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IN VITRO DEGRADATION OF THE FOUR ISOMERS OF SOMAN IN HUMAN SERUM

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Abstract—Starting from racemic soman (1,2,2-trimethylpropyl methylphosphonofluoridate), the degradation of its four stereoisomers in human serum (25°, pH 8.8), has been studied at the nM level. Phosphylation of serum proteins is eliminated by preincubation of the serum with soman. A capillary gas chromatographic method with nitrogen–phosphorous detection allows the separation of the diastereoisomers. The total hydrolysis (enzymatic and non-enzymatic) rate constants of the isomers can then be resolved indirectly on the basis of the important rate difference between P(+) and P(−) isomers. The enzymatic hydrolysis rate constants are obtained by subtracting, for each isomer, the spontaneous (non-enzymatic) rate constant from the total hydrolysis rate constant. The non-enzymatic part of the hydrolysis is obtained from experiments in serum ultrafiltrate (30,000 NMWL). Enzymatic hydrolysis of C(+) P(+) soman occurs so rapidly that only a lower limit of the rate constant can be given. The other enzymatic rate constants are 0.016 min^{-1} for C(+)P(−), 0.74 min^{-1} for C(−)P(+) and 0.028 min^{-1} for C(−)P(−).

Organophosphorus nerve agents usually display an asymmetric centre at the phosphorus atom; this leads to the existence of optical isomers which can behave differently towards biochemical reactions. With regard to the fate of some nerve agents, differences among isomers have been found: the enzymatic hydrolysis of some nerve agents in serum or in plasma has been shown to be stereospecific and a study in rat plasma of the degradation of the isomers of sarin, a nerve agent that has only one asymmetric centre, demonstrated a fast disappearance of the dextrorotatory isomer, P(+), against a slower degradation of the corresponding levorotatory isomer, P(−) [1].

The nerve agent soman has two chiral centres, the P-atom and the α-C-atom of the pinacolyl moiety, and therefore has four stereoisomers. It has been reported that these four stereoisomers have a different toxic potency, e.g. for some *in vitro* reactions, such as cholinesterase inhibition, important differences between the soman-isomers were described [2, 3]. Recently it has been reported that the four isomers of soman show a different kinetic behaviour in the mouse, only one isomer being detected in blood 17–18 hr after *in vivo* administration of 0.75 × LD 50 of C(+) soman [4].

Being interested in the toxicokinetic behaviour of soman in the mammalian body, we found in preliminary experiments in the dog that soman disappears within minutes from serum. It therefore seemed interesting to study the *in vitro* degradation phenomenon in different species starting with human serum.

One can expect that the degradation of the soman isomers in serum consists of three processes: irreversible binding to proteins, as shown for sarin [5], enzymatic hydrolysis [6] and non-enzymatic (spontaneous) hydrolysis. We report on both qualitative and quantitative aspects of these processes. Our previously described assay of soman in serum, based on capillary chromatography and nitrogen-phosphorus detection was used [7]. The chromatography allows the resolution of the diastereoisomeric pairs. Where enzymatic degradation is involved, further resolution of the antipodes is obtained from the biphasic behaviour of the degradation curves.

Most of the kinetic experiments were carried out at an initial soman concentration of 50 nM, which is in the range of physiological concentrations that can be expected to occur in poisoned mammals [4, 8].

MATERIALS AND METHODS

Materials. 1,2,2-trimethylpropyl methylphosphonofluoridate (soman) and 1,2-dimethylpropyl methylphosphonofluoridate (internal standard) were synthesized in house according to the original synthesis of sarin, starting from dimethylmethylphosphonate, as described by Saunders [9]. [^{1}H] and [^{31}P] NMR-spectroscopy showed data consistent with the structure of the compounds and a purity better than 95%.

Blood, drawn from healthy volunteers, was allowed to clot at a temperature of 37°, centrifuged and the collected supernatant was kept at 4° for a maximum of 2 days to allow a build-up of a pool of about 300 ml. Ten to fifteen millilitres of serum fractions were then prepared and stored at −18°. The stored fractions were used within eight weeks after collection. A 300-ml volume of pooled serum was obtained from about 100 blood donors.

Serum ultrafiltrate was prepared from freshly collected serum using a Minitan™ crossflow ultrafiltration system (Millipore) with a molecular weight cut-off of 30,000 NMWL; 300 ml were processed in one operation and 10 ml portions of essentially deproteinated serum were stored at −18°.

All solvents used were p.a.; butylacetate was spec-

troscopic grade and the pH 2 buffer (citrate–HCl) was prepared from Titrisol®. C_{18} cartridges were Seppak (Waters).

Degradation experiments. Samples of 1 ml serum (pH 8.7–8.8) were thermostatized at 25°. At a time $t = 0$, soman, from a concentration solution in 2-propanol (the ratio of 2-propanol/soman was 0.005), was added to the serum. At time t the degradation was stopped [1] by adding 1 ml of pH 2 buffer solution (the pH of the mixture serum–buffer varied from 4.1 to 4.4). The samples were assayed for soman and the values were transformed into a "% remaining soman" using the following equation:

$$\% \text{ remaining soman} = \frac{(\text{assay})_t}{(\text{assay})_{t=0}} \times 100 \quad (1)$$

For the assay at $t = 0$, the buffer was added to the serum prior to the addition of soman.

Assay. The assay procedure has been described in detail [7]. To the serum/buffer mixture was added an amount of 1,2-dimethylpropyl methylphosphonofluoridate (50 μl of a 2-propanol solution) as internal standard. Extraction was performed with a C_{18} modified silica cartridge, 5 ml benzene was used as an eluent. Fifty microlitres butylacetate were then added and the eluate was concentrated under a nitrogen stream to about 30 μl. A 5 μl fraction was injected splitless in a CP Wax 57 CB fused silica capillary column (25 m × 0.22 mm i.d.), installed in a Perkin Elmer Sigma 2 gas chromatograph. Further chromatographic conditions were: injector temperature 170°, detector temperature 200°, column temperature 80° for 10 min (analysis time) followed by a temperature program to clean the column. The carrier gas inlet pressure was 179 kPa. The detector was a nitrogen–phosphorus detector operating under an air pressure of 165 kPa and a hydrogen pressure of 62 kPa. The bead was heated electrically to give a baseline current of 25% of full scale at an attenuation of 32.

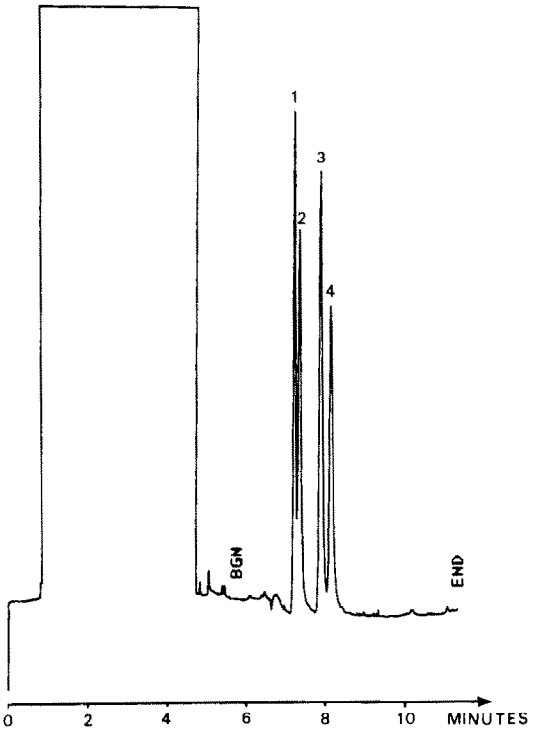

Fig. 1. Test chromatogram of a mixture of 1,2,2-trimethylpropyl methylphosphonofluoridate (soman) and 1,2-dimethylpropyl methylphosphonofluoridate (internal standard) in butylacetate (450 nM). The diastereisomers of the compounds are separated. Identification: 1,2-dimethylpropyl methylphosphonofluoridate, 1 and 2; 1,2,2-trimethylpropyl methylphosphonofluoridate, 3 [C(+)P(−); C(−)P(+)] and 4 [C(−)P(−) and C(+)P(+)]. Five microlitres are injected splitless. Attenuation 32. Further chromatographic conditions as mentioned in the Materials and Methods section.

The chromatogram, obtained under the conditions described is given in Fig. 1. Both internal standard and soman have two chiral centres and therefore display each of four possible isomers; the chromatography allows the resolution of the diastereoisomers as shown on Fig. 1. As identical achiral GLC conditions were used as by Benschop *et al.* [2] we assume that the first peak of soman contains the C(+)P(−) and C(−)P(+) isomers, the second peak contains the C(+)P(+) and C(−)P(−) isomers.

Quantitation was done by calculating the ratio of peak heights of soman and internal standard. Either total soman was quantified (sum of the two peaks = soman$_t$) or the enantiomeric pairs were quantified separately. For the calculation of the residual amount of soman versus time, eqn (1) can be used only if the ratio of peak heights of soman and internal standard is a linear function of the concentration, with a zero intercept; therefore calibration graphs were determined at regular time intervals by adding successively to 1 ml serum samples: 1 ml pH 2 buffer, various amounts of soman and a constant amount of internal standard. The samples were assayed as indicated and the ratio of peak heights of soman and internal standard were plotted in function of the concentration of soman in serum: the correlation coefficients of the graphs were better than 0.99 and the intercept was sufficiently close to zero, i.e. the absolute values of the intercepts remained smaller than 3% of the peak height ratio obtained for a soman concentration of 50 nM.

Preincubation. In those experiments in which preincubation was used, serum was spiked with soman, from a concentrated solution in 2-propanol to obtain a concentration in serum ranging from 50 nM to 5 μM. The spiked serum was kept for 17 hr in a thermostatized water bath at a temperature of 25° (±0.5°). After this period no soman could be detected above a threshold of 100 pg/ml (550 pM). Degradation experiments were then started by adding soman to the preincubated serum.

Calculation of hydrolysis rate constants. First order rate constants were determined from the plot of the logarithm of the percentage remaining soman vs time, using linear curve fitting. Biphasic hydrolysis curves, that are due to the different hydrolysis rates of optical antipodes, are resolved by the graphical method of residuals, used extensively in pharmacokinetics [10].

RESULTS

Degradation of soman in human serum

Figure 2 shows the disappearance of soman in serum as a function of time: within 5 min, more than 95% of the soman has disappeared. At 10 min no more soman could be detected. A net difference exists between the time course of the disappearance of the first and second eluting soman peak, indicating a different behaviour of each of the four isomers.

Influence of preincubation on the degradation of soman in serum

To neutralize the capacity for irreversible binding of soman, the preincubation of the serum with increasing concentrations of soman was performed under the hypothesis that this binding might become saturated. The results show that as the preincubation concentration increases, the degradation curve becomes less steep until it remains unaffected by further increase of preincubation concentration (Fig. 3). The preincubation concentration that is sufficient to obtain a constant degradation curve is estimated from a statistical evaluation based on the repetitive determination of the percentage of soman remaining after 10 min for different preincubation concentrations (Table 1): the value at a preincubation with 0.5 μM differs significantly from the value at a preincubation with 2.5 μM (one sided test of the mean with a confidence level of 95%); preincubation with 5 μM gives a value that does not differ significantly from the value at 2.5 μM. It is therefore concluded that preincubation with 2.5 μM will lead to an occupation of all adsorption sites. Confirmation of this protein binding is obtained in the experiments where serum ultrafiltrate was used (*vide infra*).

Hydrolysis of soman in serum ultrafiltrate

The contribution of spontaneous hydrolysis of soman to its total degradation in serum was evaluated by use of the serum ultrafiltrate. Figure 4 gives the decrease of the first peak and the second peak of soman vs time at 25° and for an initial concentration of 50 nM. The serum ultrafiltrate was not preincubated. It is seen that the biphasic nature has disappeared and that there is a distinct difference in hydrolysis rate between the two peaks, which accounts for the physico-chemical differences existing between diastereoisomers. From the slope the rate constants (k_w) for the hydrolysis of the isomers of soman in protein-free serum can be calculated (Table 2).

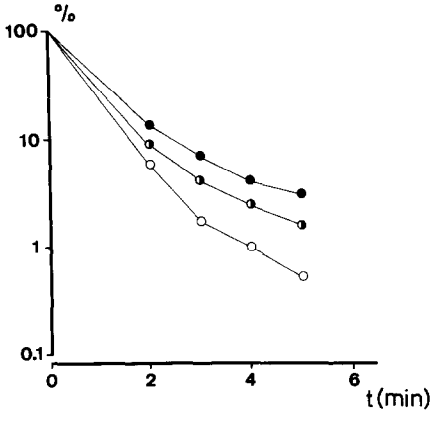

Fig. 2. Degradation of soman (50 nM) in human serum at 25° and pH ranging between 8.7 and 8.8. % remaining soman vs time is plotted. ◐ Total soman (= sum of the two peaks). ○ First eluting peak, containing C(+)P(−) and C(−)P(+) isomers. ● Second eluting peak, containing C(+)P(+) and C(−)P(−) isomers.

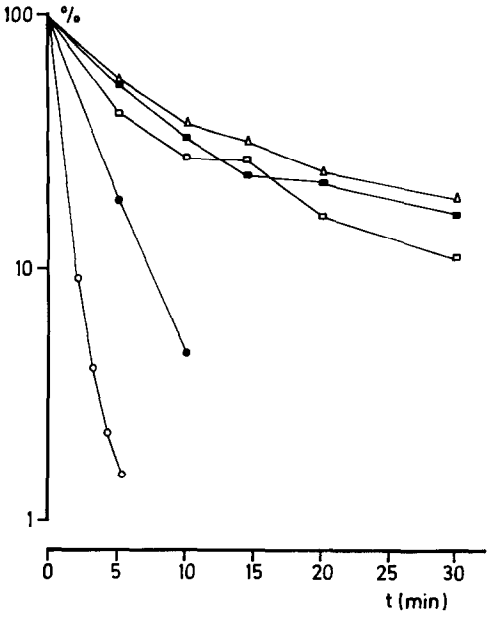

Fig. 3. Degradation of soman (50 nM) in serum at 25°. Influence of preincubation. Serum is preincubated at 25° during 17 hr with various initial concentrations of soman. The % of total soman remaining is plotted vs time. Soman preincubation concentration (μM): ○, no preincubation; ●, 0.05; □, 0.25; ■, 0.5; △, 2.5.

Hydrolysis of soman in preincubated serum

In preincubated serum both enzymatic and spontaneous hydrolysis will take place. The hydrolysis curves at 25° are shown on Fig. 5. Taking into account that the 100% point was experimentally determined, the figure shows that both peak 1 and peak 2 disappear in a biphasic way, allowing the resolution of the four rate constants (k_t) by the graphical method of residuals: the four stereoisomers of soman are hydrolysed at a different rate (Table 2). The reasoning for assigning the rate constants to the different isomers is developed in the discussion.

To verify the first order character of the total hydrolysis process for each isomer, the rate constants are determined for different initial concentrations. The degradation curves obtained with different initial concentrations are similar to Fig. 5. The results are shown in Table 3. No significant difference is found at a 95% confidence level.

According to classical theory of parallel first order reactions, the rate constants for the enzymatic process in serum can now be calculated by subtracting, for each isomer, the corresponding k_w from k_t (Table 2).

Table 1. Influence of preincubation with soman on the degradation of soman in human serum at 25°

Preincubation concentration (μM)	Remaining soman after 10 min (%)	Relative standard deviation (%)	Number of determinations
0.5	33.5	6.3	4
2.5	37.2	9.8	13
5	37.4	3.8	5

Preincubation conditions: 25° during 17 hr. Initial concentration of soman in the hydrolysis experiment: 50 nM.

Table 2. Rate constants with 95% confidence limits for the hydrolysis processes of soman in serum at 25° and pH 8.7–8.8

Soman isomer	Rate constant (min^{-1}) k_w	k_t	k_e
C(+)P(−)	0.014 ± 0.002	0.030 ± 0.004	0.016 ± 0.004
C(−)P(+)	0.014 ± 0.002	0.75 ± 0.24	0.74 ± 0.25
C(−)P(−)	0.022 ± 0.003	0.050 ± 0.003	0.028 ± 0.004
C(+)P(+)	0.022 ± 0.003	>1*	>1

* Hydrolysis rate constant of C(+)P(+) is 1.0 ± 0.4 min^{-1} at 0°.
k_w: spontaneous hydrolysis in deproteinated serum.
k_t: hydrolysis in preincubated serum.
k_e: enzymatic hydrolysis in serum, $k_e = k_t - k_w$.

DISCUSSION

From previous studies on phosphonofluoridates and phosphorofluoridates and from the soman degradation curves we obtained, it became clear that the fate of soman in human serum should be composed of mainly three processes: irreversible protein binding (phosphylation) [11, 12], spontaneous hydrolysis [1] and enzymatic hydrolysis [7].

At low soman concentrations phosphylation becomes relatively important; this is illustrated by the initial rapid decrease of soman (50 nM) in non-preincubated serum (Fig. 2) and the influence of preincubation concentration (Fig. 3). The inhibition of pseudocholinesterase (Acylcholine acylhydrolase, E.C. 3.1.1.8) should account for about 1 nM of phosphylation sites [13]. The importance of irreversible protein binding as an aid in detoxification has been mentioned by other authors: aliesterase in rodent serum can account for the higher tolerance of these species towards nerve agent intoxication [14]. However other proteins in serum are capable of binding organophosphates: DFP was shown to be irreversibly bound to serum-albumin fractions [15] and the inhibition of thrombine and plasmine by sarin was demonstrated [16].

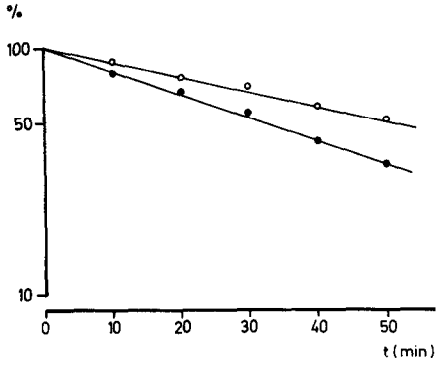

Fig. 4. Hydrolysis of soman (50 nM) in ultrafiltrated human serum at 25°. The % remaining soman vs time is plotted. ○ First eluting peak, containing C(+)P(−) and C(−)P(+) isomers. ● Second eluting peak, containing C(−)P(−) and C(+)P(+) isomers. Each point represents the mean of 4 determinations. All determinations were used for fitting.

Preincubation of serum with a sufficient amount of soman leads to a saturation of nearly all binding sites, the remaining soman being destroyed by the hydrolysis processes. Preincubation of serum with 2.5 μM soman is sufficient to obtain a reproducible degradation curve, which accounts for the remaining processes, enzymatic and non-enzymatic hydrolysis.

For total soman, the hydrolysis time course behaves in a biphasic way (Fig. 3, △). In agreement with previous observations of Christen on the soman-analogue sarin [1], we suggest that this is due to a rapid breakdown of the P(+) isomers and a much slower degradation of the P(−) isomers. Benschop *et al.* [2] further demonstrated that the first chromatographic peak of soman is composed of the C(+)P(−) and C(−)P(+) isomers and the second peak of the C(+)P(+) and C(−)P(−) isomers. The biphasic behaviour of the disappearance of the two peaks in serum can thus be used to resolve both of its components. At the experimental conditions mentioned, the C(+)P(+) isomer hydrolyses so fast that only a lower limit of k_t can be given; this limit is based on experiments at 0°, that allow a resolution

Table 3. Influence of initial concentration on the hydrolysis rate constants (±95% confidence limits) of the slowly hydrolysed isomers of soman in preincubated human serum (with 2.5 μM of soman during 17 hr at 25°)

Soman initial concentration (nM)	Hydrolysis rate constant k_t (min^{-1})		
	C(+)P(−)	C(−)P(−)	C(−)P(+)
50	0.030 ± 0.004	0.050 ± 0.003	0.75 ± 0.24
500	0.028 ± 0.003	0.047 ± 0.003	0.82 ± 0.31
5000	0.028 ± 0.005	0.048 ± 0.004	0.57 ± 0.32

of the k_t of the C(+)P(+) isomer, its value being 1.0 (±0.4) min^{-1}. The k_t values are in accordance with the qualitative *in vitro* observations of Benschop *et al.* [17], who examined the hydrolysis in mouse plasma of a much higher soman concentration (1 mM) at which phosphylation becomes a negligible phenomenon. It has been stated previously that phosphoryphosphatase in serum behaves differently from hog kidney phosphorylphospatase [18]: thus the k_e values, as far as the P(+) isomers are concerned, are in contrast with the recently published qualitative enzymatic degradation curves of the four stereoisomers of soman in purified phosphorylphosphatase from hog kidney [4]; in human serum the order of hydrolysis rates between the P(+) isomers is reversed and the difference between their rate constants is more marked.

Table 2 shows that, for the P(−) isomers, the enzymatic hydrolysis rate constants are in the same order of magnitude as the non-enzymatic rate constants, whereas for the P(+) isomers the non-enzymatic part of the hydrolysis is largely negligible. This is in accordance with the reported high selectivity of phosphorylphosphatase for the P(+) isomer of sarin [19].

Although it is more rapidly hydrolyzed than the C(+)P(−) isomer, the C(−)P(−) isomer has been shown to be the most toxic isomer of soman in mice [2]. Taking into account the faster hydrolysis rate of the C(+)P(+) isomer over its C(−)P(+) counterpart (Fig. 2) suggests that the initial irreversible adsorption to proteins is more important for the C(+)P(−) isomer than for the C(−)P(−) which might result, also in man, in a lower acute toxicity for the C(+)P(−) as compared with the C(−)P(−) isomer.

It must be stressed that the experiments were done at pH 8.8, probably due to the loss of CO_2 during the serum pool built-up time. According to Becker and Barbaro [20], this condition favours the phosphonate-esterase activity, which shows an optimum at a pH value higher than 9. It might therefore be that the processes described occur more slowly at pH 7.4. Work to investigate this is in progress.

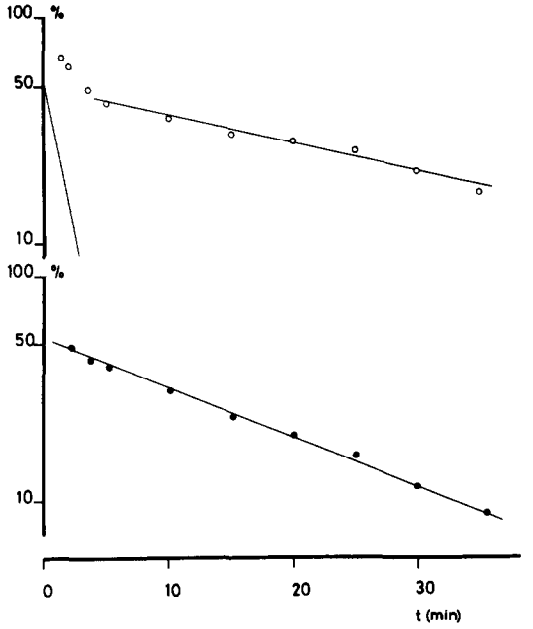

Fig. 5. Hydrolysis of soman (50 nM) in preincubated (2.5 μM during 17 hr at 25°) human serum at 25°. The % remaining soman vs time is plotted. ○ First eluting peak, containing the C(+)P(−) and C(−)P(+) isomers. ● Second eluting peak, containing the C(−)P(−) and C(+)P(+) isomers. The curves are resolved according to the method of the residuals and the best fitting is done by linear regression. Each point represents the mean of 3 or 4 determinations; fitting was done using all the determinations.

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