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Separation of geometrical *syn* and *anti* isomers of obidoxime by ion-pair high-performance liquid chromatography☆

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Abstract

Obidoxime chloride (Toxogonin) is one of the fastest reactivating oximes to restore acetylcholinesterase activity after intoxication with organophosphates. Reversed-phase HPLC analysis with ion-pairing reagents revealed the existence of three geometrical isomers, both in solid obidoxime dichloride monohydrate and in solution (Toxogonin ampoules). The *syn–syn* isomer prevails, the asymmetric *syn–anti* isomer amounts to 5% (solid) and 3% (solution) and the symmetric *anti–anti* isomer less than 0.1%. The structure of the *syn–anti* isomer isolated by ion-exchange chromatography was confined by ^{1}H NMR spectroscopy. The acidity of the oxime protons decreased from the *syn–syn* isomer (mean pK_a 8.0) to the *syn–anti* (mean pK_a 8.3) and the *anti–anti* form (mean pK_a 8.6). In addition, the absorption maximum of the oximate was shifted from 356 (*syn–syn*) to 360 (*syn–anti*) and 367 nm (*anti–anti*). Analysis of outdated Toxogonin ampoules that had been stored at about 20°C for up to 19 years indicated a shelf-life (10% decomposition) of ca. 20 years, which is less than predicted earlier from advanced decomposition studies. Cyanide formation was negligible, and the ratio of *syn–syn*- to *syn–anti*-obidoxime still remained at 3%.

1. Introduction

Mono- and bispyridinium aldoximes have been shown to be effective adjuncts to atropine in the treatment of organophosphate intoxications. Obidoxime (Toxogonin) [1] is used therapeutically in insecticide poisonings in Germany, Israel, Netherlands, South Africa, Sweden, Switzerland and in various developing countries. Obidoxime (Fig. 1) is one of the fastest reactivating oximes [2–8], although not effective in soman poisoning [9,10].

To improve the therapeutic effectiveness of obidoxime treatment in patients with intoxications by long-residing thiono organophosphate insecticides, e.g., parathion, we considered continuous infusion of obidoxime to be more appro-

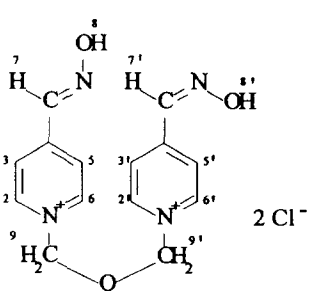

Fig. 1. Structure of *syn–anti*-obidoxime (obidoxime-I).

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priate than a single injection. Similar recommendations have recently been published for treatment with pralidoxime (2-PAM) [11]. To follow the plasma concentrations reliably, we adopted a reversed-phase HPLC method using ion-pairing reagents, as previously applied for quantitative determination of other pyridinium oximes [12–15].

When we succeeded in an improved HPLC separation of obidoxime without tailing, we became aware of a by-product (3–5%) that was found in solid obidoxime, in freshly manufactured Toxogonin ampoules and in outdated batches. Such a compound has already been noted by Benschop et al. [12], who considered it to be a decomposition product of the obidoxime bulk. More recently it has been reported that freshly prepared obidoxime solutions also contain a compound (about 4%) that was suggested to be a regioisomer of obidoxime [16].

Oximes can possess two isomeric configurations, *syn* and *anti*. Poziomek et al. [17] succeeded in isolating the two geometrical isomers of 4-PAM [4-(hydroxyiminomethyl) pyridinium methochloride], which were characterized by ^{1}H NMR spectroscopy. Interestingly, the *syn* isomer of 4-PAM has a higher reactivity towards inhibited acetylcholinesterase than the *anti* isomer: sarin-inhibited electric eel enzyme was reactivated about three times faster by the *syn* isomer.

The structure of 2-PAM as the *syn* isomer has been unequivocally proved by X-ray crystallography [18]. Until now, the pure *anti* isomer of 2-PAM has not been prepared, probably because the *anti* isomer rapidly isomerizes to give the *syn* isomer or decomposes into the nitrile and carboxamido derivative. Utley [14] was probably the first to detect small amounts of the *anti* isomer in solutions of 2-PAM by HPLC. This isomer is hardly detected in freshly prepared 2-PAM solutions but gradually increases to an equilibrium concentration of approximately 3% [14,19]. It has been suggested that the major degradation reactions proceed from the more unstable *anti* isomer of 2-PAM [20].

In the description of obidoxime synthesis, Lüttringhaus and Hagedorn [1] already mentioned that two isomers of obidoxime can be obtained which are interconvertible. During storage the more stable *syn–syn* isomer is formed. Later, preparation of the pure obidoxime isomers was claimed [21] with the *syn* isomer being twice as effective as the *anti* isomer in organophosphate-poisoned mice. Whether the mentioned *anti* isomer of obidoxime possesses a symmetrical *anti–anti* or an asymmetric *syn–anti* configuration has not been reported.

In this study, we describe the isolation and properties of a major by-product of commercial obidoxime preparations that was identified as an asymmetric *syn–anti* isomer of obidoxime. A minor by-product was tentatively described as the *anti–anti* isomer. In addition, we analysed Toxogonin ampoules that had been stored at ambient temperature for up to 19 years to determine the chemical stability and the degradation products in pharmaceutical preparations of obidoxime.

2. Experimental

Solid obidoxime dichloride monohydrate was obtained from Duphar (Amsterdam, Netherlands; batch 058646-FOA 009090). According to the analysis certificate this preparation contained 94.5% obidoxime dichloride and 4.5% water (Karl Fischer) with 4-pyridinealdoxime below 0.1%.

Toxogonin ampoules (1 ml containing 250 mg of obidoxime dichloride, 0.65 mg of methyl 4-hydroxybenzoate and 0.35 mg of propyl 4-hydroxybenzoate) were purchased from Merck (Darmstadt, Germany) and stored for up to 19 years at different places at ambient temperatures averaging between 16 and 21°C.

An attempt to separate obidoxime-I (the *syn–anti* isomer present at about 5% in the solid obidoxime preparation) by fractional crystallization of obidoxime-II, the *syn–syn* isomer, in weakly acidic (pH 4) solution was unsuccessful. However, the separation of the two isomers as monobetaine salts allowed an easy enrichment. Monobetaine formation was carried out as described [1]: about 1 g of obidoxime dichloride monohydrate was dissolved in 3 ml of water at

30°C and mixed with finely ground sodium carbonate (0.27 g) with vigorous stirring (pH 8.1). The colour of the solution changed immediately to red-orange and became turbid. Precipitation was advanced by adding 1 ml of 2-propanol dropwise. After storage in a refrigerator for 1 h, the orange precipitate was centrifuged and the dark supernatant, which had an obidoxime-I to obidoxime-II ratio of about 1:3, was removed. Further crystallizations increased the portion of obidoxime-I in the supernatant only marginally. Therefore, we took advantage of their differences in acidity and separated the two isomers in the supernatant by ion-exchange chromatography.

The monobetaines (12.5 mg of obidoxime-I in 200 ml) were chromatographed on CM_{50}-Sephadex (18 × 5 cm I.D. column; equilibrated with 10 m*M* ammonium acetate, pH 8.5). Obidoxime-II was eluted first when a linear gradient (500 ml of 10 m*M*–500 ml of 20 m*M* ammonium acetate, pH 8.5) was applied, followed by obidoxime-I and a small additional peak eluting last. The first cut, containing obidoxime-II, was pure as judged by HPLC. Cut 2, containing obidomine-I was >99% pure, but tended to isomerize slowly upon lyophilization (5% obidoxime-II). The amount of the material eluting last was too small for NMR analysis but allowed characterization by UV spectroscopic titration (obidoxime-0).

For ^1H NMR spectroscopy, both lyophilized isomers were dissolved in ^2H_2O and measured in a 500-MHz Bruker instrument with H_2O set to 4.80 ppm.

2.1. Analytical procedure

The pK_a values of the oxime functions were investigated by spectroscopic titration at 20°C. Oximate formation was followed at around 360 nm. According to the Henderson–Hasselbalch equation, log ($[A^-]/[HA]$) was plotted versus pH, which gives pK_a at $\log([A]/[HA]) = 0$. On changing the pH, perfect isosbesticity was observed throughout. For spectroscopic determinations of more concentrated solutions (up to 30 m*M*), cuvettes with shorter light paths were used (0.01, 0.1, 1 and 10 mm; Hellma, Freiburg, Germany). Electronic spectra were recorded with a UV-265 spectrophotometer (Shimadzu, Duisburg, Germany).

The obidoxime isomers and some of their decomposition products were determined by HPLC on LiChrospher 60 RP-select B (5 μm) (Merck) with an L-6200A pump (Merck) at a flow-rate of 1.2 ml/min. The mobile phase consisted of methanol–PIC-B7–PIC-A (12:4:0.5 ml, diluted to 100 ml with water). The ion-pairing reagents PIC-B7 and PIC-A were obtained from Waters–Millipore (Eschborn, Germany). PIC-A was included to reduce peak tailing of obidoxime. Peaks were quantified with an SPD-6AV UV–Vis detector or an SPD M6A diode-array detector (Shimadzu) and a D-2500 chromato-integrator (Merck) calibrated with authentic standards. The detection wavelength was set to 285 nm.

Formaldehyde was determined as formaldimedone [22]. Hydrocyanic acid was determined spectrophotometrically by the Zincke–König reaction [23]. The ampoules were cooled on ice before opening and the contents transferred immediately into Fernbach flasks for microdiffusion of hydrocyanic acid [23].

3. Results and discussion

When freshly prepared obidoxime solutions were analysed by HPLC (Fig. 2), several by-products were observed that were also found in Toxogonin ampoules. The major by-product (peak I) and the main compound (peak II) had virtually the same UV spectra (Fig. 3). Rechromatography of the cuts from several HPLC runs showed that the compounds did not change their individual retention times. Hence, the by-product giving peak I was tentatively assumed to be a regioisomer of obidoxime. Similarly, a compound eluting earlier (by-product giving peak 0, Fig. 2) had spectroscopic properties similar to those of the main compound. The material eluting after 2.2 min had an identical retention time (sample spiking) and UV spectrum as 4-pyridinealdoxime and amounted to less than

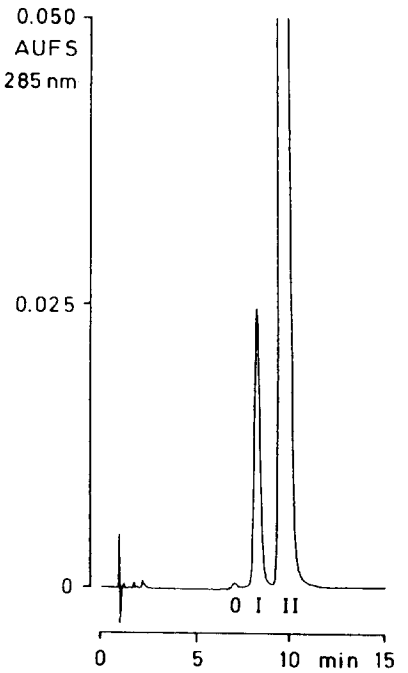

Fig. 2. HPLC analysis of freshly dissolved obidoxime dichloride. Peaks: 6.84 min = *anti–anti*-obidoxime (0.07%); 7.94 min = *syn–anti*-obidoxime (5.1%); 9.32 min = *syn–syn*-obidoxime (94.8%).

0.1%. This material probably originated from obidoxime synthesis.

Spectroscopic titration of the tentative obidoxime isomers that were obtained from several HPLC runs (cf., Fig. 2) showed different pK_a values of the oxime functions. We intentionally exploited this behaviour to separate the compounds on a larger scale by anion-exchange chromatography.

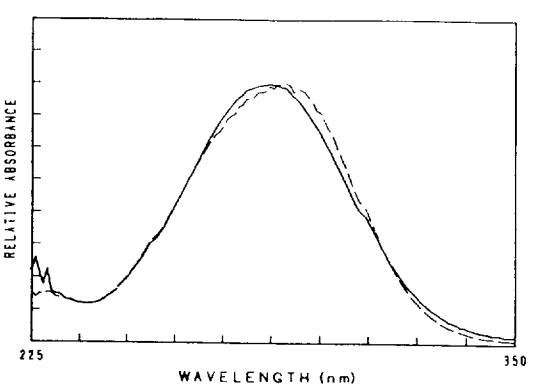

Fig. 3. UV spectra of two obidoxime isomers. Spectra of the HPLC eluate (pH 4) were recorded with a diode-array detector and normalized to the same relative absorbance. Solid line, *syn–syn*-obidoxime; dashed line, *syn–anti*-obidoxime.

The structure (Fig. 1) of the isolated obidoxime-I and obidoxime-II was confirmed by ^1H NMR spectroscopy. The data summarized in Table 1 indicate that obidoxime-II possesses the symmetric *syn–syn* configuration whereas obidoxime-I showed some asymmetry with four different aromatic proton signals, two different methine resonances and two different methylene signals. Particularly striking were the differences in the proton signals 3′ and 5′, which were shifted by 0.39 ppm to lower field in the *anti* configuration, while the signal of the methine proton (7′) was shifted by 0.47 ppm to higher field. Such a behaviour has been repeatedly described in the literature for other oximes [17,24,25]. The reason for this phenomenon might be the missing coplanarity of the aromatic ring and the aldoxime group in the case of the *anti* configuration, while the diamagnetic anisotropy of the oxime group has been suggested to strengthen the outer field at the methine proton in the *syn* configuration [24,25]. The isolated material of obidoxime-0 was too small for ^1H NMR analysis.

UV spectroscopic titration of the three obidoxime isomers indicated a systematic difference in acidity of the oxime function, being highest in the *syn–syn* and lowest in the assumed *anti–anti* configuration (Table 2). Such a phenomenon has been discussed previously [24,25]. Moreover, the absorption maximum of the oximate was shifted to longer wavelengths from *syn–syn* to *anti–anti*. This behaviour has been previously described for *syn–anti* isomerism of other oxime compounds [24,25].

HPLC analysis of outdated Toxogonin ampoules that had been stored up to 19 years indicated several additional products (Fig. 4). The material that eluted first (M-1; 1.89 min) had similar spectroscopic characteristics to obidoxime. The oxime maximum at 284 nm changed to 354 nm on alkalinization. In addition a second maximum was present at 262 nm that

Table 1
^{1}H NMR data for obidoxime isomers

Position	Obidoxime-II (*syn–syn*)			Obidoxime-I (*syn–anti*)		
	δ (ppm)	*J* (Hz)	Relative intensity	δ (ppm)	*J* (Hz)	Relative intensity
2	8.88	6.9	1	8.91	6.5	1
3	8.20	6.9	1	8.22	6.5	1
5	8.20	6.9	1	8.22	6.5	1
6	8.88	6.9	1	8.91	6.5	1
7	8.33		1	8.34		1
9	6.17		2	6.19		2
2′	8.88	6.9	1	9.07	6.6	1
3′	8.20	6.9	1	8.59	6.6	1
5′	8.20	6.9	1	8.59	6.6	1
6′	8.88	6.9	1	9.07	6.6	1
7′	8.33		1	7.86		1
9′	6.17		2	6.24		2

^{1}H NMR spectra were recorded in ^2H_2O (H_2O = 4.80 ppm). For assignments, see Fig. 1.

did not show any pH dependence. These figures indicate that one oxime function had remained whereas the other pyridinium ring probably had a 4-carboxy or carboxamido function [22]. Because of the high polarity of the compound, we favour an isonicotinic acid structure and tentatively assign the compound as 4′-carboxy-4-(hydroxyiminomethyl)-1,1′-oxydimethylenedipyridinium dichloride. Such a degradation pathway has already been described [26,27].

The material eluting at 2.2 min was 4-pyridinealdoxime. The amount of the material eluting at 4.72 min was too small for further analysis. The compounds eluting at 7.55, 8.9 and 10.55 min belong to the regioisomers of obidoxime. As formaldehyde and cyanide can be formed during degradation of obidoxime [26,28], we analysed the different batches accordingly. Table 3 gives the amounts of compounds found in the various batches.

Visual examination of the outdated Toxogonin ampoules that had been stored between 16 and 20°C on average showed that the oldest preparations had the most intense orange colour, whereas freshly prepared solutions were almost colourless. As the pH of the old batches was lower (pH 3.6) than in fresh solutions (pH 4.0) a change in colour due to the formation of the orange betaine form [1] was unlikely. In fact, spectroscopy of the solutions at pH 4 between 800 and 500 nm excluded a charge-transfer complex [1]. Rather, the absorbance gradually increased without the formation of distinct maxima or shoulders in the visible spectrum. All batches revealed the typical obidoxime maximum at 285 nm.

Table 2
UV spectroscopic data and pK_a values for obidoxime isomers

Compound	Obidoxime-II	Obidoxime-I	Obidoxime-0
Tentative structure	*syn–syn*	*syn–anti*	*anti–anti*
Absorption maximum of the betaine (nm)	356	360	367
pK_a	8.0	8.3	8.6

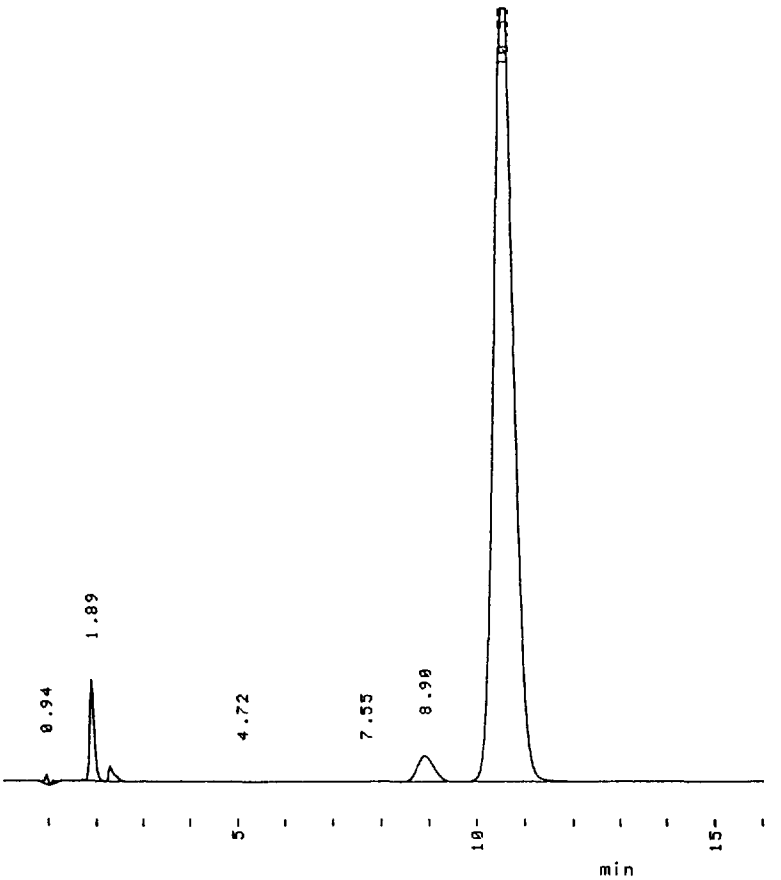

Fig. 4. HPLC analysis of an outdated Toxogonin ampoule. The following products were tentatively assigned: 1.89 min = 4′-carboxy-4-(hydroxyiminomethyl)-1,1′-oxydimethylenedipyridinium dichloride; 2.20 min = 4-pyridinealdoxime; 7.55 min = *anti–anti*-obidoxime; 8.90 min = *syn–anti*-obidoxime; 10.55 min = *syn–syn* obidoxime.

Table 3
Analysis of Toxogonin ampoules

Storage (years)	pH	Colour[a]	M-1 (%)	4-PAO (%)	CH_2O (%)	HCN (10^{-3} %)	Obi-1 (%)	Obi-2 (%)
19	3.57	0.54	2.4	0.83	0.16	0.32	2.90	89.0
14	3.62	0.37	2.3	0.97	0.08	0.27	3.04	88.5
14	3.62	0.41	2.3	0.93	0.10	0.35	3.00	89.3
14	3.70	0.30	1.8	0.63	0.08	0.29	3.03	91.7
14	3.63	0.34	0.8	0.73	0.12	0.29	3.04	90.0
14	3.54	0.16	2.2	0.40	0.06	0.20	3.12	90.9
2	4.00	0.05	0.2	0.06	0.04	0.11	3.04	95.9

Mean values of three determinations per batch. Abbreviations: M-1 = 4′-carboxy-4-(hydroxyiminomethyl)-1,1′-oxydimethylenedipyridinium dichloride; 4-PAO = 4-pyridinealdoxime; Obi-1 = *syn–anti*-obidoxime; Obi-2 = *syn–syn*-obidoxime.
[a] Colour: molar absorptivity of a 1% solution at 400 nm.

It has been mentioned [1] that the molar absorptivity of the oximate did not follow the Lambert–Beer law. This observation was confirmed by Schoene [29], who reported on bathochromic and hypsochromic shifts of the oximate spectra in increasingly concentrated solutions. These experiments were performed in 0.5–50 m*M* obidoxime solutions (pH 7.5). To allow readings at these high concentrations, Schoene used cuvettes with a 1-mm light path and made use of difference spectroscopy, e.g., sample cuvette 50 m*M*, reference cuvette 37.5 m*M* obidoxime [29]. Of course, such measurements are detrimentally falsified by stray light (50 m*M* obidoxime at pH 7.5 has a molar absorptivity at 334 nm of about 100 $l \cdot mol^{-1} \cdot cm^{-1}$ with a 1-mm light path!). When we repeated these measurements using quartz cuvettes with 0.01–10-mm light paths at pH 3, 7.5 and 8.0, the spectra followed the Lambert–Beer law perfectly. No bathochromic shifts were observed. Hence, we cannot confirm spectroscopic anomalies that had been interpreted as indicative of charge-transfer complex formation [29]. Thus, the orange colour probably stems from an unknown decomposition product.

Overall, this study has shown that obidoxime solutions contain about 3% of the asymmetric *syn–anti* isomer and about 96% of the *syn–syn* isomer. Solid obidoxime dichloride contains almost 5% of the *syn–anti* isomer. In addition, we could confirm the high stability of obidoxime solutions with a shelf-life of almost 20 years when stored around 20°C. The stability, however, is less than predicted from advanced decomposition studies (calculated shelf-life of about 100 years at 25°C [30]).

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