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Simple high-performance liquid chromatographic method for assessing the deterioration of atropine-oxime mixtures employed as antidotes in the treatment of nerve agent poisoning

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ABSTRACT

A set of reversed-phase HPLC conditions for determining the degradation of atropine and the oxime (pralidoxime, obidoxime, or HI-6) in autoinjectors designed for use against nerve agent poisoning is described. The assay conditions for atropine do not require its prior separation from the large molar excess of oxime since both the atropine and tropic acid peaks elute well clear of the oxime and its degradation products and the phenolic preservatives. Further dilution of the sample and simple changes to the mobile phase then provide conditions for the oxime and its major degradation products to be quantitated.

INTRODUCTION

A mono-quaternary-pyridinium or bis-quaternary-pyridinium oxime with atropine is the basis of the currently preferred treatment for poisoning by organophosphate compounds [1]. Autoinjectors containing these materials are provided to military personnel for self-aid against nerve agent poisoning.

Despite the many reports of HPLC methods for the analysis of atropine and the above oximes, particularly those that also consider their degradation products [2–11], we have found few if any that can be applied directly to both these components in injection mixtures [12,13]. Typically the autoinjectors contain oxime:atropine millimolar ratios of between 100:1 and 1100:1 and the tailing of the large oxime peak at the solvent front has made the direct assay of atropine and its breakdown products in such mixtures difficult.

Of the oximes, HI-6 is less stable in aqueous solution than either obidoxime or pralidoxime chloride [6,14,15]. Also solutions of HI-6 or pralidoxime chloride are less stable than atropine sulphate [6,13,16]. However, both the atropine and the oxime in injectors could be susceptible to the effects of high ambient temperatures [4,5,10,14,17,18] as might be found under some field conditions.

This paper describes a relatively simple HPLC method for determining the extent of degradation of the oxime and atropine in autoinjectors that does not involve a prior separation of the solution components. The method allows the

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detection and assay of one or more of the known breakdown products as well as the assay of the parent compounds.

EXPERIMENTAL

Materials

Atropine sulphate, pralidoxime chloride [2-((hydroxyimino)methyl)-1-methyl pyridinium chloride] (2-PAM-Cl) and pralidoxime methanesulphonate (P2S) were obtained from Sigma (USA). Obidoxime chloride [1,1'-(oxy-bis(methylene)) - bis(4 - (hydroxyimino)methyl) - pyridinium dichloride] (Toxogonin) was obtained from E. Merck (Germany) and HI-6 {1-[((4-(aminocarbonyl)-pyridino)methoxy)methyl]-2[(hydroxyimino)methyl]-pyridinium dichloride} from Drs. P.A. Lockwood and J.G. Clement, Defense Department, Canada. The structures are detailed in Fig. 1.

Other compounds, used as standards, were DL-tropic acid, pyridine-Zaldoxime, pyridine-4-aldoxime, isonicotinic acid (pyridine-4-carboxylic acid), isonicotinamide (pyridine-4-carboxylic acid amide) and methyl paraben (*p*-hydroxybenzoic acid methyl ester) (all from Sigma). Phenol was obtained from International Biochemistry Industries (CT, USA) and α-picolinamide (pyridine-2-carboxylic acid amide) from the Tokyo Chemical Industry Company, Japan.

Tetramethylammonium chloride (TMA) was obtained from Fluka (Germany) and 1-octanesulphonic acid sodium salt (l-OSA) from Sigma.

Other reagents were analytical grade.

Acetonitrile was HPLC grade purchased from Waters Division of Millipore (USA), and distilled water was deionized by a Milli-Q water-purification system (Millipore, USA).

Samples of autoinjectors containing a solution of atropine sulphate and obidoxime chloride (Combopen, Duphar, Amsterdam, Netherlands) or atropine sulphate and HI-6 (Astra Meditec, Mölndal, Sweden) were obtained from the Australian Department of Defence, Canberra, Australia.

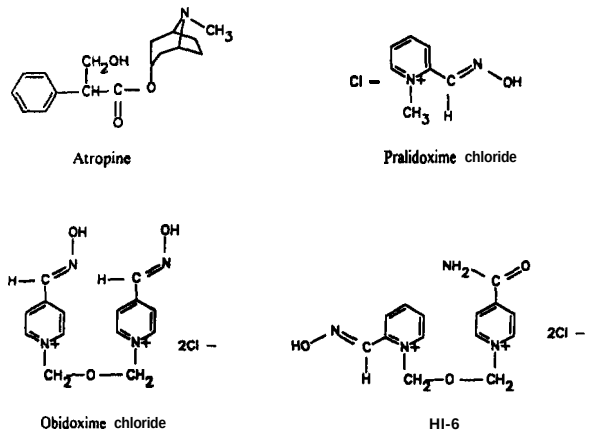

Fig. 1. Structures of atropine and the oximes.

Chromatography

Isocratic, reversed-phase HPLC was carried out using a Waters system comprising a 510 pump, 712 WISP autoinjector, 481 variable-wavelength UV detector and 730 data module. A Zorbax RX-C,, column (25 cm x 4.6 mm I.D.) and guard column (1.25 cm × 4.0 mm I.D.) (particle size 5 μm) (Rockland Technologies, USA) was chosen for the analyses. The column temperature was maintained at 25°C.

The limits of composition of the mobile phase, which were optimized for each particular component and its degradation products, were: 50 m*M* sodium dihydrogenorthophosphate, l-5 m*M* TMA, 0.5-l m*M* l-OSA and between 3.0 and 20% (v/v) acetonitrile in Milli-Q water. The pH was adjusted to 3.5 with concentrated orthophosphoric acid. Finally the mobile phase was filtered through a 0.45-μm Millipore filter. During analyses the flow-rate was 1 ml/min. Details of the specific composition of each mobile phase used for estimating the extent of degradation of the different oximes or atropine are given in Table I.

Detection wavelengths (Table II) were selected that would increase the sensitivity of the HPLC analyses to the degradation products of the oximes or better allow the detection of atropine and tropic acid in the presence of up to a 1100-fold excess of oxime. Information in this regard was obtained from absorbtion spectra of 0.1 m*M* solutions of the oximes and atropine and known degradation products in a mobile phase (pH 3.5) containing 50 m*M* sodium dihydrogen-

TABLE I

MOBILE PHASE COMPOSITIONS FOR DEGRADATION STUDIES OF OXIME-ATROPINE MIXTURES

Column: Zorbax RX-C,,. Buffer: 50 m*M* NaH_2PO_4, mobile phase pH 3.5.)

Heated mixture (m*M*)	Component assayed	TMA (m*M*)	1-OSA (m*M*)	Acetonitrile (%, v/v)	Mobile phase
2-PAM-Cl–atropine (1740:1.5)	Atropine	1	0.5	20.0	A
Obidoxime-atropine (209:1.4)	Atropine	1	0.5	20.0	A
HI-6-atropine (350:1.5)	Atropine	1	0.5	20.0	A
2-PAM-Cl-atropine (1740:1.5)	2-PAM-Cl	1	1.0	3.0	B
Obidoxime-atropine (209:1.4)	Obidoxime	5	1.0	13.0	C
HI-6-atropine (350:1.5)	HI-6	1	1.0	10.0	D

orthophosphate, 5 m*M* TMA, 1 m*M* l-OSA and 20% (v/v) acetonitrile. (The instrument used was a Pye-Unicam PU 8800 UV-Vis spectrophotometer). This mobile phase did not account for more than 6% of the absorbance of the oximes or atropine at any of the wavelengths selected and not more than 14% of the absorbance of the degradation standards.

Sample preparation

Concentrated standard solutions of atropine sulphate (0.1 M), pyridinium oximes (0.2-1.74 M) and certain of their degradation products (see ***Materials)*** (0.1 M) were prepared in Milli-Q water and stored frozen until required. HI-6 was prepared just prior to use.

Aqueous OS-ml samples of an oxime and/or

TABLE II

HPLC CONDITIONS FOR DETERMINING THE EXTENT OF DEGRADATION OF THE OXIME AND ATROPINE IN OXIME-ATROPINE MIXTURES

Column and millimolar ratio of oxime-atropine as in Table I. Injection volume 20 μl. Peak heights of the parent compounds were between 50 and 100% of full scale. Temperature 25°C)

Mixture	Component assayed	Detector wavelength (nm)	Dilution factor	Detector gain (AUFS)
2-PAM-Cl–atropine	Atropine	203	10	0.2
Obidoxime-atropine	Atropine	203	10	0.2
HI-6-atropine	Atropine	203	10	0.2
2-PAM-Cl–atropine	2-PAM-Cl	203	10000	0.1
Obidoxime-atropine	Obidoxime	200	1000	0.2
HI-6-atropine	HI-6	208	3500	0.1

atropine (for concentrations see Table I) were sealed in glass ampoules and heated in an oven at 80°C for various time periods between 19 and 120 h (approx. l-5 days). The heated samples and unheated controls (mixtures or single components) were diluted with Milli-Q water so that the concentration of the material to be assayed (oxime or atropine) was between 0.1 and 0.2 m*M*. The HPLC injection volume was 20 μl.

RESULTS

Measurement of atropine and tropic acid

Controls. In experiments using aqueous pralidoxime chloride-atropine sulphate mixtures in the millimolar ratio of 1740:1.5 (Fig. 2), a complete resolution of the atropine (peak 5) from the broad pralidoxime peak (peak 1) beginning at the solvent front was achieved using mobile phase A (Table I). Tropic acid eluted earlier (peak 4, Fig. 2d) but was also resolved from the oxime peak.

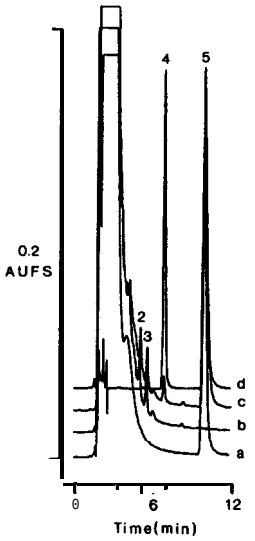

Fig. 2. Chromatograms showing the effect of 5 days at 80°C on an aqueous mixture of 1.5 m*M* atropine sulphate and 1.74 *M* 2-PAM-Cl. HPLC conditions for atropine: see Tables I (mobile phase A) and II. Traces: (a) control (2-PAM-Cl and atropine sulphate), (b) heated 2-PAM-Cl, (c) heated 2-PAM-Cl and atropine sulphate and (d) control (atropine sulphate and tropic acid, each 0.15 m*M*). Peaks: 1 = 2-PAM; 2, 3 = unidentified compounds from the degradation of 2-PAM; 4 = tropic acid; 5 = atropine.

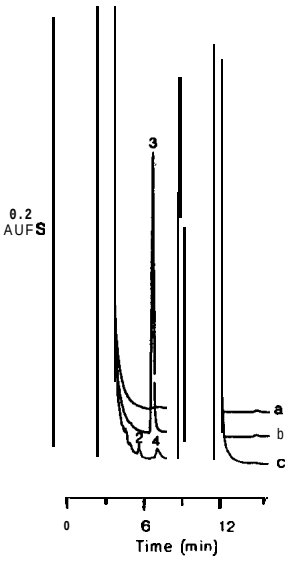

Fig. 3. Chromatograms showing separation of atropine and tropic acid in the presence of excess obidoxime (millimolar ratio of atropine:tropic acid:obidoxime = 1.4:1.0:209). HPLC conditions for atropine: see Tables I (mobile phase A) and II. Traces: (a) obidoxime chloride, (b) obidoxime chloride, atropine sulphate and tropic acid and (c) Combopen contents. Peaks: 1 = obidoxime; 2, 4 = unknowns; 3 = tropic acid; 5 = atropine; 6 = preservative (phenol).

The complete resolution of both atropine and tropic acid (peaks 5 and 3, Fig. 3b) from a 150-fold millimolar excess of obidoxime (peak 1, Fig. 3b) was also obtained using mobile phase A (Table I). The preservative (phenol) (peak 6, Fig. 3c) present in a commercially available solution of obidoxime chloride and atropine sulphate (Combopen) was well separated from atropine and any tropic acid that might form. Traces of unidentified compounds (peaks 2 and 4) occurred in the Combopen sample (Fig. 3c) which were not present in a freshly prepared mixture of obidoxime chloride, atropine sulphate and tropic acid (Fig. 3b).

A similar separation of the components in a mixture of HI-6, atropine sulphate and tropic acid in a millimolar ratio of 350:1.5:1.0 was obtained under the same conditions (peaks 1, 4 and 2, Fig. 4a). The analysis of a freshly constituted injectable solution of HI-6 and atropine

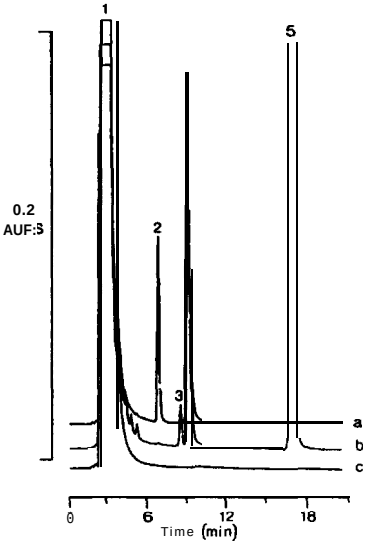

Fig. 4. Separation by HPLC of atropine and tropic acid from HI-6 (millimolar ratio of atropine:tropic acid:HI-6 = 1.5:1.0:350). HPLC conditions for atropine: see Tables I (mobile phase A) and II. Traces: (a) standard mixture, (b) Astra autoinjector contents and (c) HI-6. Peaks: 1 = HI-6; 2 = tropic acid; 3 = unknown; 4 = atropine; 5 = preservative (methyl paraben).

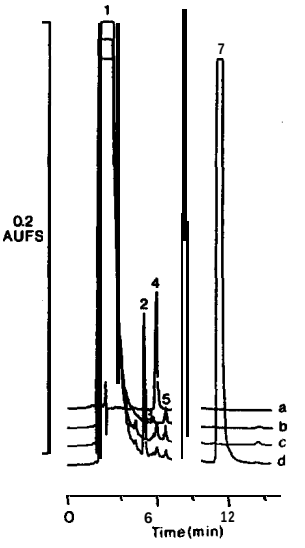

Fig. 5. Chromatograms showing effect of heating an aqueous mixture of 1.4 m*M* atropine sulphate and 209 m*M* obidoxime chloride for 5 days at 80°C. HPLC conditions for atropine: see Tables I (mobile phase A) and II. Traces: (a) heated atropine sulphate, (b) heated obidoxime chloride, (c) heated atropine sulphate and obidoxime chloride and (d) heated Combopen contents. Peaks: 1 = obidoxime; 2 = unknown from Combopen; 3, 5 = unknowns from obidoxime; 4 = tropic acid; 6 = atropine; 7 = preservative (phenol).

sulphate (Astra Meditec) gave an extra peak (peak 5, Fig. 4b), due to the preservative (methyl paraben) [13], as well as several small peaks (e.g. peak 3, Fig. 4b) which were not present in our standard mixture (Fig. 4a).

Heat-degraded samples. Atropine sulphate (1.5 m*M*) in unbuffered aqueous solution (pH about 7) was degraded by heating at 80°C over 1–5 days in a sealed ampoule. This is illustrated in Fig. 5a where peak 4 has the same retention time (t_R) (about 6.7 min) as tropic acid in control (unheated) samples (see peak 4, Fig. 2d; peak 3, Fig. 3b and peak 2, Fig. 4a).

However, in the presence of a high concentration of pralidoxime, obidoxime or HI-6 at an initial pH of between 3 and 4, the degradation of atropine sulphate after 4 or 5 days at 80°C appears to be much less. This is illustrated in Figs. 2c and 5c for heat-treated mixtures of atropine sulphate with pralidoxime chloride and obidoxime chloride respectively. In all cases the pH had dropped to between 2 and 3 and only a small amount of material with the retention time of tropic acid was formed.

After heating the contents of a Combopen under the same conditions (Fig. 5d), peak 2, which was a trace component of the injector contents before heating (see peak 2, Fig. 3c), was now increased. This peak was absent from the standard mixture of obidoxime chloride and atropine sulphate heated in the same way (Fig. 5c).

The products of the degradation of pralidoxime chloride (e.g. peaks 2 and 3, Fig. 2b) and obidoxime chloride (e.g. peaks 3 and 5, Fig. 5b) do not interfere with the measurement of tropic acid or atropine peaks.

The breakdown of HI-6 is much more extensive than that of pralidoxime and especially of obidoxime after 4 days at 80°C. Many rider peaks, which are degradation products of HI-6, appeared on what was now the broader trailing edge of the peaks at the solvent front. Nevertheless it was still possible to distinguish the peaks due to atropine and tropic acid in the heated mixture.

Measurement of oximes

The HPLC separation of each of the three oximes (pralidoxime and the two bis-pyridinium oximes, obidoxime and HI-6) from several of their respective substituted pyridine breakdown products was achieved using mobile phases B, C and D respectively (Table I) and the appropriate sample dilutions (Table II).

Controls. For example, using mobile phase C, isonicotinic acid (t_R 2.4 min), isonicotinamide (t_R 3.1 min) and pyridine-4-aldoxime (t_R *4.2* min) elute well before obidoxime (t_R 10.4 min). Chromatograms of a standard mixture of these four components contained an extra peak (t_R 8.9 min) that was also present in chromatograms of a solution of the original sample of obidoxime and of the contents of a Combopen. NMR data from this laboratory (unpublished results) suggest that this peak is due to the presence of a small amount (cu. 4 mol%) of a regioisomer of obidoxime.

Fig. 6a similarly illustrates, using mobile phase D, that isonicotinamide (peak 1, t_R 3.7 min), picolinamide (peak 2, t_R 6.6 min) and pyridine-2-aldoxime (peak 3, t_R 7.3 min) elute well before HI-6 (peak 6, t_R 4.4 min). Peak 4 in this trace is an unknown component of the sample of pyridine-Zaldoxime used in the standard mixture .

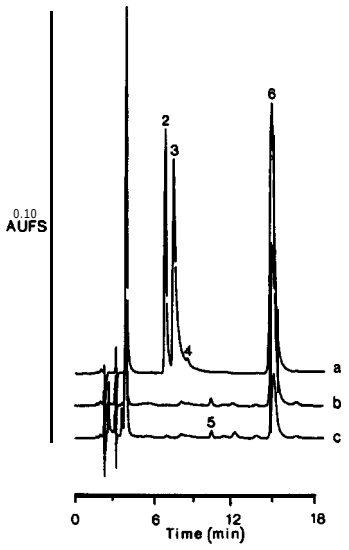

Fig. 6. Degradation of 350 m*M* HI-6 during 4 days at 80°C. HPLC conditions: see Tables I (mobile phase D) and II. Traces: (a) control (unheated) mixture of HI-6, isonicotinamide, picolinamide and pyridine-2-aldoxime, all at 0.1 m*M*, (b) HI-6 heated for 1 day and (c) HI-6 heated for 4 days. Peaks: 1 = isonicotinamide; 2 = picolinamide; 3 = pyridine-Zaldoxime; 4 = unknown from pyridine-Zaldoxime in control mixture; 5 = unknown; 6 = HI-6.

Heat-degraded samples. In chromatograms of heated pralidoxime chloride-atropine sulphate mixtures the breakdown products of the oxime were not specifically identified. However the degradation of 2-PAM-Cl over 5 days at 80°C was clearly demonstrated by the decrease in size of the oxime peak at 12.6 min as well as the progressive growth of four new peaks with retention times of 1.8, 6.2, 7.0 and 9.4 min. Similar results were obtained with P2S.

After 5 days of heating, the breakdown of obidoxime chloride was less extensive than for 2-PAM-Cl. According to the retention times, isonicotinamide (t_R 3.1 min) and pyridine-4-aldoxime (t_R 4.2 min), but not isonicotinic acid (t_R 2.4 min), appear to be the main degradation products. The preservative (phenol) in the Combopen contents elutes at 19.1 min.

The progressive degradation of HI-6 (peak 6, Fig. 6) under similar conditions is greater than that of 2-PAM-Cl and obidoxime chloride. One of the main products again appears to be isonicotinamide (peak 1, t_R 3.7 min, Fig. 6).

DISCUSSION

The major problem in this work was to find a set of HPLC conditions that would allow atropine and tropic acid to be estimated in the presence of a large molar excess of a pyridinium oxime and its breakdown products. We have found few previous references to HPLC methods that could be applied in this way and even these [13,19] lack sufficient detail regarding the extent to which tailing of the oxime peak and the presence of degradation products of the oxime cause any problem for the detection and estimation of the breakdown of atropine.

Atropine salts have long been known to be more stable in acid solution than in neutral or alkaline solution. Their maximum stability has been reported to lie between pH 3 and 4 [2,17,20]. This is presumably due to the higher

susceptibility of atropine to alkaline hydrolysis in comparison with acidic hydrolysis [17,21,22]. At ambient temperatures (< 40°C) and acidic pH (2.8-6.0) dilute atropine sulphate solutions (3-15 m*M*) have been reported to be stable (i.e. ≤ 1% breakdown) for several years [3,16,23]. However, considerable breakdown has been reported when similar solutions of atropine sulphate have been heated at 80–100°C for several days [17] or autoclaved for a few hours [24].

In the current experiments the increased rate of hydrolysis of atropine sulphate in an aqueous unbuffered solution when compared to the rate in a solution containing 0.2–1.7 *M* oxime may be explained by the initial pH. At a pH of around 7.0 atropine is likely to be subject to substantial base-catalysed hydrolysis, whereas at a pH of between 3 and 4 (as in the presence of the oxime) this would be much less [20]. Acid-catalysed hydrolysis at pH 4.0 would also be slow [21].

In acidic solution the major degradation products of the pyridinium oximes detected by HPLC methods are the acid amide and/or carboxylic acid derivatives [4–10,14,25,26]. In the current HPLC measurements a major breakdown product of obidoxime or HI-6 after 4-5 days at 80°C was isonicotinamide. Pyridine-4-aldoxime appeared as a minor breakdown product of obidoxime.

The pK_a values for the three pyridinium oximes lie between 7.28 and 7.80 [27] and their pH in aqueous solution is between 3.5 and 4.0. If one considers the pH–rate profile for the hydrolysis of atropine [20] (see also ref. 21) it might be expected that the decomposition of the oximes and a lowering of the pH to < 3.0 could cause an increased rate of hydrolysis of the atropine sulphate also present. Certainly a small amount of tropic acid was produced after 5 days at 80°C in the presence of degraded oxime. Whether this was due to the direct effect of heat or of a shift in pH on the rate of hydrolysis is unknown.

Under the assay conditions for atropine, the breakdown of the oxime upon heating causes the generation of a broader, composite peak extending from the solvent front. Furthermore, there are small peaks representing traces of oxime degradation products which have retention times in the vicinity of those for atropine and tropic acid. This is also the likely explanation for the trace amounts of unidentified compounds observed in the atropine assay of the contents of the autoinjectors even prior to heat treatment. Nevertheless, neither these factors nor the presence of peaks due to the phenolic preservatives added to the commercial preparations interferes with the analysis of atropine.

Thus the HPLC method outlined in detail in this paper appears to be suitable for estimating the extent of degradation of both atropine and the pyridinium oximes in a binary injection mixture without the need for any prior separation.

REFERENCES

1 P.M. Luody, AS. Hansen, B.T. Hand and C.A. Boulet, Toxicology, 72 (1992) 99.
2 N.B. Brown and H.K. Sleeman, 3. ***Chromatogr.,*** 150 (1978) 225.
3 U. Lund and S.H. Hansen, 3. ***Chromatogr.,*** 161 (1978) 371.
4 D. Utley, 3. ***Chromntogr.,*** 265 (1983) 311.
5 P. Fyhr, A. Brodin, L. Ernerot and J. Lindquist, 3. ***Pharm. Sci., 75 (1986) 608.***
6 A.C. Schroeder, J.H. DiGiovanni, J. von Bredow and M.H. Heiffer, 3. ***Pharm. Sci.,*** 78 (1989) 132.
'7 P. Eyer and W. Hell, ***Arch. Pharm. (Weinheim),*** 318 (1985) 938.
8 P. Eyer, W. Hell, A. Kawan and H. Klehr, ***Arch. Toxicof., 59 (1986) 266.***
9 N.D. Brown, L. Kazyak, B.P. Doctor and I. Hagedorn, 3. Chromatogr., 351 (1986) 599.
10 P. Eyer, I. Hagedom and B. Ladstetter, ***Arch. Toxicol., 62 (1988) 224.***
11 R.E. Mdachi, W.D. Marshall, D.J. Ecobichon, F.M. Fouad and C.E. Connolley-Mendoza, ***Chem. Res. Toxicol.,*** 3 (1990) 413.
12 N.D. Brown, L.L. Hall, H.K. Sleeman, B.P. Doctor and G.E. Demaree, ***J. Chromatogr.,*** 148 (1978) 453.
13 J.W. Schlager, T.W. Dolzine, J.R. Stewart, G.L. Wannarka and M.L. Shih, ***Pharm. Res.,*** 8 (1991) 1191.
14 I. Christenson, ***Acta Pharm. Suec., 5 (1968) 249.***
15 J.G. Clement, K.J. Simons and C.J. Briggs, ***Biopharm. Drug Dispos.,*** 9 (1988) 177.
16 E. Linden and G. Schill, ***Acta Pharm. Suec.,*** 4 (1967) 327.
17 T. Jira and R. Pohloudek-Fabini, ***Pharmazie, 8 (1983) 520.***
18 L. Leadbeater, ***Technical Note, No. 171,*** Chemical Defense Establishment, Porton Down, UK, 1973.

19 T. Okuda, M. Nishida, I. Sameshima, K. Kyoyama, K. Hiramatsu, Y. Takehara and K. Kohriyama, *J. Chromatogr.*, 567 (1991) 141.
20 W. Lund and T. Waaler, *Acta Chem. Scand.*, *22* (1968) 3085.
21 G.M. Loudon, *J. Chem. Educ.*, *12* (1991) 973.
22 E. von Bjerkelund, F. Gram and T. Waaler, *Pharm. Acta Helv.*, *44* (1969) 745.
23 A. Richard and G. Andermann, *Pharmazie, 39 (1984) 866.*
24 S.A.H. Khalil, Y. El-Beltagy and S. El-Mast-y, *J. Pharm. Pharmacof.*, 23 (1971) 214.
25 J. Lin and D.L. Klayman, *J. Pharm. Sci., 75* (1986) 797.
26 I. Christenson, *Acta Pharm. Suec.*, *5 (1968) 23.*
27 I. Hagedorn, I. Stark and P. Lorenz, *Angew. Chem.*, *Znt. Ed. Engl.*, 11 (1972) 307.