995.

RIBEIRO MC, ÁVILA DS, SCHNEIDER CYM, HERMES FS, FURIAN AF, OLIVEIRA MS, RUBIN MA, LEHMANN M, KRIEGLSTEIN J, MELLO CF. α-Tocoferol protects against pentylenetetrazol and methylmalonate-induced convulsions. **Epylep. Res.** 66:185-194, 2005.

RICE DC. Effects of exposure to 3,3',4,4',5-pentachlorobiphenyl (PCB 126) throughout gestation and lactation on behavior (concurrent random interval-random interval and progressive ratio performance) in rats. **Neurotoxicol. Teratol.** 21:59-69, 1999.

ROBERTS SA. Drug metabolism and pharmacokinetics in drug discovery. Curr Opin **Drug Discov. Dev.** 6:66-80, 2003.

ROBINSON M.D, DOWD LA. Heterogeneity and functional subtypes of sodiumdependent glutamate transporters in the mammalian central nervous system. Adv. Pharmacol. 37:69-115, 1997.

ROCINHOLI L.F, ALMEIDA SS, DE-OLIVEIRA LM. Response threshold to aversive stimuli in stimulated early protein-malnourished rats. **Braz. J. Med. Biol. Res.** 30:407-413, 1997.

ROSA RM, FLORES DG, APPELT HR, BRAGA AL, HENRIQUES JAP, ROESLER R. Facilitation of long-term object recognition memory by pretraining administration of diphenyl diselenide in mice. **Neurosci. Lett.** 341:217-220, 2003.

ROSA RM, ROESLER R, BRAGA AL, SAFFI J, HENRIQUES JA. Pharmacology and toxicology of diphenyl diselenide in several biological models. **Braz. Med. Biol. Res.** 40:1287-1304, 2007.

ROSSATO JI, KETZER LA, CENTURIÃO FB, SILVA SJN, LUDTKE DS, ZENI G, BRAGA AL, RUBIN MA, ROCHA JBT. Antioxidant propeties of new chalcogenides against lipid peroxidation in rat brain. **Neurochem. Res.** 27:297-303, 2002.

ROTTA NT. Convulsões neonatais. In: Miura E. (ed). Neonatologia: Princípios e Prática. Artes Médicas, Porto Alegre 307-310, 1991.

ROYES LFF, FIGHERA MR, FURIAN AF, OLIVEIRA MS, FIORENZA NG, PETRY JC, COELHO RC, MELLO CF. The role of nitric oxide on the convulsive behavior and oxidative stress ionduced by methylmalonate: An eletroencephalographic and neurochemical study. **Epilepsy Research** 73:228-237, 2007.

SANKAR R, SHIN DH, WASTERLAIN CG. GABA: Metabolism during status epilepticus in the developing rat brain. Developmental **Brain Research** 98:60-64, 1997

SANTOS FW, ORO T, ZENI G, ROCHA JBT, NASCIMENTO PC, NOGUEIRA CW. Cadmium induced testicular damage and its response to administration of succimer and diphenyl diselenide in mice. **Toxicol. Lett.** 152:255-263, 2004.

SANTOS FW, ZENI G, ROCHA JBT, NASCIMENTO PC, MARQUES MS, NOGUEIRA CW. Efficacy of 2,3-dimercapto-1-propanesulfonic acid (DMPS) and diphenyl diselenide on cadmium induced testicular damage in mice. **Food Chem. Toxicol.** 431:723-1730, 2005.

SAVEGNAGO L, TREVISAN M, ALVES D, ROCHA JBT, NOGUEIRA CW, ZENI G. Antisecretory and antiulcer effects of diphenyl diselenide. **Environ. Toxicol. Pharmacol.** 21:86–92, 2006.

SAVEGNAGO L, JESSE CR, PINTO LG, ROCHA JBT, NOGUEIRA CW. Diphenyl diselenide attenuates acute thermal hyperalgesia and persistent inflammatory and neuropathic pain behavior in mice. **Brain Res.** 1175:54-59, 2007a.

SAVEGNAGO L, PINTO LG, JESSE, CR, ALVES D, ROCHA JBT, NOGUEIRA CW., ZENI G. Antinociceptive properties of diphenyl diselenide: Evidences for the mechanism of action. **Eur. J. Pharmacol.** 555:129-138, 2007b.

SAVEGNAGO L, PINTO LG, JESSE CR, BARANCELLI DA, ROCHA JBT, NOGUEIRA CW, ZENI G. Diphenyl diselenide exerts antidepressant-like and anxiolytic-like effects in mice: Involvement of L-arginine-nitric oxide-soluble guanylate cyclase pathway in its antidepressant-like action. **Pharmacol. Biochem. Be.** 88:418-426, 2008.

SCHEUER ML, PEDLEY TA. The evaluation and treatmant of seizures. **N. Engl. J. Med**. 323:1468-1474, 1990.

SCHEWE T. Molecular Actions of Ebselen-an Antiinflammatory Antioxidant. **Gem Phnrmoc.** 26(6)1153-1169, 1995.

SCHOUSBOE A. Role of astrocytes in the maintenance and modulation of glutamatergic and GABAergic neurotransmission. **Neurochem. Res.** 28:347-352, 2003.

SCHOUSBOE A, SARUP A, BAK LK, WAAGEPETERSEN HS, LARSSON OM. Role of astrocytic transport processes in glutamatergic and GABAergic neurotransmission. **Neurochem. Int.** 45:521-527, 2004.

SCHWARTZ K, FOLTZ G. Selenium as an integral part of factor 3 against diety necrotic liver degeneration. **J. Am. Chem. Soc.** 79:3292-3293, 1957.

SHIOBARA Y, SHIOBARA T, YOSHIDA A, SUZUKI KT. Effects of dietary selenium species on Se concentrations in hair, blood and urine. **Toxicol. Appl. Pharmacol.** 152:309-314, 1998.

SNYDER SH, BREDT DS. Biological roles of nitric oxide. **Scientific American** 28-35, 1992.

SOUSA SC, MACIEL EN, VERCESI AE, CASTILHO RF. Ca^{2+}-induced oxidative stress in brain mitochondria treated with the respiratory chain inhibitor rotenone. **FEBS Letters** 543:179-183, 2003.

SPALLHOLZ JE, SHRIVER JE, REID TW. Dimethyldiselenide and methylselenide acid generates superoxide in an in vitro chemiluminescence assay in the presence of glutathione: implications for the anticarcinogenic activity of l-selenomethionine and L-Se-methylselenocysteine. **Nutr. Cancer** 40:34-41, 2001.

SPERK G, SCHWARZER C, TSUNASHIMA K, FUCHS K, SIEGHART W. GABA(A) receptor subunits in the rat hippocampus I: immunocytochemical distribution of 13 subunits. **Neuroscience** 80:987-1000, 1997.

STADTMAN TC. Selenium-dependent enzymes. **Annu. Rev. Biochem.** 49:93-110, 1980.

SUZUKI KT, ITOH M. Metabolism of selenite labelled with enriched stable isotope in the bloodstream. **J. Chromatogr. B. Biomed. Sci. Appl.** 692:15-22, 1997.

SUZUKI KT. Metabolomics of selenium: Se metabolites based on speciation studies. **Journal of Health Science** 51(2):107-114, 2005a.

SUZUKI KT, KURASAKI K, OKAZAKI N, OGRA Y. Selenosugar and trimethylselenonium among urinary Se metabolites: dose- and age-related changes. **Toxicology and Applied Pharmacology** 206(1):1-8, 2005b.

SUZUKI KT, DOI C, SUZUKI N. Metabolism of 76Se-methylselenocysteine compared with that of 77Se-selenomethionine and 82Se-selenite. **Toxicology and Applied Pharmacology** 217(2):185-195, 2006.

SUZUKI KT, TSUJI Y, SUZUKI, N. Preferential organ distribution of methylselenol source Se-methylselenocysteine relative to methylseleninic acid. **Toxicology and Applied Pharmacology** 227:76–83, 2008.

TIMBRELL J. Principles of Biochemical Toxicology, 3ª ed, London: Taylor & Francis, 2000.

TREIMAN DM. Electroclinical features of status epilepticus. **J Clin. Neurophysiol.** 12:343-362, 1995.

TREIMAN DM. GABAergic mechanisms in epilepsy. **Epilepsia** 42:8-12, 2001.

ULLRICH V, WEBER P, MEISCH F, WON APPEN F. Ebselen-Binding Equilibria Between Plasma and Target Proteins. Biochemical Pharmacology 52:15-19, 1996.

UMEMURA A, MABE H, NAGAI H. A phospholipase C inhibitor ameliorates postischemic neuronal damage in rats. **Stroke** 23:1163-1166, 1992.

URSINI F, HEIM S, KIESS M, MAIORINO M, ROVERI A, WISSING J, FLOHÉ L. Dual function of the seleno-protein PHGPx during sperm maturation. **Science** 285:1393-1396, 1990.

VAN DE WATERBEEMD H. Which in vitro screens guide the prediction of oral absorption and volume of distribution? **Basic Clin. Pharmacol. Toxicol.** 96:162-166, 2005.

VAN DEN ANKER JN. Pharmacokinetics and renal function in preterm infants, Acta Paediatr. 85:1393-1399, 1996.

VARJU P, KATAROVA Z, MADARÁSZ E, SZABÓ G. GABA signaling during development: new data and old questions. **Cell Tissue Res.** 305:239-246, 2001.

VASILETS LA, SCHWARZ W. Struture-function relationships of cation binding in the Na^+, K^+-ATPase. **Biochim. Biophys. Acta** 1154:201-222, 1993.

VINGERHOETS G. Cognitive effects of seizures. Seizure, 15, 221-226, 2006.Volatile anesthetics and glutamate activation of N-methyl-D-aspartate receptors. **Biochem. Pharmacol.** 49:809-817, 1995.

WAGNER G, SCHUCH G, AKERBOOM TP, SIES H. Transport of ebselen in plasma and its transfer to binding sites in the hepatocyte. **Biochem. Pharmacol.** 15(6):1137-44, 1994.

WAJNER M, LATINI A, WYSE AT, DUTRA-FILHO CS. The role of oxidative damage in the neuropathology of organic acidurias: insights from animal studies. **Journal Inherited Metabolic Disease** 27:427-448, 2004.

WANG J, URBAN L. The impact of early ADME profiling on drug discovery and development strategy. **Drug Discov. World** 5:73-86, 2004.

WENDEL A, OTTER R, TIEGS G. Inhibition by ebselen of microsomal NADPH-cytochrome P450-reductase in vitro but not in vivo. **Biochem Pharmacol** 35:2995-2997, 1986.

WHITE HS, SARUP A, BOLVIG T, KRISTENSEN AS, PETERSEN G, NELSON N, PICKERING DS, LARSSON OM, FROLUND B, KROGSGAARD-LARSEN P, SCHOUSBOE A. Correlation between anticonvulsant activity and inhibitory action on glial gamma-aminobutyric acid uptake of the highly selective mouse gamma-aminobutyric acid transporter 1 inhibitor 3-hydroxy-4-amino-4,5,6,7-tetrahydro-1,2-benzisoxasole and its N-alkylated analogs. **J. Pharmacol. Exp. Ther.** 302:636-644, 2002.

WILBER CG. Toxicology of selenium: A review. **Clin. Toxicol.** 17:171-230, 1980.

WINGLER K, BRIGELIUS-FLOHÉ R. Gastrointestinal glutathione peroxidase. **Biofactors** 10:245-249, 1999.

YAN Z, CALDWELL GW. Methods in pharmacology and toxicology: Optimization in Drug Discovery: In vitro Methods. New Jersey: Humana Press p. 1, 2004.

YAN L, SPALLHOLZ JE. Generation of reactive oxygen species from the reaction of selenium compounds with thiols and mammary tumor cells. **Biochem. Pharmacol.** 45:429-437, 1991.

ZEEVALK GD, BERNARD LP, SINHA C. Excitotoxicity and oxidative stress during inhibition of energy metabolism. **Development of Neuroscience** 20:444-453, 1998.

ZIEGLER DM, GRAF P, POULSEN LL, STAHL W, SIES H. NADPH-dependent oxidation of reduced ebselen, 2-selenylbenzanilide, and of Z-(methylseleno)benzanilide catalyzed by pig liver flavin-containing monooxygenase, **Chem. Res. Toxic.** 15:163-166, 1992.

ZIGMOND MJ, BLOOM FE, LANDIS SC, ROBERTS JL, SQUIRE LR. Eds Fundamental. Neuroscience, 2nd ed. Academic Press: New York. Glimcher, PW., 2003.

8.0 ANEXO

8.1 Influência do pré tratamento com MK 801 e DNQX nas convulsões induzidas por glutamato (4 g/kg) e $(PhSe)_2$ (50 mg/kg).

Os resultados apresentados na tabela abaixo são complementares aos resultados apresentados no **artigo 2** e têm como objetivo avaliar o papel desempenhado pelo receptor glutamatérgico NMDA nas convulsões induzidas por $(PhSe)_2$ em ratos bebês. A metodologia utilizada é a mesma utilizada no **artigo 2**, página 49.

Table 1: Influence of pretreatment with MK 801 and DNQX in glutamate (4 g/kg) and $(PhSe)_2$ (50 mg/kg)-induced seizures in rat pups.

Groups	Appearance of Seizures[a]	Latency[b] (min)
Control	0/8	Ns
Glutamate 4	8/8	33.87 ± 4.64
$(PhSe)_2$ 50	3/8	45.00 ± 14.55
Glutamate 4+ MK 801	0/8**	Ns
Glutamate 4+DNQX	3/8	47.00 ± 6.80*
$(PhSe)_2$ 50 +MK 801	$0/8^{\#\#}$	Ns
$(PhSe)_2$ 50 +DNQX	2/8	52.5 ± 3.53

[a] Number of animals which presented seizures/N of animals per group.

[b]time (min) to the appearance for the first seizure episode. "ns" animals which did not present seizure (in 60 minutes of observation). $(PhSe)_2$ MK 801 and DNQX (mg/kg). Glutamate (g/kg). Data are reported as mean ± S.D.; (**)Denoted $p < 0.05$ as compared to the Glutamate 4 group (X^2 method and Fischer's exact probability test); ($^{\#\#}$) Denoted $p< 0.05$ as compared to the $(PhSe)_2$ 50 + MK 801 group (X^2 method and Fischer's exact probability test); (*) Denoted $p < 0.05$ as compared to the Glutamate 4 group (one-way ANOVA/Duncan).

Observou-se que a pré administração de MK 801 protegeu completamente das convulsões induzidas por glutamato (4 g/kg) e por $(PhSe)_2$ (50 mg/kg). Enquanto que a pré administração de DNQX aumentou a latência para o início do primeiro episódio convulsivo induzido por glutamato, porém não causou alteração no latência para o início do primeiro episódio convulsivo induzido por $(PhSe)_2$. Estes resultados corroboram com os apresentados no **artigo 2** e reforçam o envolvimento do receptor glutamatérgico do tipo NMDA nas convulsões induzidas por $(PhSe)_2$.