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Toxicokinetics of tabun enantiomers in anaesthetized swine after intravenous tabun administration

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ABSTRACT

In the present study, we report the first *in vivo* toxicokinetic study of tabun (O-ethyl-N,N-dimethylphosphoramidocyanidate). The toxicokinetics of the enantiomers of tabun were investigated in anesthetized swine after intravenous administration of 3 × LD_{50} (161.4 μg/kg) tabun. Blood samples were taken for gas chromatographic–mass spectrometric determination of the tabun enantiomers and for measurement of the activity of red blood cell acetylcholinesterase (AChE) and plasma butyrylcholinesterase (BChE). The tabun enantiomers could be quantified in swine blood to a minimum concentration of 3.0 pg/ml (18.5 pM) and could be detected to a minimum concentration of 1.0 pg/ml (6.2 pM). The concentration–time profiles of both tabun enantiomers were best described by a bi-exponential equation. The elimination of (+)-tabun and (−)-tabun were comparable in the initial phase. In the terminal phase a remarkable difference was found, with terminal half lives of 11.5 min for (+)-tabun and 23.1 min for (−)-tabun. (+)-Tabun showed a markedly longer persistence *in vivo* than (+)-enantiomers of other G-type nerve agents and could be detected in all swine at least up to 30 min post-injection, (−)-tabun at least up to 90 min post-injection. These results demonstrate a rather rapid elimination of tabun enantiomers *in vivo* and may provide a toxicokinetic basis for the further development and optimization of medical countermeasures against this nerve agent.

1. Introduction

Highly toxic derivates of methylphosphonic and phosphoric acids, such as sarin, cyclosarin, soman, VX and tabun (O-ethyl-N,N-dimethylphosphoramidocyanidate) are representatives of the most important group of chemical warfare agents (nerve agents). Organophosphorus (OP) nerve agents are characterized by an asymmetrical P-atom and consist of at least two stereoisomers. The (+)-P and (−)-P stereoisomers exhibit different biological and toxicological characteristics (Benschop and de Jong, 1991). (−)-P isomers of sarin, cyclosarin, soman, VX and tabun are much more toxic compared to (+)-P isomers (Benschop and de Jong, 1988; Reiter et al., 2008; Tenberken et al., 2010b). Determination of toxicological and toxicokinetic parameters of nerve agents is essential for the development of medical countermeasures and demands consideration of different biochemical and physiological characteristics of its stereoisomers. Hence, in order to assess toxicokinetic and toxicodynamic properties, reliable bioanalytical methods are required for the specific and selective determination of individual isomers of OP in blood and plasma (Benschop and de Jong, 1988; Reiter et al., 2008), thereby providing the basis for an approach to the qualities, timing and formulation of medical countermeasures.

To date, several methods have been used or proposed for the investigation of the stereoselective toxicokinetics of the OP nerve agents sarin (Spruit et al., 2000; Spruit et al., 2001), soman (Benschop and de Jong, 2001; Benschop et al., 1987, 1995), cyclosarin (Reiter et al., 2007) and VX (Reiter et al., 2008). However, no sensitive method appropriate for in vivo toxicokinetic studies of tabun stereoisomers had been established until recently. Tenberken et al. (2010a) developed a fully validated, fast and reliable method for the quantification of tabun enantiomers in hemolysed blood and plasma of different species and proved its applicability by monitoring the *in vitro* hydrolysis of the tabun enantiomers in plasma of different species. In the present study, this analytical method has been applied for the first time for the quantification and determination of toxicokinetic parameters of tabun enantiomers in swine blood samples after challenge by intravenous tabun.

2. Materials and methods

2.1. Chemicals and reagents

O-Ethyl-N,N-dimethylphosphoramidocyanidate (>98% by ^{1}H NMR and ^{31}P NMR) and the internal standard (IS) O-propyl-N,N-dimethylphosphoramidocyanidate (>97% by ^{1}H NMR and ^{31}P NMR) were

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supplied by the German Ministry of Defence. Tabun of same quality for in vivo experiments was synthesized at the Canadian National Single Small Scale Facility at DRDC Suffield. Ethylenediaminetetraacetic acid tripotassium salt dihydrate (99%), acetylthiocholine iodide (ATCh), S-butyrylthiocholine iodide (BTCh) and 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) were purchased from Sigma–Aldrich Chemie (Taufkirchen, Germany). Isolute C18 (EC) cartridges (octadecyl end-capped sorbent; 100 mg, 10 ml) were obtained from Separtis (Grenzach-Wyhlen, Germany). Ammonia (6.0) was supplied by Linde (Unterschleißheim, Germany), and Helium (6.0) from Air Liquide Germany (Düsseldorf, Germany). Chloroform (HPLC grade) was purchased from VWR (Darmstadt, Germany). Isopropanol (SupraSolv, for gas chromatography), all other chemicals (analytical grade) and liquid reagents (HPLC grade) were obtained from Merck (Darmstadt, Germany).

2.2. Domestic swine experiments

Swine experiments were carried out at Defence Research and Development Canada-Suffield (DRDC Suffield) Canada. Castrated male York-Landrace cross pigs (Sus scrofa domestica) weighing approximately 25 kg were purchased from a local supplier and housed indoors in the DRDC Suffield vivarium. The animals were allowed to acclimatise for at least one week prior to experimental use and were fed until the evening prior to nerve agent exposure. Tap water was available ad libitum. In conducting this research the authors adhered to the "Guide to the Care and Use of Experimental Animals" and "The Ethics of Animal Experimentation" published by the Canadian Council on Animal Care (CCAC). The animal experiments were approved by the Animal Care Committee at DRDC Suffield. At the end of the experiment animals were euthanized by intravenous (i.v.) injection of 8 ml (540 mg/ml) sodium pentobarbital (Euthanyl Forte, Nimeda-MTC, Cambridge, ON, Canada).

2.3. Anaesthesia

The animals underwent an inhalation induction with 5% isoflurane in a carrier gas of 100% oxygen (O_2) at a flow rate of 8 l min^{-1}. Post-induction, the animals were placed in the dorsal recumbent position on a heated operating table. Core body temperature was maintained at approximately 38 °C. Animals were intubated with a 6.5 mm internal diameter cuffed oral endotracheal tube (Ruschelt, Willy Rusch AG, Kernen, Germany). After intubation, the isoflurane concentration was reduced to 3% in 100% O_2 at a flow rate of 2 l min^{-1}. Once all monitors and catheters were placed and affixed, isoflurane was maintained at ~2% in medical air supplemented with oxygen to a fraction of inspired O_2 of 0.3. Animals were allowed to stabilize for at least 30 min, during this time a steady state anaesthesia (SSA) was established. The animals received 0.9% normal saline at a flow rate of 9.5 ml kg^{-1} h^{-1} via an i.v. line for fluid replacement and cystostomy was performed in order to monitor urinary output. Continuous physiological parameters, pulse oximetry, end-tidal capnography, arterial pressure, 3 lead EKG and rectal and surface temperatures were monitored using a Siemens SC 7000 patient monitor (Dynamed, Markham, ON, Canada). If required following tabun exposure, mechanical ventilation with oxygen-supplemented air was provided at a rate of 150–200 ml per cycle at 20 cycles per minute using a Hallowell EMC Model 2000 (Hallowell Engineering and Manufacturing, Pittsfield, MA, USA).

2.4. Tabun administration

A 3 × LD_{50} dose of tabun, 161.4 μg/kg, was administered as an intravenous (i.v.) infusion over 5 min in a total volume of 3 ml. The nerve agent was diluted in isopropanol prior to a final dilution in saline immediately prior to administration. The final concentration of isopropanol was less than 1%.

2.5. Medical countermeasures

In pig No. 1 and 2, atropine sulphate (AS; 2 mg) was injected i.m. at 180 min post-agent injection. In pig No. 3, AS was injected i.m. at 5 min, 30 min and 60 min post-exposure. In pig No. 4, AS was administered i.m. at 5 min, 30 min, 60 min and 116 min post-exposure. Assisted ventilation was provided as required at a rate of 3–4 l min^{-1}.

2.6. Blood sampling

For the gas chromatographic–mass spectrometric determination of the tabun enantiomers, blood samples (8 ml) were obtained from an indwelling catheter (20 gauge) placed in a branch of the saphenous artery at 5 min prior to tabun injection (blank sample) and at 2 min, 4 min, 6 min, 10 min, 15 min, 20 min, 30 min, 45 min, 60 min, 90 min, 120 min, 180 min, 240 min, 300 min, 360 min after start of tabun injection samples were collected in EDTA tubes. Stabilization of the tabun enantiomers was achieved by immediate treatment with two formate buffers as described before (Tenberken et al., 2010a): 2 ml of blood were mixed with 6.0 ml of 50 mM sodium formate buffer (pH 3.75) and incubated on ice for 1 min before adding 4.0 ml of 100 mM sodium formate buffer (pH 3.75). All processed samples were immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Blood samples from 4 animals were investigated.

In addition, blood samples were collected in EDTA tubes at the same time intervals for the photometric determination of red blood cell acetylcholinesterase (AChE, E.C. 3.1.1.7) and plasma butyrylcholinesterase activity (BChE, E.C. 3.1.1.8). Whole blood samples for cholinesterase determination (200 μl) were immediately diluted in 3.8 ml ice-cold distilled water. Plasma samples were prepared by centrifugation of whole blood (3800 × g, 10 min). All samples were immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

2.7. Sample preparation and extraction procedure for GC–MS

For the gas chromatographic–mass spectrometric determination of the tabun enantiomers, the samples were thawed and centrifuged at 4000 rpm (Rotina 35 r, Hettich Zentrifugen, Tuttlingen, Germany) and 4 °C for 5 min. Each sample (6 ml) was used for further treatment while the remainder was instantly frozen again to serve as control. After the addition of 0.05 ml IS solution (200 ng/ml O-propyl-N,N-dimethylphosphoramidocyanidate in isopropanol), the samples were mixed (15 s) on a rotary shaker and loaded on SPE cartridges previously conditioned with 1 ml methanol and 2.5 ml deionized water. After loading, the cartridges were washed with 2 ml deionized water. Vacuum was applied until the cartridges were dry, and the analytes were eluted with 1 ml chloroform into 5 ml glass tubes. The eluate was evaporated to a final volume of about 50 μl in a TurboVap LV workstation (Caliper Life Sciences, Rüsselsheim, Germany) under a gentle stream of nitrogen (5 psi) at 30 °C. The residue was transferred to an autosampler vial with a glass insert for analysis. A 40 μl aliquot of this solution was injected into a GC–MS system.

2.8. GC–MS conditions

The processed samples were analyzed as described before (Tenberken et al., 2010a) using an Agilent Technologies (Waldbronn, Germany) HP 6890N gas chromatographic system and a 5975N MS detector with ammonia positive ion chemical ionisation mass spectrometry (GC-PCI-MS). The system was equipped with a cold injection system CIS 4plus (Gerstel, Mülheim an der Ruhr, Germany). Chromatographic separation was performed on a Supelco BetaDex® 225 column (30 m length, 0.25 mm i.d., 0.25 μm film thickness, Sigma–Aldrich Chemie).

2.9. Enzyme assays

After thawing the whole blood dilutions and the plasma samples AChE and BChE activities were measured spectrophotometrically (Cary 3Bio, Varian, Darmstadt, Germany) at 436 nm with a modified Ellman assay (Worek et al., 1999). The assay mixture (3.16 ml) contained 0.45 mM ATCh (AChE) or 1.0 mM BTCh (BChE) as substrate and 0.3 mM DTNB as chromogen in 0.1 M phosphate buffer (pH 7.4).

The selective determination of AChE activity in whole blood samples was achieved by adding the selective BChE inhibitor ethopropazine (20 μM) to the cuvette. The specific activity of erythrocyte AChE activity in whole blood samples was calculated from the quotient of AChE activity and hemoglobin content (mU/μmol Hb_{Fe}). Hemoglobin was determined by a cyanmethemoglobin method at 546 nm (Worek et al., 1999). BChE activity (mU/ml) was determined in plasma samples. Assays were run at 37 °C. AChE and BChE activities were referred to the individual baseline activities (−5 min) and are given as % of control. The LOQ for the determination of AChE and BChE activities was approx. 1%.

2.10. Curve fitting of the data

Curve-fitting of the quantified tabun enantiomers was performed by nonlinear regression analysis using GraphPad®Prism 4.0 (GraphPad, San Diego, CA, USA) in order to obtain toxicokinetic parameters.

3. Results

3.1. Blood levels of tabun enantiomers after intravenous tabun administration

After intravenous administration of racemic tabun (3 × LD_{50}, 161.4 μg/kg over 5 min) to anaesthetized pigs both enantiomers were unequivocally detectable in all blood samples up to 30 min post-injection. Whereas (+)-tabun was found in two pigs 45 and 60 min after injection, (−)-tabun was present in all pigs up to 90 min post-injection. (+)-Tabun was present in traces in one pig up to 90 min post-injection. On the other hand, (−)-tabun was found in three pigs up to 120 min after injection. Neither (+)- nor (−)-tabun could be detected in samples taken 180 min post-injection or later. No relation between the different atropine therapeutic schedules and the period during which (+)-tabun could still be detected was observed. Having received identical antidotal treatment, (+)-tabun