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Selective system of identification and determination of antidepressants and neuroleptics in serum or plasma by solid-phase extraction followed by high-performance liquid chromatography with photodiode-array detection in analytical toxicology

Marie Balíková

Institute for Forensic Toxicology, Medical Faculty 1, Charles University, Na bojišti 3, 121 08 Prague 2 (Czechoslovakia)

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ABSTRACT

A selective off-line solid-phase isolation of antidepressants, neuroleptics and other structurally related basic drugs from plasma or serum prior to high-performance liquid chromatography was tested and optimized for general use in toxicological analyses where concomitant drugs can be encountered. The sequential elution preseparated drug mixtures and simplified the subsequent analytical steps. High isolation efficiencies of cyano-bonded silica cartridges from Baker for fifteen amine drugs were determined. Isocratic chromatography on octadecylsilica proved to be very suitable for broad practical applications in complicated cases. The identification of an unknown peak was supported by photodiode-array detection in the range 200–400 nm with a resolution of 2 nm. The linearity of the assay from therapeutic to toxic concentrations was attained. Sufficient sensitivities covering low therapeutic levels of parent drugs and their demethylated metabolites were reached. The system is flexible and allows various methods of quantitative assay to be devised according to the conditions of a particular case in clinical or forensic toxicology.

INTRODUCTION

Many recent cases of drug overdose in Czechoslovakia have been due to benzodiazepines and tricyclic psychopharmaca. The latter term covers drugs of a similar chemical structure but with different pharmacological effects, *e.g.* antidepressants, neuroleptics, hypnotics and antihistaminics. The identification of the drugs responsible for poisoning, and the monitoring of plasma or serum concentrations during therapy are important, especially with tricyclic antidepressants because of their severe toxic effects [1–3]. Many high-performance liquid chromatographic (HPLC) assays for tricyclic antidepressants and related drugs in blood material have been reported. However, a majority of them are restricted to therapeutic monitoring of a known drug and its metabolites only [4–7]. The toxicological method, even if targeted to a certain group of drugs, must consider possible interferences, be able to confirm the identity, and detect the compound both in the therapeutic and the toxic range and in mixtures with other drugs and their metabolites in biological samples.

Our previous attempts to make use of the classic solvent extraction of tricyclic antidepressants and phenothiazines with hexane–isoamyl alcohol

Correspondence to: Dr. M. Balíková, Institute for Forensic Toxicology, Medical Faculty 1, Charles University, Na bojišti 3, 121 08 Prague 2, Czechoslovakia.

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resulted in poor recovery of secondary amines and some phenothiazines, in particular. Some of these drawbacks are also mentioned in the literature [8–10], though not unambiguously [11–13]. Solid-phase extraction (SPE) is an alternative approach, which has found broad application recently [4,7,8,14–17]. Mush and Massart [15] developed a general procedure for the isolation of various basic drugs from plasma on cyano-bonded silica.

This paper describes a method for the isolation recoveries of fifteen amine drugs at therapeutic and toxic serum concentrations from cyano-bonded silica cartridges. The sequential elution of drugs was introduced to separate concomitant acids, neutral compounds and weak bases for waste or other analytical steps. SPE on cyano-bonded silica was followed by analytical separation on octadecyl-silica slightly modified from the methods of Kabra *et al.* [18] and Gill and Wanogho [19], and diode-array detection was used. It proved to be a reliable and selective technique for the identification and quantitation of tricyclic psychopharmaca and structurally related basic drugs in a blood sample of a toxicological case.

EXPERIMENTAL

Chemicals

Organic-free water was obtained from a Milli-Q standard system (Millipore, Milford, MA, USA). Acetonitrile Chromasolv was purchased from Riedel de Haen (Seelze, Germany). Methanol (p.a.), sodium dihydrogenphosphate dihydrate (p.a.) and phosphoric acid (85% p.a.) were provided by Lachema (Brno, Czechoslovakia). Nonylamine (98%) came from Aldrich Chemie (Steinheim, Germany) and triethylamine (p.a.) from Fluka (Buchs, Switzerland). Baker-bond CN cartridges (500 mg/3 ml) were obtained from Baker Chemikalien (Gross Gerau, Germany).

All drug standards were of pharmaceutical purity and were kindly donated by the Research Institute of Pharmacy and Biochemistry (Prague, Czechoslovakia), Slovakopharma (Hlohovec, Czechoslovakia), Pharmamed (Dresden, Germany) or Ciba-Geigy (Basel, Switzerland).

Preparation of standard solutions

Stock standard solutions of hydrochloride of each drug (2 mg/10 ml) were prepared in methanol and stored at 4°C. Working standard solutions were prepared by appropriate dilution with 0.1 *M* hydrochloric acid.

HPLC conditions

The chromatographic system, which was operated in isocratic mode with recycle, consisted of commercial components: a Waters 510 pump, a Waters U6K injector, a Waters 990 photodiode-array detector, an APC IV NEC Power Mate 2 computer and a Waters 990 plotter. UV spectra were obtained in the 200–400 nm range with 2 nm resolution.

Guard (30 mm × 3 mm I.D.) and analytical (150 mm × 3 mm I.D.) reversed-phase columns packed with octadecylsilica Separon SGX C_{18}, particle size 7 μm, were the products of Tessek (Prague, Czechoslovakia).

The mobile phase, acetonitrile–buffer (1:3, v/v), was filtered with a Durapore filter (Millipore) and degassed by vacuum. The buffer was made from 1 l of 0.01 *M* phosphate and 1.2 ml of nonylamine, and the pH was adjusted to 3.0 with 1 *M* phosphoric acid. The flow-rate of the mobile phase was set to 0.7 ml/min, and time of analysis was 30 min. A 1-l volume of the mobile phase was recycled for *ca.* one week, depending on the number of samples analysed.

Solid-phase extraction

A 1-ml volume of serum was mixed with 0.2 ml of 1 *M* hydrochloric acid and 50 μl of internal standard (1 μg) chosen according to the particular circumstances of a case.

A solid-phase cartridge was conditioned with 5 ml of acetonitrile and 5 ml of 0.1 *M* hydrochloric acid. Then the acidified serum sample was slowly applied to the cartridge, together with the rest of the sample which was washed from the container twice with 2 ml of 0.1 *M* hydrochloric acid. The remaining proteins and acids were washed from the cartridge with 5 ml of water, and neutral and slightly basic drugs were eluted with 5 ml of acetonitrile–water (1:1, v/v). Then air was drawn

through the cartridge for 1 min to dry it to some extent. The elution of amine drugs was achieved with acetonitrile modified by triethylamine (100:0.15, v/v). The eluate was collected into a glass tube with a conical bottom, and evaporated under nitrogen. The evaporate was reconstituted into 0.2 ml of the mobile phase, 0.15 ml of which was injected into the chromatograph.

Recovery tests

Recovery tests were performed with 1 ml of blank rat serum spiked with various mixtures of standards to the levels 200 or 2000 ng/ml. A 1-μg amount of the internal standard (selected to be separated well from other drugs) was added to the eluate just after extraction. The recovery values were calculated by means of the internal standard method by comparing the actual content of the standards in the extracts with those theoretically expected.

Linearity of calibration and detection limit

Two working mixtures of standards, 25 and 2.5 μg/ml, were used to spike the pool of blank rat serum to different levels to test the linearity of the assay in the range 20–4000 ng/ml: (1) haloperidol, nortriptyline, amitriptyline, chlorpromazine and clomipramine (internal standard protriptyline, 1 μg/ml); (2) promethazine, levopromazine, perphenazine, chlorpromazine and chlorprothixene (internal standard imipramine, 1 μg/ml).

RESULTS AND DISCUSSION

Chromatographic system

The idea of an isocratic reversed-phase HPLC method with the addition of a competitive amine to the acid mobile phase was accepted [18,19] for the analysis of the basic psychopharmaca. Two years of routine work proved the method to be very useful. Because the mobile phase can be recycled the isocratic system is economical and suitable for practical operation. A retention time window of 20% was adopted. The variability of retention data during aging and changing of the column were compensated by altering the flow of the mobile phase to keep the retention time of protriptyline in the range 11.00 ± 1 min.

The conditions of the mobile phase were set with respect to the octadecylsilica column to achieve optimal retention and separation of the group of drugs of interest within 30 min (Table I). The majority of current tri- and tetracyclic antidepressants and phenothiazines can be analysed by this system. However, some of them require a stronger eluent, *e.g.* trifluoperazine and thioridazine. The HPLC system was also tested for interferences from other drugs commonly encountered in toxicological samples, and the selectivity of the SPE procedure was verified simultaneously (Table II).

The SPE procedure described here allows the selective isolation of amines: acid, neutral and slightly basic drugs, *e.g.* barbiturates, phenacetin, caffeine and benzodiazepines, are discarded during washing steps. These could be analysed by other methods if desired.

TABLE I

CAPACITY FACTORS AND SPECTRAL MAXIMA OF SELECTED BASIC DRUGS

T_o = 1.58 min.

Drug	k'	λ (nm)
Pericyazine	4.13	204, 226, 268
Promethazine	4.25	200, 250, 300
Haloperidol	4.38	215, 246
Northiepin	4.63	208, 228, 304
Desipramine	5.14	212, 252
Dithiaden	5.39	200, 306
Desmethylmaprotiline	5.52	205, 252, 264, 274
Protriptyline	5.58	210, 290
Dothiepin	5.74	210, 230, 302
Imipramine	6.44	212, 252
Nortriptyline	6.66	206, 238
Maprotiline	6.97	205, 252, 264, 274
Amitriptyline	8.46	206, 240
Levopromazine	9.03	200, 252, 310
Perphenazine	9.22	200, 256, 310
Chlorpromazine	12.92	204, 254, 308
Clomipramine	15.90	216, 252
Chlorprothixene	16.15	206, 230, 268, 326
Trifluoperazine	>20.00	200, 258, 308
Thioridazine	>20.00	264, 310

TABLE II

COMMONLY ENCOUNTERED DRUGS TESTED FOR INTERFERENCES IN THE HPLC SYSTEM

T_o = 1.58 min.

k' = 0–1.5	k' = 1.5–15.00	k' > 15.00
Barbiturates[a]	Aminoflunitrazepam	Oxazepam[a]
Glutethimide[a]	Phenacetin[a]	Diazepam[a]
Amphetamine	Cinchocaine	Nitrazepam[a]
Ephedrine	Dextropropoxyphene	Clonazepam[a]
Morphine[a]	Methadone	Flunitrazepam[a]
Codeine[a]	Carbamazepine[a]	Triazolam[a]
Naloxone[a]	Flumazenil[a]	Alprazolam[a]
Pholcodine[a]		Prazepam[a]
Pethidine		Midazolam[a]
Tilidine		Medazepam[a]
Pentazocine		
Dibenzepine		
Cocaine		
Papaverine		
Benzooctamine		
Chloroquine		
Quinine		
Quinidine		
Amidopyrine		
Nialamide		
Aminodesmethylflunitrazepam		
Quaiphenesine[a]		
Caffeine[a]		
Theophyline[a]		
Theobromine[a]		

[a] Did not interfere with HPLC after selective SPE.

Spectral system

The circumstances of toxicological analysis require the identity and purity of each solute of interest in a chromatogram to be confirmed. UV spectrophotometry is a useful aid in identifying tricyclic and tetracyclic antidepressants, phenothiazines and structurally related drugs and in distinguishing among them and their metabolites. The advantages and the restrictions of diode-array detection in the confirmation of various drugs and their metabolites in biological material have been widely described elsewhere [20–30].

The degree of certainty in the identification of a solute depends on its concentration in the biological fluid, as demonstrated in Fig. 1. There is no substantial difference in the quality of spectra at the concentrations 4000 and 200 ng/ml. Significant deterioration occurred at values lower than 100 ng/ml and was greater when the retention time of a solute was longer.

The maxima in the UV spectra of tri- and tetracyclics are concentrated at short wavelengths (205–210 nm), whereas the majority of phenothiazines display maxima around 250 nm (Table I, Fig. 2). Thus the detection wavelength is selected according to the nature of the drug to be analysed. The selection of a suitable internal standard for the assay of a particular group of drugs must also take into consideration the spectral criteria.

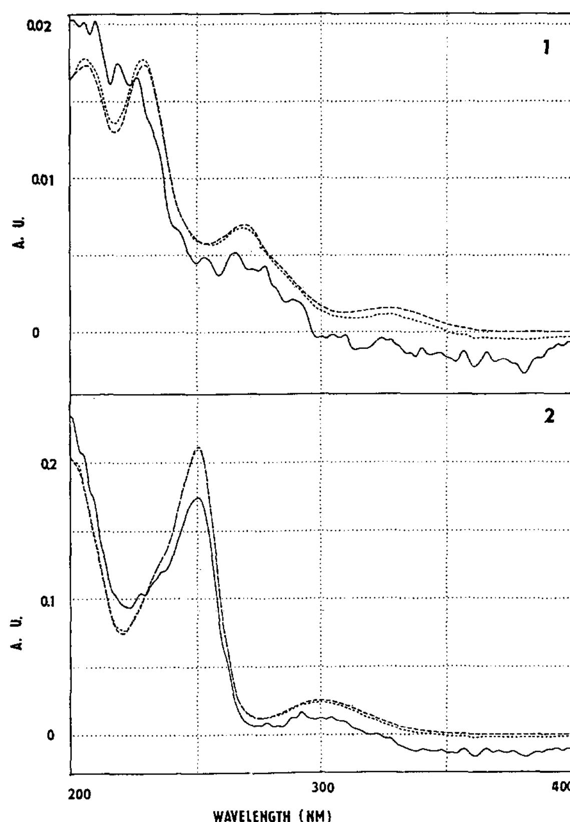

Fig. 1. UV spectra of chlorprothixene (1) and promethazine (2) isolated from 1 ml of rat serum spiked to different levels: 4000 ng/ml (dashed lines), 200 ng/ml (dotted lines), 25 ng/ml (full lines).

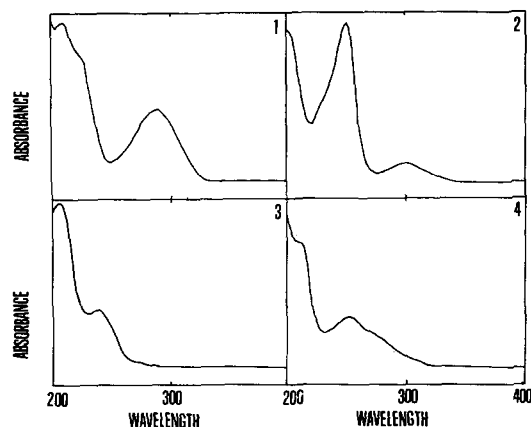

Fig. 2. UV spectra of some tricyclic antidepressants and phenothiazines: protriptyline (1), promethazine (2), amitriptyline (3), imipramine (4).

TABLE IV

ESTIMATE OF SPE RECOVERY

Drug	Recovery at 200 ng/ml[a] (%)	Internal standard	Detection wavelength (nm)
Northiepin	89	Amitriptyline	205
Dothiepin	102		
Maprotiline	78		
Desipramine	89	Chlorpromazine	250
Imipramine	102		
Perphenazine	79		
Promethazine	98	Imipramine	250
Levopromazine	95		
Chlorpromazine	94		
Chlorprothixene	92		

[a] $n = 1$.

SPE recovery

To achieve maximal recoveries of the group of drugs tested from the Bakerbond SPE cartridges, 5 ml of acetonitrile–triethylamine were experimentally found to be sufficient for elution. The recoveries of drugs tested at therapeutic concentrations in serum (200 ng/ml) did not differ significantly from those at toxic levels (2000 ng/ml) (Table III). The recoveries of drugs from cyano-bonded silica (Baker) were surprisingly high, uniform and stable in the range 93–102%, when compared with the values obtained with the Sep-Pak CN cartridges (Waters) previously used in our laboratory, which were only in the range 45–75%, and with those obtained with Presep CN (Tessek), which were in the range 24–93% for the same set of drugs. The quality of cyano-bonded silicas from various sources, the stability of bonds of cyano groups, and the amount of residual silanols on the surface proved to be very important factors affecting SPE recovery of basic drugs of the amine type.

TABLE III

PERCENTAGE RECOVERY FROM SPE

Internal standard, protriptyline (1 μg/ml serum); detection wavelength, 205 nm.

Drug	Recovery (mean ± S.D., n = 5) (%)	
	200 ng/ml	2000 ng/ml
Haloperidol	98 ± 3	102 ± 3
Nortriptyline	96 ± 2	93 ± 4
Amitriptyline	101 ± 4	96 ± 3
Chlorpromazine	98 ± 3	101 ± 4
Clomipramine	98 ± 6	101 ± 5

Table IV lists the recoveries for a broader spectrum of drugs. The values for secondary amines and hydroxylated drugs (perphenazine) are somewhat lower than those for tertiary amines. We did not find any significant difference in the competitive effects of triethylamine and diethylamine during elution.

Linearity of the assay and detection limit

The uniform recovery of drugs at therapeutic and toxic levels indicates the broad linearity of the assay. For example, the calibration curve obtained from the peak-area ratio of amitriptyline to the internal standard protriptyline (y) at 205 nm in the concentration range 25–4000 ng/ml (x) yielded the linear regression equation $y = 0.0036 + 0.0053x$ and the correlation coefficient $r = 0.9988$. The analogous calibration curve obtained for promethazine and the internal stan-

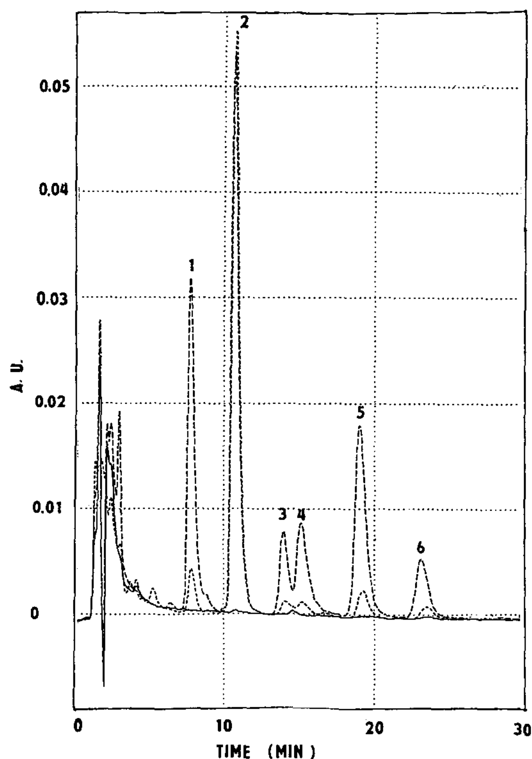

Fig. 3. Chromatograms obtained from 1 ml of blank rat serum (full line), spiked to 25 ng/ml (dotted line) or to 200 ng/ml (dashed line). Internal standard, imipramine (1 μg); detection wavelength, 250 nm. Peaks: 1 = promethazine; 2 = imipramine; 3 = levopromazine; 4 = perphenazine; 5 = chlorpromazine; 6 = chlorprothixene.

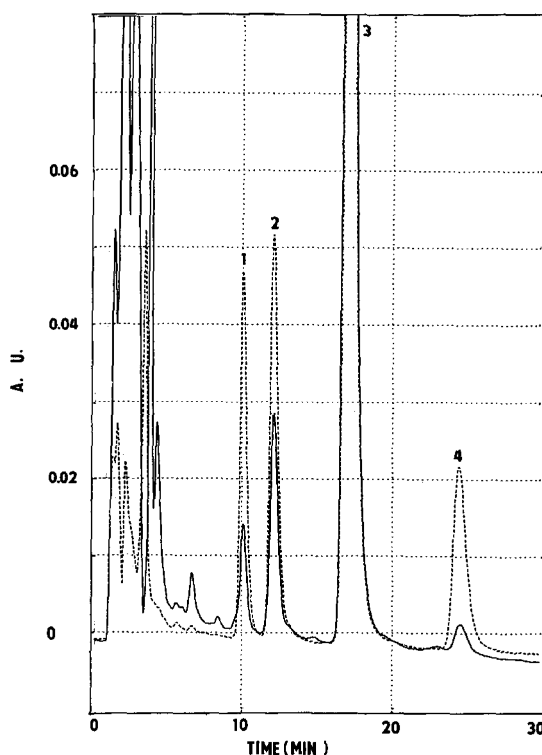

Fig. 4. Chromatograms obtained from a case of human intoxication (full line) and rat serum (dotted line) spiked with 500 ng/ml northiepin (1), dothiepin (2) and chlorpromazine (4), and 1 μg/ml amitriptyline as internal standard (3). Detection at 205 nm.

dard imipramine at 250 nm yielded the linear regression equation $y = 0.0014 + 0.0019\,x$ and the correlation coefficient $r = 0.9999$.

The estimated limit of detection in serum is 25 ng/ml on average (Fig. 3), but the positive identification of a solute based only on UV spectra at this low serum concentration is rather uncertain, as mentioned previously.

Examples of human intoxication

Fig. 4 shows a chromatogram obtained from a serum sample from a suspected case of intoxication with dothiepin, chlorpromazine and ethanol. The assay of 1 ml of serum was performed with amitriptyline (1 μg) as the internal standard. The calculation was made at 205 nm, and concentrations of 113, 205 and 49 ng/ml were found for northiepin, dothiepin and chlorpromazine, respectively.

Fig. 5 concerns another case of drug overdose in which promethazine was expected. The assay of 1 ml of serum was performed with 1 μg of imipramine as the internal standard, and the calculation was made at 250 nm. Only a low concentration (35 ng/ml) of promethazine was found, and it was proved by other methods (thin-layer chromatographic screening and identification of drugs in urine) that intoxication was also due to flunitrazepam, phenobarbital and allobarbital.

CONCLUSION

The combination of SPE on cyano-bonded silica and reversed-phase HPLC on octadecylsilica

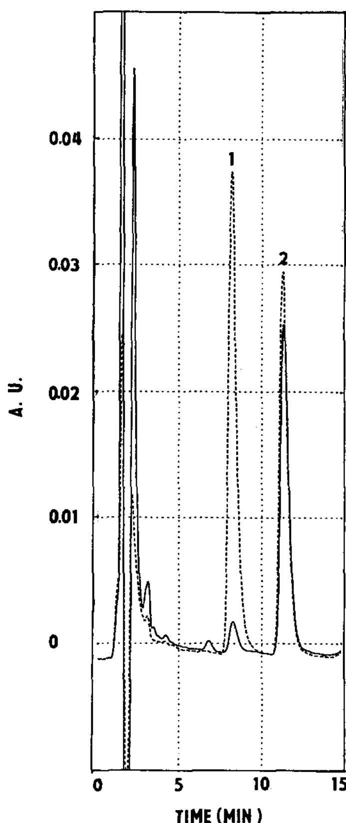

Fig. 5. Chromatograms obtained from a case of human intoxication (full line) and rat serum (dotted line) spiked with 500 ng/ml promethazine (1) and 1 μg/ml imipramine as internal standard (2). Detection at 250 nm.

makes a selective system for the determination of basic psychopharmaca of the amine type. Antidepressants and neuroleptics in various mixtures with other drugs common in blood samples after drug overdose can be assayed in this way. The quality of the cyano-bonded silica used for the isolation of drugs of this type markedly affects the final analytical results. The procedure allows a reliable distinction between drugs assayed on the basis of retention data and UV spectra, and it can be used to monitor serum or plasma levels in the therapeutic range as well as in the range corresponding to heavy or fatal poisoning.

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REFERENCES

1 S. H. Preskorn and G. S. Jerkowich, *J. Clin. Psychopharmacol.*, 10 (1990) 88.
2 K. R. Labrosse and H. G. McCoy, *Clin. Chem.*, 34 (1988) 859.
3 P. J. Orsulak, *Ther. Drug Monit.*, 11 (1989) 497.
4 R. Bouileu, J. L. Bonnefous and S. Ferry, *J. Chromatogr.*, 528 (1990) 542.
5 T. Loennechen, A. Andersen, P. A. Hals and S. G. Dahl, *Ther. Drug Monit.*, 12 (1990) 574.
6 Ch. Svensson, G. Nyberg and E. Martensson, *J. Chromatogr.*, 432 (1988) 363.
7 C. S. Smith, S. L. Morgan, S. V. Greene and R. K. Abramson, *J. Chromatogr.*, 423 (1987) 207.
8 S. H. Wong, *Clin. Chem.*, 34 (1988) 848.
9 J. Fekete, P. del Castilho and J. C. Krak, *J. Chromatogr.*, 204 (1981) 319.
10 J. S. Kiel, R. K. Abramson, C. S. Smith and S. L. Morgan, *J. Chromatogr.*, 383 (1986) 119.
11 P. O. Lagerström, J. Marle and B. A. Persson, *J. Chromatogr.*, 273 (1983) 151.
12 S. Yang and M. A. Evenson, *Anal. Chem.*, 55 (1983) 994.
13 P. M. Edelbroek, E. J. M. de Haas and F. A. de Wolff, *Clin. Chem.*, 28 (1982) 2143.
14 G. L. Lensmeyer and M. A. Evenson, *Clin. Chem.*, 30 (1984) 1774.
15 G. Mush and D. L. Massart, *J. Chromatogr.*, 432 (1988) 209.
16 R. J. Hughes and M. D. Osselton, *J. Anal. Toxicol.*, 13 (1989) 77.
17 M. Mazhar and S. R. Binder, *J. Chromatogr.*, 497 (1989) 201.
18 P. M. Kabra, N. A. Mar and L. J. Marton, *Clin. Chim. Acta*, 111 (1981) 123.
19 R. Gill and S. O. Wanogho, *J. Chromatogr.*, 391 (1987) 461.
20 T. Alfredson, T. Sheenan, T. Lenert, S. Aamodt and L. Correia, *J. Chromatogr.*, 385 (1987) 213.
21 E. I. Minder, R. Shaubhut, C. H. E. Minder and D. J. Vonderschmitt, *J. Chromatogr.*, 419 (1987) 135.
22 K. Jino, M. Kuwajima, M. Hayashida, T. Watanabe and T. Hondo, *J. Chromatogr.*, 436 (1988) 11.
23 T. D. Wilson, W. J. Trompeter and H. F. Gartelman, *J. Liq. Chromatogr.*, 12 (1989) 1231.
24 H. Engelhardt and T. König, *Chromatographia*, 28 (1989) 341.
25 E. I. Minder, *Clin. Toxicol.*, 27 (1989) 117.
26 R. B. Logan, D. T. Stafford, I. R. Tebbett and C. M. Moore, *J. Anal. Toxicol.*, 14 (1990) 154.
27 A. Turcant, A. Premel-Cabie, A. Cailleux and P. Allain, *Clin. Chem.*, 37 (1991) 1210.
28 P. R. Puopolo, S. A. Volpicelli, D. MacKeen Johnson and J. G. Flood, *Clin. Chem.*, 37 (1991) 2124.
29 S. R. Binder, A. K. Adamo, M. Regalia, H. Essien and R. Rosenblum, *J. Chromatogr.*, 550 (1991) 449.
30 M. Bogusz and M. Wu, *J. Anal. Toxicol.*, 15 (1991) 188.