RETROSPECTIVE DETECTION OF EXPOSURE TO ORGANOPHOSPHATES: ANALYSES IN BLOOD OF HUMAN BEINGS AND RHESUS MONKEYS

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Abstract

The terrorist attacks with nerve agents in Japan as well as the ongoing debate on a possible relationship between the so-called Gulf War Syndrome and accidental exposure to traces of nerve agents have made clear that improved methods for detection of exposure to organophosphates are needed. Low level exposures can not be detected by the methods which, are presently available. In addition, these methods have a limited value for retrospective analysis. We developed a method for biomonitoring of human exposure based on organophosphate-inhibited butyrylcholinesterase (BuChE) as the most persistant and abundantly available marker in blood. High concentrations of fluoride ions release the organophosphate from BuChE with formation of a phosphofluoridate, which is specific for the organophosphate used except for its leaving group. The phosphofluoridate can be identified and quantitated, e.g., by means of gas chromatography, in order to determine origin and extent of poisoning. In the present work we studied during which period of time the new method can be used subsequent to sublethal exposure to tabun or sarin (1-4 μg/kg, i.v.) of atropinized rhesus monkeys, as a model for humans. It appeared that exposure could be monitored in these animals for 3-5 weeks at inhibition levels of BuChE $\geq$ 0.01%. In addition, a method was developed for LC-MS-MS analysis of the hydrolysis product of sarin in blood. The usefulness of the new procedures was demonstrated by our preliminary analysis of serum samples from victims of the terrorist attack in the Tokyo subway and in Matsumoto.

1. Introduction

The use by Iraq of sulfur mustard in the war with Iran and of nerve agents against the Kurdish opposition is well documented. In terroristic attacks at Osaka, Matsumoto and in the Tokyo subway, nerve agents were used by members of the AUM

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Shinriyko sect (1). These events, as well as the controversy over exposure coalition troops to nerve agents in the Gulf War, demonstrate the need for adequate methodology for retrospective detection of exposure to chemical warfa agents. The presently used methods are of limited value. The intact compound a metabolites can only be measured shortly after exposure, whereas measurement cholinesterase inhibition in blood does not provide reliable evidence at inhibit levels less than 20%.

Heilbronn (2) has shown that fluoride ions reactivate the activity organophosphate (OP)-inhibited acetylcholinesterase (AChE) and butyr cholinesterase (BuChE), while De Jong and Van Dijk (3) found that fluoride ion: acidic pH (ca pH 4.8) regenerate rapidly the organophosphate moiety from inhibi carboxylesterases with formation of the corresponding phosphofluoridate. Assum that these two reactivation reactions are analogous, this would mean that reactior OP-inhibited BuChE in human blood with fluoride ions and quantitation of generated phosphofluoridate might provide a sensitive "fingerprint" for retrospec biomonitoring of exposure, based on the most abundantly available (80 nM) persistent marker for OP exposure in blood (4). Both the origin and extent of the poisoning can be determined in this way. Based on the minimal concentration: phosphofluoridate that can be analyzed in blood, it can be calculated that inhib levels $\geq$ 0.01% of inactivated BuChE should be quantifiable. Since rhesus monk like humans, do not have carboxylesterases in blood, these animals are presuma good model for exposure of humans to OP anticholinesterases.

In this paper we confirm the analogy of the reactions between OP-inhib carboxylesterase and BuChE and fluoride ions, and report on quantitative meth for (i) *in vitro* reactions with fluoride ions in plasma of rhesus monkeys and hun after incubation with tabun and sarin, (ii) analysis of blood samples of rh monkeys exposed to these agents, (iii) application of the procedure to serum sam from victims of terrorist attacks in Tokyo and Matsumoto (4) and (iv) additi semi-quantitative LC-MS-MS analysis of hydrolyzed agent, i.e., ^{i}PrO(Me)P(O (IMPA) in these serum samples.

Previous investigations on qualitative methods to detect exposure to n agents, developed for forensic purposes after the Tokyo incident, involved inhil AChE from erythrocytes (5) and brain slices (6) of fatally exposed victims, w was trypsinized and subsequently treated with alkaline phosphatase in ord release IMPA and methylphosphonic acid. These acids were trimethylsilylated analyzed by means of GC-MS. Obviously, this procedure is more laborious fluoride-induced release of phosphofluoridate fom inhibited BuChE. identification of IMPA was reported in urine samples of deceased victims (7).

2. Experimental

2.1. *IN VITRO* EXPERIMENTS

After inhibition of plasma from rhesus monkeys or humans with excess tab sarin and removal of excess inhibitor, the plasma was incubated at pH 7.5, 3

Samples were taken at regular intervals and added to acetate buffer in order to obtain pH 4-6. After addition of internal standard (O,O-diethyl phosphorofluoridate and d_7-isopropyl methylphosphonofluoridate for tabun and sarin, respectively), the mixture was incubated with potassium fluoride. Generated phosphofluoridate and internal standard were extracted over a C_{18} Sep-Pak column, eluted with ethyl acetate and analyzed gas chromatographically (alkali flame or mass spectrometric detection). Additional plasma samples were taken to determine BuChE activity.

2.2. EXPERIMENTS WITH RHESUS MONKEYS

The study for each nerve agent was performed with two unanesthetized rhesus monkeys (*M. mulatta*) from the colony of the Biomedical Primate Research Center, Rijswijk, The Netherlands, after written approval of the protocol by the ethical committee. Atropine sulfate (28.5 µg/kg) was administered (i.m., hind leg) 10 min before administration of nerve agents in the *vena saphena* of the right hind leg whereas blood samples were taken in the same way from the other hind leg. Nerve agents were administered portionwise, guided by cholinesterase activity measurements after each administration, until ca 40% inhibition of BuChE was reached.

2.3. REACTIVATION WITH FLUORIDE IONS AND OXIME IN SERUM OF JAPANESE VICTIMS

These experiments were described in a previous paper (4).

2.4. ANALYSIS OF ISOPROPYL METHYLPHOSPHONIC ACID (IMPA) IN SERUM OF JAPANESE VICTIMS

The diluted serum sample was acidified to pH 1-2 and subsequently extracted with isobutanol/toluene. LC-MS-MS analysis of IMPA was performed with negative ion electrospray ionisation and multiple reaction monitoring ($M\text{-}H^-$, *m/z* 137, → $M\text{-}H^- - C_3H_6$, *m/z* 95) or single ion recording ($CH_3PO_3H^-$, *m/z* 95). Quantitation was based on comparison with external standard. In selected cases, CD_3-IMPA was used as an internal standard.

3. Results and Discussion

3.1. *IN VITRO* EXPERIMENTS

Experiments in plasma from rhesus monkeys and humans were performed in order to develop optimal reaction conditions for fluoride-induced reactivation of samples obtained in the *in vivo* experiments. BuChE in plasma from both species was completely inhibited with tabun or sarin. The plasma samples were incubated with potassium fluoride in order to release, isolate and analyze (GC/NPD) the

corresponding phosphofluoridate. The reaction conditions (pH, temperatur concentration of KF, reaction time) were varied systematically in order to optimiz the yield of phosphofluoridate. It appeared that an acidic pH (4-6) is important t obtain a rapid and complete reactivation. Final reaction conditions were 2.0 and 0.2 M KF for tabun-BuChE and sarin-BuChE, respectively, whereas a reaction time o 15 min at 25 °C was optimal for both inhibitors. It appeared that the maximu binding capacity for tabun was ca 16 ng/ml plasma (98 pmol/ml) and 22 ng/r plasma in humans and rhesus monkey, respectively, whereras maximal binding sarin was ca 7 ng/ml (50 pmol/ml) plasma in both species. These data are in the san range as the total binding capacity (80 pmol/ml) of BuChE (4). The identity of (ethyl N,N-dimethylphosphoramidofluoridate (fluorotabun) and of sarin regenerate from inhibited BuChE was confirmed by GC-MS analysis.

The yield of reactivated phosphofluoridate decreases gradually with tim Concomitantly, an increase in BuChE actvity with time is also observed. The phenomena are probably due to spontaneous reactivation and ageing of the inhibit BuChE. Assuming that both processes are first-order, half lives for (spontaneo reactivation + ageing) can be calculated. Results are summarized in Table 1, fro which it is evident that these half lives in humans and rhesus monkey are rath similar for tabun, while the half life for sarin-inhibited BuChE in human plasma is order of magnitude shorter than in monkey plasma.

TABLE 1. Half lives for *in vitro* (ageing + spontaneous reactivation) at pH 7.5, 37 °C, after inhibition of BuChE in human and rhesus monkey plasma with tabun or sarin, based on reactivatibility with fluoride ions (duplicate experiments)

Compound	*In vitro* half life of (ageing + spontaneous reactivation)	
	Human plasma (h)	Rhesus monkey plasma (h)
Tabun	52, 74	70, 74
Sarin	13, 16	113, 148

3.2. *IN VIVO* EXPERIMENTS IN RHESUS MONKEYS

Rhesus monkeys were used for experiments to determine the *in vivo* life span of C inhibited BuChE that can be analyzed after fluoride-induced formation of phospl fluoridate. In this primate species, as in humans, cholinesterases are supposed to the only binding sites in blood for OP anticholinesterases. Tabun or sarin w administered to unanesthetized, atropinized rhesus monkeys until ca. 40% inhibiti of BuChE was obtained. No clinical signs were observed at this level of BuC

inhibition. Blood samples were taken at appropriate time intervals and analyzed f activities of BuChE and AChE, and for fluoride-reactivatible OP-BuChE in plasma

Intravenous administration of 4.1 and 3.2 µg/kg of tabun to a female and a m rhesus monkey, respectively, resulted in approximately the same degree of inhibiti of AChE and BuChE in both animals. However, in the course of 28 days, BuC activity was significantly restored, whereas AChE activity was hardly restor Initially, preferential inhibition of AChE relative to BuChE was observed af administration (i.v.) of 1.0 and 3.2 µg/kg of sarin to a female and a male rhe monkey, respectively. In the course of 50 days, a remarkable lack of restoration activity was observed for both enzymes.

As illustrated in *Figure 1*, the amount of fluoride-released fluorotabun fr tabun-inhibited BuChE decreased gradually with time, but could be quantified u almost 30 days after administration of tabun to both rhesus monkeys. The decre could be described with a mono-exponential function, with half lives of 86 and 3 in the female and male rhesus monkey, respectively. After administration of sa the decrease in the amount of fluoride-induced (re)formation of sarin from sa inhibited BuChE had to be described with a two-exponential function (confer *Fig 2*), with half lives of 20/167 h in the female monkey and 15/326 h in the m monkey. Altogether, the release of sarin from inhibited BuChE could be quantif for 15 and 35 days in the female and male monkey, respectively.

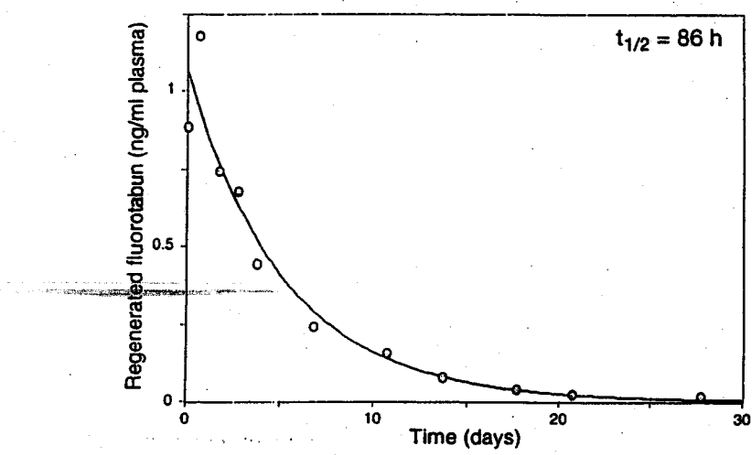

Figure 1. In vivo decrease of fluoride-induced release of fluorotabun from inhibi BuChE in plasma of a rhesus monkey after administration (i.v.) of tabun (4.1 µg/

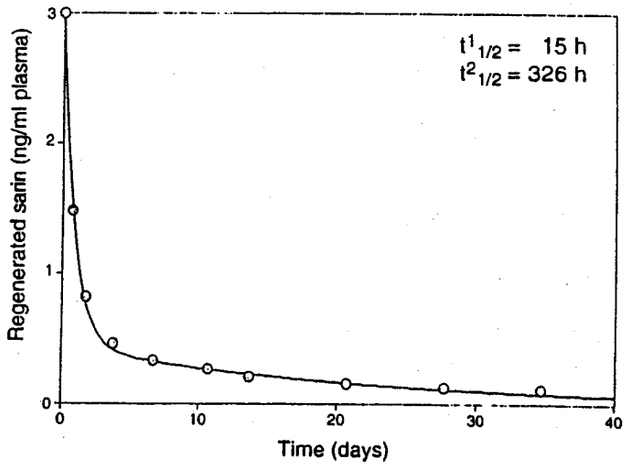

Figure 2. In vivo decrease of fluoride-induced release of sarin from sarin-inhil BuChE in plasma of a rhesus monkey after administration (i.v.) of sarin (3.2 μg

3.3. ANALYSIS OF SERUM SAMPLES FROM JAPANESE VICTIMS

In a recent paper (4) we published preliminary results on the analysis of s samples from victims of terrorist attacks in the Tokyo subway (March 20, 1995 in Matsumoto (June 27, 1994). Blood samples from victims of the Tokyo attack taken within 1.5 h after the incident, while the victims were not treated with ox The samples were sent to TNO Prins Maurits Laboratory in February, 1996 analyzed by means of fluoride-induced regeneration of sarin from inhibited Bu Regenerated sarin was identified by means of GC-high resolution MS, thus prov a conclusive fingerprint for exposure to an anticholinesterase with the stru *i*PrO(Me)P(O)X, with probably X=F. Moreover, the samples were reactivatec obidoxime. As expected, the oxime-induced change in BuChE activity shov satisfactory correlation with the amount of fluoride-regenerated sarin. Resul summarized in Table 2.

We have now analyzed the serum samples for a second fingerprint of exp to sarin, i.e., by means of semi-quantitative analysis of the primary hydr product IMPA in the serum samples. After acidification, the samples were extr with isobutanol/toluene. Using O-isopropyl [^{14}C]methylphosphonic acid, the av recovery of this procedure appeared to be 85 ±15%. The amount of isolated I was analyzed by means of LC-MS-MS analysis with multiple reaction monitor single ion recording. In selected cases a quantitative analysis was perform addition of an internal standard before work-up. Results of these analyses are

in the last column of Table 2. Obviously, substantial amounts of IMPA are present i most of the serum samples, which provides additional evidence for exposure to nerve agent with the structure ^{i}PrO(Me)P(O)X. This second fingerprint for exposu to nerve agents can only be observed in blood for a rather limited period of time aft exposure, since the highly polar IMPA is rapidly excreted into urine. Since IMP must have entered systemically as intact nerve agent and in view of the rapid *in vi* hydrolysis of sarin to IMPA, it might be expected that the degree of initial inhibit of BuChE correlates with the amount of IMPA in the serum samples, which we taken within 1.5 h after exposure. As shown in *Figure 3*, a high content of IMPA serum appears indeed to correlate with a low residual BuChE activity and *vice vers*

TABLE 2. Analyses in serum samples from victims of terrorist attacks in Tokyo and in Matsumoto

BuChE[a] (AU)	Δ BuChE after 10 μM obidoxime[b] (ng/ml serum) (IU/l)	Sarin equiv.	IMPA (ng/ml serum)
Tokyo			
21	+ 865	1.0	127
126	+ 1995	3.3	16
126	+ 1948	2.3	41
126	+ 1145	2.3	14
583	+ 1397	1.7	4
818	+ 462	1.1	2
1100	- 508	0.2	n.d.
1131	+ 1428	2.6	3
804	- 290	n.d.	12
66	+ 3022	4.1	19
172	+ 2257	3.2	27
Matsumoto			
166	+ 1223	2.7	53
52	+ 1474	1.8	107
224	- 219	n.d.	3
1460	- 387	n.d.	n.d.
761	- 963	n.d.	9
1172	- 338	n.d.	n.d.
1186	- 162	n.d.	2

[a] BuChE activity measured in Japan within 1.5 h after exposure (arbitrary units). [b] Change in BuChE activity due to incubation with obidoxime, measured at TNO Prins Maurits Laboratory after long term storage of the samples.

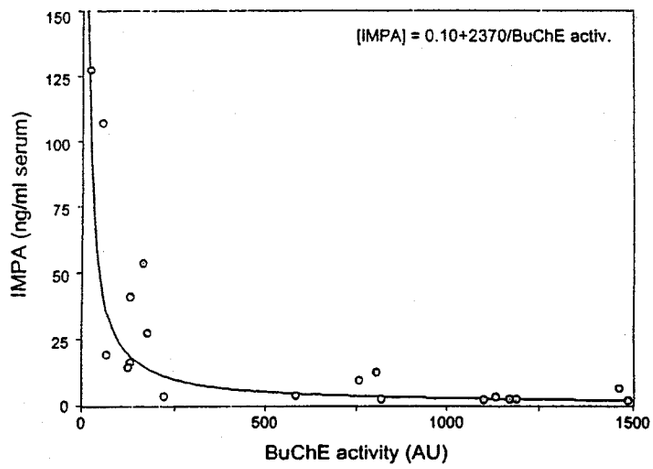

Figure 3. Residual activity of BuChE in serum samples of victims of terrorist attacks with sarin in Tokyo and Matsumoto, measured within 1.5 h after exposure, and concentration of hydrolyzed sarin (IMPA) in these samples.

4. Conclusions

- Reactivation of phosphylated BuChE in plasma with fluoride ions to generate and analyze the corresponding phosphofluoridate represents a rapid and sensitive new method for retrospective detection, identification and quantitation of exposure to nerve agents, which can probably also be adapted for exposure to organophosphate pesticides.
- The retrospectivity of the procedure is only limited by spontaneous reactivation, ageing, and further sequestration of the inhibited BuChE.
- After sublethal administration of tabun and sarin to rhesus monkeys, the regeneration of phosphofluoridate from inhibited BuChE in plasma could be followed for 30 and 15-35 days, respectively, at estimated inhibition levels of BuChE $\geq$ 0.01%.
- Application of the new procedure to serum samples from victims of the terroris attacks with sarin released quantifiable amounts of sarin from BuChE in 10 ou of the 11 cases from the Tokyo subway and 2 of the 7 samples from Matsumot city.
- Analysis of hydrolyzed sarin, i.e., isopropyl methylphosphonic acid, in bloo samples by direct analysis with LC-MS-MS provides a second, but les retrospective, fingerprint of exposure to sarin.

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