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Use of high-performance liquid chromatography–diode array detection in forensic toxicology

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Abstract

A comprehensive approach to the analysis for many drugs in postmortem blood and biological fluids using high-performance liquid chromatography and diode array detection has been developed. To reduce the likelihood of co-eluting interference components of postmortem blood or other drugs, selective back-extraction was also used to screen and quantitate drugs in blood and biofluids. An isocratic mobile phase (acetonitrile, phosphoric acid and triethylamine buffer, pH 3.4) was developed and found stable, reliable and convenient for general drug screening and quantitation. A library of drug spectra in the ultraviolet wavelength range (210–367 nm) was established for 272 drugs on two reversed-phase columns: Supelcosil (biphenyl) and LiChrospher RP-8.

The application of several methods to whole blood, the analysis of complex cases and the use of multicomponent analysis for qualitative and quantitative analysis is discussed.

1. Introduction

High-performance liquid chromatography (HPLC) has developed into one of the most useful and widely used analytical techniques in the last two decades. The range and variety of compounds that can be analysed by HPLC has led to its application in environmental, clinical and forensic laboratories. In the early days, HPLC was primarily a quantitative technique, retention times being the only criteria for identification. With the advent of diode array detection (DAD) for HPLC, the ultraviolet (UV) spectrum becomes accessible as a three-dimensional data matrix. Data are available not only for retention time and peak height, but also for wavelength, thus, allowing identification from UV spectral properties in addition to retention behaviour.

DAD dramatically improves the selectivity of HPLC and offers many capabilities, previously enjoyed only by gas chromatography–mass spectrometry. HPLC–DAD has proven itself for screening anabolics [1], neuroleptics [2], diuretics [3], basic drugs [4] and many other compounds [5–12]. A survey of the literature showed however, that a great variety of HPLC columns and mobile phases were required. In view of the wide range of conditions reported for the chromatographic analysis of drugs, the goal of this work was to establish standard chromatographic conditions that could be used both for basic and acidic drugs. In this paper, four years of experience with HPLC–DAD systems is presented. The application of several methods to whole blood, the analysis of complex cases and the use of multicomponent analysis for qualitative and quantitative analysis is also illustrated.

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2. Experimental

2.1. Liquid chromatography system

The components of an LC system have been described previously [13]. The system includes a Model 9010 pump, a Model 9095 autosampler, a Model 9065 photodiode array detector, a Compaq 386 computer with the LC Star 9020 workstation and with the PolyView (version 2.02) software, all from Varian, Georgetown, Canada. The LC Star 9020 workstation software is used for system control and chromatographic data handling and the PolyView software is used for automated post-run spectral evaluation and for multicomponent analysis.

Chromatographic separation was achieved isocratically at ambient temperature on reversed-phase columns. The detailed analytical conditions are listed in Table 1. Mobile phase was continuously degassed with helium during use. After the system was equilibrated (approximately 60 min), the mobile phase was recycled back to the reservoir. Drugs were monitored at 229 nm and, if required, were quantitated at their UV maxima. Injection was 25 μl out of a final volume of 300 μl.

2.2. Chemicals and reagents

Drug standards were of the highest possible purity and obtained from pharmaceutical manufacturing companies. Stock solutions containing 0.4 mg/ml of free base/acid of each drug in methanol were stored at −20°C. Acetonitrile and methanol were distilled-in-glass quality (spectra analysed) and supplied by Caledon Labs. (Georgetown, Canada). Triethylamine (TEA) was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical-

Table 1
Chromatographic conditions for drugs on reversed-phase HPLC columns

Column type and conditions	Comment
(I) Supelcosil LC-DP, 5 μm, 250 mm × 4.6 mm I.D. (Supelco Canada, Oakville, Canada) Mobile phase CH_3CN–0.025% (v/v) H_3PO_4–TEA buffer, pH 3.4 (25:10:5, v/v/v) Flow-rate 0.6 ml/min	Used for quantitation and confirmation of drugs and for screening acidic drugs
(II) LiChrospher 100 RP-8, 5 μm, 250 mm × 4.0 mm I.D. (Hewlett-Packard, Mississauga, Canada) Mobile phase CH_3CN–0.025% (v/v) H_3PO_4–TEA buffer, pH 3.4 (60:25:15, v/v/v) Flow-rate 0.6 ml/min	Used for quantitation of drugs (mostly phenothiazines) and for screening basic and acidic drugs
(III) APEX ODS, 5 μm, 250 mm × 4.6 mm I.D. (Mandel Scientific, Guelph, Canada) Mobile phase methanol–water (50:50, v/v)[a] or (40:60, v/v)[b] Flow-rate 1.0 ml/min[a] or 0.4 mL/min[b]	Used for quantitation of barbiturates, phenytoin, salicylic acid, acetaminophen and theophylline
(IV) Nova-Pak Phenyl, 5 μm, 150 mm × 3.9 mm I.D. (Waters, Division of Millipore, Mississauga, Canada) Mobile phase methanol–water (60:40, v/v)[c] or CH_3CN–0.025% (v/v) H_3PO_4 (50:50, v/v) Flow-rate 0.7 ml/min[c] or 0.6 ml/min	Used for quantitation of some benzodiazepines and for the confirmation of some drugs

[a] Barbiturate and phenytoin analysis.
[b] Salicylic acid, acetaminophen and theophylline analysis.
[c] Benzodiazepines.

reagent grade and solvents distilled-in-glass quality. Water was purified using a Millipore Milli-Q Plus water-purification system (Millipore, Mississauga, Canada).

TEA buffer was prepared by adding 9 ml of concentrated phosphoric acid and 10 ml of TEA to 900 ml of water. After the pH was adjusted to 3.4 with diluted phosphoric acid, the volume was made up to 1 l with water and stored at 4°C. Before it was used to make up the mobile phase, it was kept at room temperature overnight and filtered through a Millipore 0.45-μm HA filter.

2.3. Methods

Extraction of acidic drugs

Extraction and chromatographic conditions have been developed in this laboratory and reported elsewhere [14,15]. The method is outlined in Fig. 1. Blood samples were rotated with Amberlite XAD-2 resin-slurry, then the mixture was transferred into an empty Brinkmann column (containing a small plug of cotton), filtered and washed. The air-dried resin was rotated with dichloromethane and filtered. Acidic drugs were back-extracted into 0.45 *M* NaOH, acidified with 6 *M* HCl and re-extracted with isopropyl ether. The final extract was washed, evaporated and reconstituted with mobile phase.

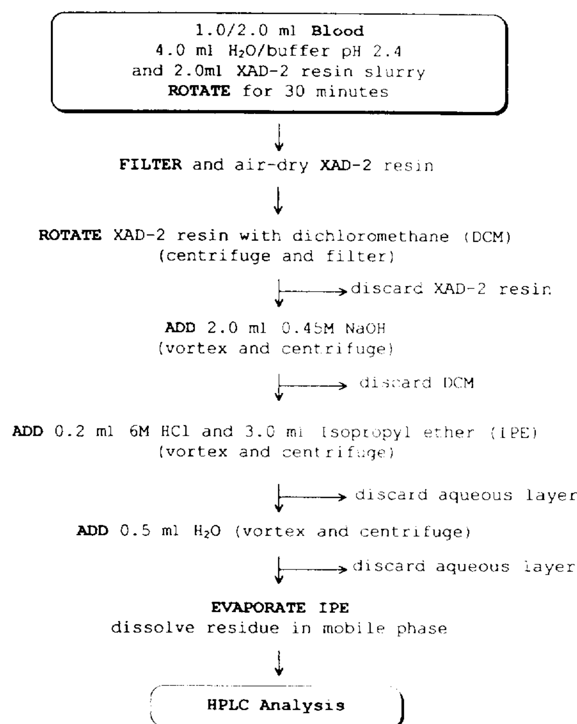


Fig. 1. Flow diagram of method for acidic drug extraction from blood.

Extraction of basic drugs

In 1992 we reported on a method for the detection and quantitation of basic drugs in postmortem blood using HPLC–DAD [13]. The method is based on selective extraction of basic and weakly basic drugs, by back-extraction into 0.1 *M* sulphuric acid and 6 *M* hydrochloric acid after the initial extraction from 2.0 ml of blood with toluene under basic conditions. The extraction procedure is outlined in Fig. 2, fractions B and C. Only minor modification of the method was necessary for the quantitation of acid- or alkaline-sensitive drugs such as diltiazem: instead of 0.1 *M* H_2SO_4, back-extracted into 0.01 *M* H_2SO_4 and the acid fraction was made alkaline with 0.5 *M* K_2CO_3 before it was re-extracted with toluene.

A specific extraction procedure has been developed for the quantitation of some benzodiazepines such as nitrazepam, oxazepam, clonazepam, demoxepam and lorazepam [16]. These drugs were separated from the other basic drugs by using diluted NaOH solution for back-extraction after the initial extraction with toluene (Fig. 2, fraction A).

Spectral libraries

Dilutions of the stock solutions with the mobile phase (1:100) were used to establish retention times and create a library of spectra in the ultraviolet range (210–367 nm). The spectra were acquired under the same conditions as for sample analysis and were stored with the absolute retention times. Eight group standards were also prepared in methanol at a concentration of 0.4 mg/ml for each drug. They covered a variety of basic, acidic and amphotheric drugs (a total of 60 drugs). These group standards were stored at −20°C and were diluted with mobile phase

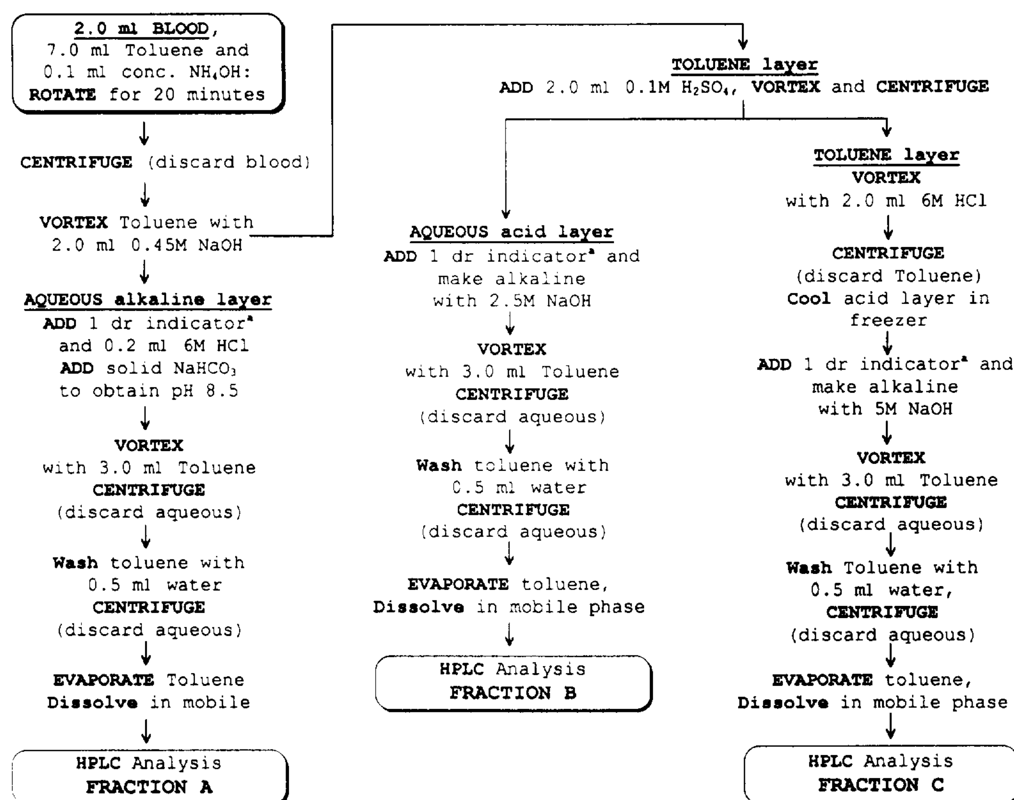


Fig. 2. Flow diagram of method for basic drug extraction from blood. [a]1% Bromothymol blue (w/v) in methanol–water (1:1, v/v). dr = drop.

(1:100) to monitor column performance and the validity of identifications based on the library data. The complete directory of the library's contents on two reversed-phase columns is listed in Table 2.

A separate library was created for multicomponent analysis. Group standards were prepared at a concentration of 0.8 mg/ml. These group standards were diluted with mobile phase (1:100) and injected on the LiChrospher column. The area spectrum obtained for each drug (a total of 245 drugs), in addition to identification, retention time and the amount of the injected drug, made up the library.

Data analysis

The PolyView spectral processing software and library search employed in this work have been described by Sheenan et al. [17]. The data libraries included five data files, containing 272 and 280 drugs on the Supelcosil and LiChrospher columns, respectively (Table 2). The PolyView Report included: (a) a continuous plot of UV absorbance as a function of time from 0 to 20 min at 229 nm, (b) a continuous plot of purity parameter (PUP) from 0 to 20 min over a wavelength range of 210 to 367 nm and (c) a library search report with overlay graphic. A retention time window of ± 5% and a PUP of ± 1 nm were set as primary search criteria. A dissimilarity value of 0.06 was set to the confirm peak identity and peak homogeneity.

Multicomponent analysis

Twelve group standards, containing 76 drugs were prepared to evaluate the applicability of the PolyView multicomponent analysis (MCA) software to the quantitation

Table 2
Retention times (t_R) for drugs on reversed-phase HPLC columns

Drug	t_R [a] (min)	t_R [b] (min)	Drug	t_R [a] (min)	t_R [b] (min)
Acebutolol	6.51	3.72	Cisapride	10.90	5.81
Acebutolol metabolite 1	5.71	3.41	Clobazam	7.20	7.11
Acebutolol metabolite 2	6.08	–	Clomipramine	17.90	8.53
Acepromazine	12.10	5.81	Clonazepam	6.45	6.02
Acetaminophen	4.75	3.68	Clonidine	7.97	4.25
Acetanilide	5.47	4.90	Clopenthixol	14.10	6.81
Acetazoleamide	4.77	3.68	Clozapine	10.90	5.15
Acetophenazine	9.83	4.89	Cocaine	10.00	4.99
Albuterol	5.57	3.27	Codeine	6.09	3.42
Alprazolam	6.40	6.44	Colchicine	5.09	4.15
Aminochlorobenzophenone	9.48	13.10	Coumarine	6.27	6.09
Aminopromazine	17.20	8.10	Creatinine	5.15	3.11
Amitriptyline	15.80	7.27	Cyclizine	12.40	5.78
Amobarbital	5.94	5.59	Cyclobenzaprine	15.50	7.05
Amoxapine	11.40	5.55	Dantrolene	6.04	5.23
Anileridine	11.70	5.92	Demoxepam	5.75	5.05
Antazoline	11.50	5.91	Desacetyldiltiazem	9.28	4.59
Antipyrine	5.39	4.54	Desalkylflurazepam	6.51	6.56
Atenolol	5.33	3.11	Desalkylhydroxyflurazepam	5.91	5.42
Atropine	6.95	3.75	Desipramine	13.00	6.26
Azacyclonal	8.70	4.52	Desmethylchlordiazepoxide	5.75	4.69
Azatadine	10.70	4.55	Desmethylchlordiazepoxide[c]	6.30	5.05
Baclofen	5.71	3.47	Diazepam	7.71	8.83
Benzocaine	6.25	6.23	Dichloroisoproterenol	8.43	4.84
Bromazepam	5.79	5.06	Diclofenac	8.68	9.97
Bromocriptine	10.80	6.21	Diethazine	15.10	7.38
Bromodiphenhydramine	14.70	7.48	Diflunisal	5.91	5.95
Brompheniramine	11.10	5.44	Dihydroxybenzoic acid	4.28	3.46
Brotizolam	7.43	7.90	Diltiazem	11.10	5.42
Bupivacaine	10.60	5.79	Diltiazem metabolite 1	10.60	5.09
Buspirone	9.07	4.98	Diltiazem metabolite 3	8.74	4.37
Butabarbital	5.60	4.92	Diltiazem metabolite 4	8.13	7.49
Butalbital	5.77	5.12	Dinitro-*o*-cresol	7.09	7.49
Caffeine	4.79	3.84	Diphenhydramine	12.20	6.01
Carbamazepine	6.15	5.49	Diphenidol	12.10	6.05
Carbamazepine metabolite	–	10.30	Diphenoxylate	dne	14.10
Cetrizine	8.89	5.29	Dipyridamol	8.58	4.79
Chlophendianol	10.60	5.33	Disopyramide	9.56	4.89
Chlorcyclizine	14.70	6.89	Dobutamine	6.58	3.80
Chlordiazepoxide	6.85	5.26	Doxapram	8.67	4.64
Chlormezanone	6.03	5.33	Doxepine	12.90	6.06
Chloroquine	12.70	3.55	Doxepine[c]	13.40	–
Chlorpheniramine	10.80	5.25	Droperidol	8.63	4.71
Chlorpromazine	17.00	7.75	Encainide	9.42	4.91
Chlorpromazine sulfoxide	8.37	4.26	Ethopropazine	16.60	8.25
Chlorpropamide	6.40	6.34	Ethyldiazepam	8.40	10.50
Chlorprothixene	17.60	8.29	Ethylmorphine	6.74	3.63
Chlorthalidone	5.14	4.03	Fenethazine	12.50	5.98
Chlorzoxazone	6.01	5.87	Fenoprofen	8.00	8.87
Cimetidine	5.47	3.16	Fenoterol	5.74	3.38

(*Continued on p. 108*)

Table 2 (*continued*)

Drug	t_R [a] (min)	t_R [b] (min)	Drug	t_R [a] (min)	t_R [b] (min)
Fentanyl	11.40	6.03	Mephenytoin	5.99	5.40
Flavoxate	11.30	5.94	Mepivacaine	7.71	4.20
Floctafenine	6.57	5.84	Mepyramine	12.95	6.06
Fluoxetine	12.20	7.07	Mesoridazine	10.12	5.02
Flupenthixol	13.70	7.53	Metaproterenol	5.41	3.18
Flupenthixol[c]	14.20	7.68	Metformine	5.68	3.25
Fluphenazine	13.60	7.22	Methadone	16.58	8.43
Fluphenazine sulfoxide	7.51	4.07	Methaqualone	6.82	7.44
Flurazepam	10.50	5.53	Methdilazine	15.16	6.66
Flurbiprofen	8.01	8.91	Methocarbamol	5.02	3.94
Fluspirilene	18.30	9.79	Methotrexate	4.72	2.93
Fluvoxamine	9.97	5.94	Methotrimeprazine	15.23	7.19
Fonazine	12.50	6.02	Methoxiamine	6.41	3.62
Frusemide	5.78	5.03	Methoxypromazine	14.36	6.54
Glutethimide	6.60	6.24	Methyldopa	4.05	2.82
Glyburide	8.51	9.83	Methylenedioxyamphetamine	6.89	3.83
Guaifenesine	4.94	3.93	Methylphenidate	8.61	4.65
Haloperidol	11.10	6.17	Metoclopramide	7.92	4.26
Heroin	7.90	4.11	Metolazone	6.05	5.16
Homidium Bromide	13.70	6.28	Metoprolol	7.12	4.19
Homotropine	6.77	3.63	Metronidazole	4.83	3.86
Hydralazine	6.46	3.49	Midazolam	10.21	6.30
Hydralazine[c]	–	5.16	Moclobemide	6.86	3.85
Hydrochlorothiazide	5.09	3.98	Morphine	5.60	3.20
Hydrocodone	6.93	3.71	Nadolol	5.64	3.39
Hydromorphone	5.84	3.42	Nalbuphine	6.56	3.61
Hydroxychloroquine	9.60	3.23	Naloxone	6.16	3.51
Hydroxyethylflurazepam	6.21	5.94	Naphazoline	9.13	4.77
Hydroxyzine	11.40	6.27	Naproxen	6.97	7.18
Ibuprofen	8.12	10.50	Nifedepine	7.42	7.91
Imipramine	14.70	6.78	Nifedepine[c]	7.66	8.28
Indomethacin	8.45	9.22	Nitrazepam	6.27	5.97
Indoxyl	3.82	3.19	Nizatidine	5.57	3.13
Isopromethazine	13.60	6.65	Nordiazepam	6.70	6.81
Ketazolam	7.72	9.23	Norepinephrine	4.90	–
Ketazolam[c]	8.32	8.85	Norfluoxetine	10.78	6.43
Ketoconazole	11.30	5.92	Normeperidine	8.29	4.46
Ketoprofen	7.02	7.04	Nortriptyline	13.70	6.75
Ketorolac	6.32	5.55	Oxazepam	6.00	4.52
Labetalol	7.68	4.22	Oxprenolol	8.27	3.69
Levorphanol	–	4.26	Oxycodone	6.51	5.82
Lidocaine	8.04	4.50	Oxymetazoline	10.42	5.82
Loratadine	10.90	13.25	Parathion	11.15	16.34
Lorazepam	6.14	5.78	Paroxetine	11.06	5.77
Lovastatine	10.49	14.79	Pemoline	4.91	3.84
Loxapine	13.78	6.26	Pentazocine	9.91	5.47
Mazindol	9.71	5.06	Pentobarbital	5.92	5.58
Mefenamic acid	9.77	13.08	Pentoxifylline	5.01	4.15
Mepazine	15.28	7.04	Pericyazine	10.24	5.13
Meperidine	9.21	4.83	Perphenazine	13.16	6.33
Mephenesine	5.34	4.55	Phenacetin	5.63	5.24

Table 2 (*continued*)

Drug	t_R [a] (min)	t_R [b] (min)	Drug	t_R [a] (min)	t_R [b] (min)
Pheniramine	9.49	4.49	Sotalol	5.96	3.41
Phenobarbital	5.61	4.74	Spironolactone	8.50	9.12
Phenol	5.85	5.29	Strychnine	7.49	3.88
Phenolphthalein	6.05	5.66	Sulfinpyrazone	5.71	5.33
Phentolamine	9.05	4.79	Sulindac	6.17	5.99
Phenylbutazone	10.15	12.11	Temazepam	8.86	6.70
Phenyltoloxamine	13.20	6.31	Terbutaline	5.62	3.27
Phenytoin	6.11	5.26	Terfenadine	dne	12.24
Pimozide	14.36	7.96	Tetrabenazine	–	4.08
Pindolol	7.02	4.07	Tetrabenazine[c]	–	6.00
Pipamazine	11.22	5.63	Tetracaine	10.81	5.44
Pipotiazine	10.94	5.66	Theophylline	4.52	3.45
Piroxicam	6.84	6.67	Thiethylperazine	dne	9.45
Pizotyline	15.78	6.84	Thiopental	7.04	7.33
Pramoxine	11.16	6.25	Thioproperazine	15.39	6.34
Prazepam	10.49	12.77	Thiopropazate	16.64	8.49
Prazosine	7.38	4.21	Thioridazine	dne	9.82
Probenecid	7.49	–	Thiothixene	16.49	6.57
Procaineamide	6.32	3.52	Thymol	7.93	10.45
Procaine	7.25	4.03	Tiaprofenic acid	6.84	6.65
Prochlorperazine	dne	8.18	Timolol	7.04	3.94
Procyclidine	dne	4.65	Tocainide	6.28	3.60
Promazine	14.24	6.34	Tolbutamide	6.74	6.94
Promethazine	13.20	6.40	Tolmetine	6.45	6.29
Promethazine sulfoxide	–	4.50	Trazodone	8.37	4.58
Propafenone	12.20	6.37	Triamterene	6.73	3.90
Propantheline	7.08	–	Triazolam	6.43	6.65
Propantheline[c]	8.03	–	Trifluoperazine	dne	9.32
Propiomazine	14.08	7.10	Trifluoperazine sulfoxide	9.08	4.45
Propofol	10.07	15.24	Triflupromazine	17.28	8.93
Propranolol	9.19	4.91	Triiodothyroacetic acid	8.15	8.40
Protriptyline	13.20	6.33	Trimebutine	12.21	6.21
Quazepam	11.94	17.69	Trimeprazine	14.91	7.07
Quinidine	8.72	4.55	Trimethoprim	6.67	3.65
Quinidine[c]	9.29	4.83	Trimipramine	15.49	7.66
Quinine	8.34	4.47	Verapamil	13.28	6.96
Quinine[c]	8.90	4.75	Verapamil metabolite 1	12.60	6.66
Racemethorphan	11.47	5.92	Verapamil metabolite 2	11.10	5.88
Ranitidine	5.88	3.27	Verapamil metabolite 3	8.63	4.68
Remoxipride	8.84	4.64	Verapamil metabolite 4	7.92	4.41
Risperidone	9.12	4.63	Warfarin	8.01	8.29
Salicylic acid	5.20	4.35	Xylometazoline	12.68	7.16
Scopolamine	6.95	3.70	Yohimbine	8.15	4.37
Secobarbital	6.19	5.91	Zomepirac	6.96	7.02
Sertraline	14.50	7.68	Zopiclone	7.50	3.79
SKF	19.27	10.72			

dne = Did not elute on this column within 20 min.
[a] Supelcosil LC-DP, particle size 5 μm, 25 cm × 4.6 mm I.D.
[b] LiChrospher 100 RP-8, particle size 5 μm, 25 cm × 4.0 mm I.D.
[c] Secondary peak.

of unresolved peaks. Within the twelve groups, the three sub-groups contained the same set of drugs in three combinations. In general, the ratios for the three drugs under the same retention time were 0.5:1.0:2.5, 1.0:0.5:2.5 and 2.5:1.0:0.5. This experiment was performed with a resolution factor of $R_s = 0.0$ (completely fused).

2.4. Analysis of forensic samples

Quantitation

Calibration standards were prepared by spiking outdated Red Cross blood which was artificially aged at room temperature for approximately 3–4 weeks. The blood was preserved with 1 g/100 ml of sodium fluoride. Quantitation of a drug (basic, acidic or others) was based on the peak areas, representing concentrations likely to be encountered at the high end of therapeutic use. A standard curve was constructed using duplicate samples at each concentrations. Drug-free blood was included in the assay to produce blank chromatogram. The efficiency of the extraction was calculated by comparing the peak areas at each point on the blood standard curve with those of standards prepared for spiking and diluted to the appropriate concentrations with mobile phase. Chromatographic conditions, final volume and peak areas of blood and straight standards were documented for each assay, which allowed the variability of recovery for each drugs to be followed.

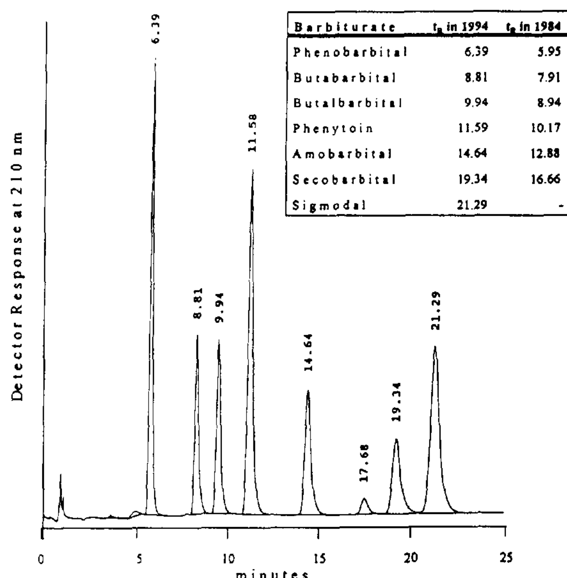
Screening

Assays for screening postmortem blood and urine samples were performed as outlined in Fig. 1 and 2. A procedure for extraction of gastric contents has also been developed in this laboratory [18]. A portion of the gastric content was mixed with ethanol and kept on a water bath for 15 min. After filtration, the ethanol was evaporated, the residue was dissolved in 10 ml of water and filtered. A 1-ml volume was extracted to obtain three fractions: amphotheric/neutral, basic and acidic. The final extract was evaporated and reconstituted with 0.5 ml of mobile phase. An aliquot of 25 μl was injected onto the column.

3. Results and discussion

3.1. HPLC columns and libraries

The APEX ODS column has been used for the chromatographic analysis of salicylic acid, acetaminophen, theophylline and barbiturates for ten years. The reproducibility of the retention times using different batches of columns of the same brand was very good. Experience has shown that this column is suitable for the analysis of barbiturates using a methanol–water mobile phase. The chromatogram of an extract of blood containing six barbiturates, including sigmodal as an internal standard and phenytoin is shown in Fig. 3. In addition, retention times obtained for the same set of barbiturates (except the internal standard sigmodal) in 1984 are listed. Originally, the barbiturate assay was carried out on an SP8100 chromatography system, later it was transferred to the LC Star system,



Barbiturate	t_R in 1994	t_R in 1984
Phenobarbital	6.39	5.95
Butabarbital	8.81	7.91
Butalbarbital	9.94	8.94
Phenytoin	11.59	10.17
Amobarbital	14.64	12.88
Secobarbital	19.34	16.66
Sigmodal	21.29	-

Fig. 3. Chromatogram obtained from extract of spiked blood sample on APEX ODS column. Concentration of each barbiturate was 5.0 μg/ml of blood. t_R = retention time in min.

thus accounting for the difference of approximate 10% in the retention times between 1984 and 1994.

In the previous study [13] it was demonstrated that the APEX ODS column has been successfully used for the chromatographic analysis of basic and other drugs for two years. Reproducibility, however, between two columns of the same brand but different batches was unsatisfactory when the mobile phase was a mixture consisting of acetonitrile and buffer. The problems included tailing peaks, poor reproducibility of retention times and change in elution order for basic drugs. To replace the APEX ODS column, two other columns were investigated. The LiChrospher column was previously used for the analysis of phenothiazine and the Supelcosil column (diphenyl), was chosen for better selectivity. The long-term reproducibility of the retention time on both columns was very good. The selectivity and elution patterns of a second set of the columns (LiChrospher and Supelcosil) of different batches were nearly identical when tested with seven group standards. The retention times in the data base had to be slightly corrected for routine use, but this can be completed within a week. There was no difficulty using the same libraries for two LC systems; the detectors were calibrated, the same columns and mobile phase were used and the length of the stainless-steel tubing on the two instruments was identical. The HPLC system was verified at the beginning of the analysis using group standards. The two test groups are illustrated in Fig. 4.

The multicomponent analysis employed in this work was described by Excoffier et al. [19] and reviewed by Keller et al. [20]. Since 1986, the performance of the system has been continuously monitored and it was found that the UV absorbance of a drug remained within ± 10% for the same column, mobile phase and instrument used. Therefore an MCA library containing 245 drugs was created to investigate the long-term use of a permanent library to determine the identity and concentration of drugs in co-eluting peaks. The MCA software is limited in its ability to determine the individual drugs from the spectrum of a mixture if it is composed of more than six drugs at the same retention time. Therefore small sub-libraries containing approximate

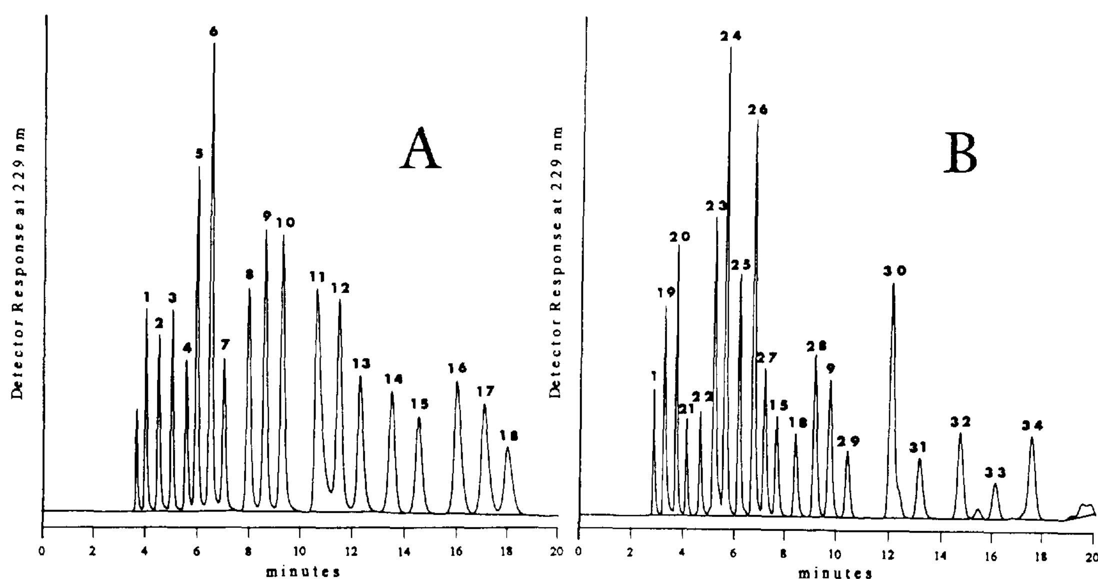


Fig. 4. Chromatograms obtained on Supelcosil (A) and LiChrospher (B) columns with standard solutions containing a mixture of drugs. Peaks: 1 = methyldopa; 2 = theophylline; 3 = methocarbamol; 4 = nizatidine; 5 = sotalol; 6 = acebutolol; 7 = ketoprofen; 8 = flurbiprofen; 9 = glyburide; 10 = propranolol; 11 = midazolam; 12 = diltiazem; 13 = fluoxetine; 14 = verapamil; 15 = sertraline; 16 = amitriptyline; 17 = chlorpromazine; 18 = clomipramine; 19 = hydroxychloroquine; 20 = acetaminophen; 21 = metoprolol; 22 = trazodone; 23 = clozapine; 24 = lorazepam; 25 = chlorpropamide; 26 = temazepam; 27 = trimeprazine; 28 = indomethacin; 29 = ibuprofen; 30 = phenylbutazone; 31 = diphenoxylate; 32 = propofol; 33 = parathion; 34 = quazepam.

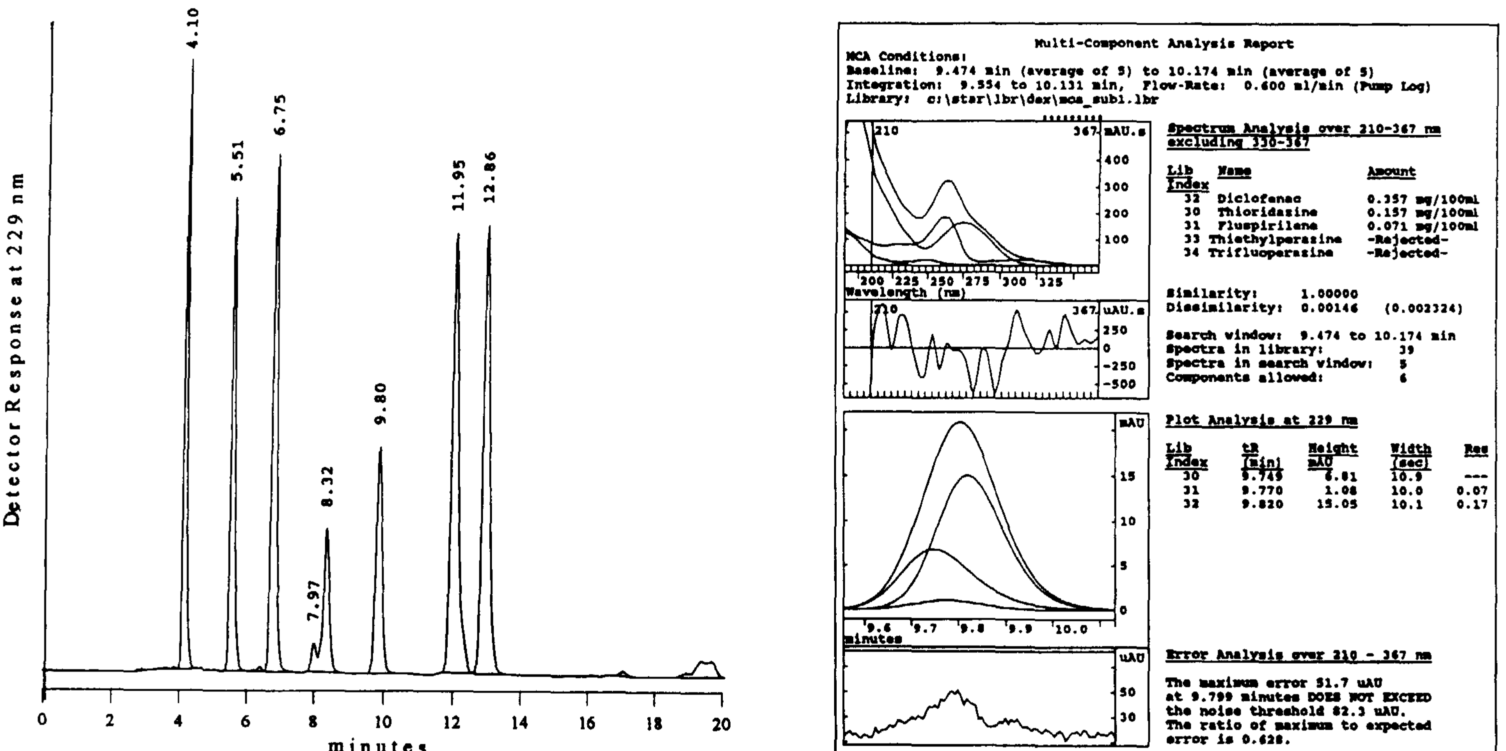


Fig. 5. Multicomponent analysis of a mixture of 20 drugs. Target concentration of diclofenac, thioridazine and fluspirilene were 0.360, 0.159 and 0.072 mg/100 ml, respectively, at $t_R = 9.80$ min.

Fig. 6. Accuracy of multicomponent analysis for the quantitation of co-eluting drugs.

40 drugs were created from the main library and the number of drugs with similar or identical retention time in those sub-libraries was six. The chromatogram in Fig. 5 represents one of the mixtures containing 20 drugs. Fig. 6 shows that 93% of the drugs, totally or partially fused into the chromatographic peak of another drug, could be quantitatively isolated with ± 15% accuracy. This study has confirmed the potential usefulness of MCA but more work has to be completed before it can be used on a routine bases. At present, the following guidelines are used: (a) previous information on the probable co-eluting drugs in quantitative MCA is required. This information can be obtained from GC–nitrogen–phosphorus detection screening, case history or the types of drugs submitted with the case, (b) limit the number of candidates by using selective extraction i.e. excluding the presence of acidic, amphotheric and neutral drugs from a basic extract, (c) obtain qualitative information by manually inspecting the eluted peak and (d) establish fit criteria based on error analysis and inject a standard of known concentration concurrently with the MCA analysis.

3.2. Extraction of acidic drugs

Direct acidic extracts of biological specimens prepared for drug screening purposes often contain other substances which interfere with HPLC analysis. The XAD-2 procedure has been in use over ten years in this laboratory for the quantitation of barbiturates and phenytoin. It has been extended to the screening and quantitation of other acidic drugs such as naproxen, ibuprofen, ketorolac, piroxicam, flurbiprofen, warfarin etc. The addition of a back-extraction step into 0.45 *M* NaOH increased selectivity of the procedure and provided an extract with no background peaks.

Salicylic acid, acetaminophen and theophylline were extracted with ethyl acetate. Because of the lyophilic properties of acetaminophen and theophylline, purification using back-extraction was not feasible. However, the high therapeutic concentrations of these drugs allowed the direct extraction of whole blood with ethyl acetate using 0.5 ml of blood and a final volume of 2.0 ml. Interference from blood impurities had no effect on the identification and quantitation. Evaluation of solid-phase extraction instead of direct extraction with ethyl acetate is in progress.

3.3. Extraction of basic drugs

One of the most demanding problems in forensic toxicology is the analysis of complex cases when a variety of drugs and metabolites are present which interfere with each other in the chromatographic analysis. The method de-

scribed eliminates some of the problems encountered in the analysis of "multiple drug overdose" cases. The benefit of selective extraction is illustrated in Fig. 7. A 2-ml volume of blood was spiked with 10 drugs at a concentration of 0.1 mg/100 ml each; after the initial extraction with toluene under basic conditions, it was extracted with 0.45 *M* NaOH, 0.1 *M* H_2SO_4 and 6 *M* HCl. As shown in Fig. 7A, the poorly resolved or totally fused peaks obstruct the accurate quantitation of these drugs. By contrast, using the method outlined in Fig. 2, all drugs can be easily quantitated and identified in the three fractions (Fig. 7B, C and D). Figs. 8 and 9 show chromatograms of basic extracts of forensic case blood samples with multiple drugs findings.

3.4. Analysis of forensic samples

Two cases (Fig. 8A and B) were analysed for diltiazem and a third case (Fig. 8C) for verapamil. In each case, unexpected drugs (bromazepam, acebutolol and trimethoprim, respectively) were identified by the library search. The first case had a limited amount of blood sample and could not be re-extracted to determine the concentration of bromazepam. A quantitative bromazepam standard was injected

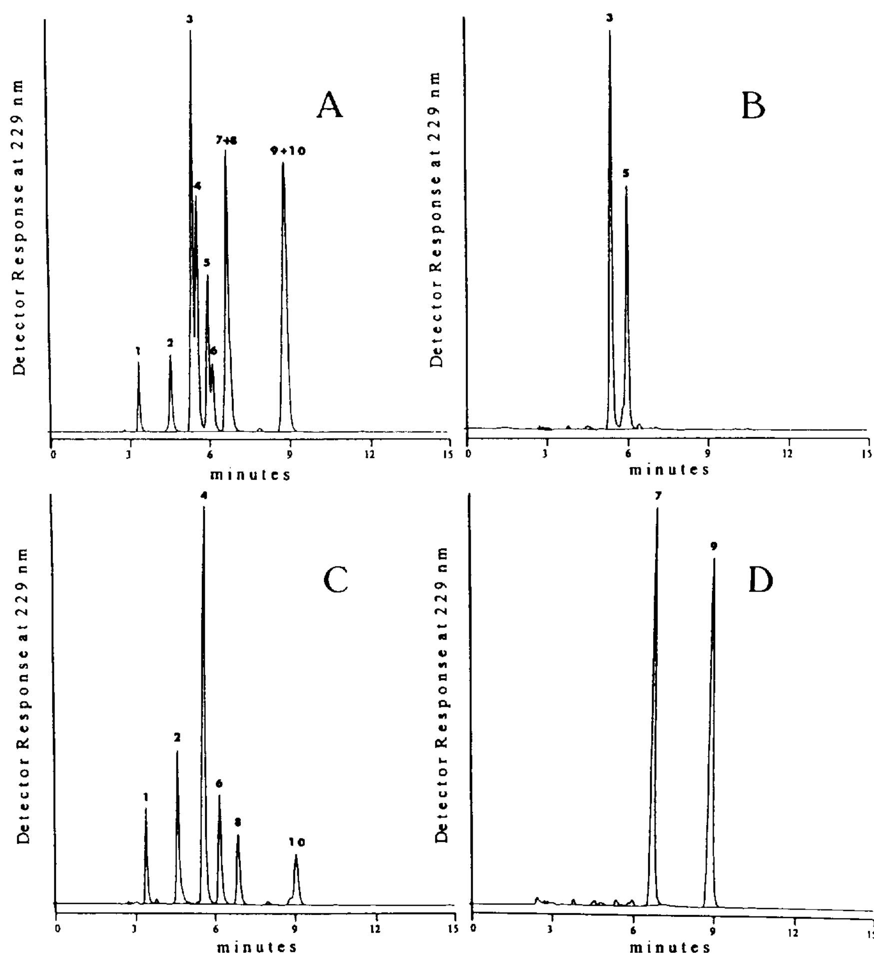


Fig. 7. Chromatograms obtained of the basic extracts of a spiked blood sample: (A) spiking standard, (B) fraction A, (C) fraction B and (D) fraction C. Peaks: 1 = codeine; 2 = trazodone; 3 = oxazepam; 4 = flurazepam; 5 = clonazepam; 6 = haloperidol; 7 = temazepam; 8 = imipramine; 9 = diazepam; 10 = trifluopromazine.

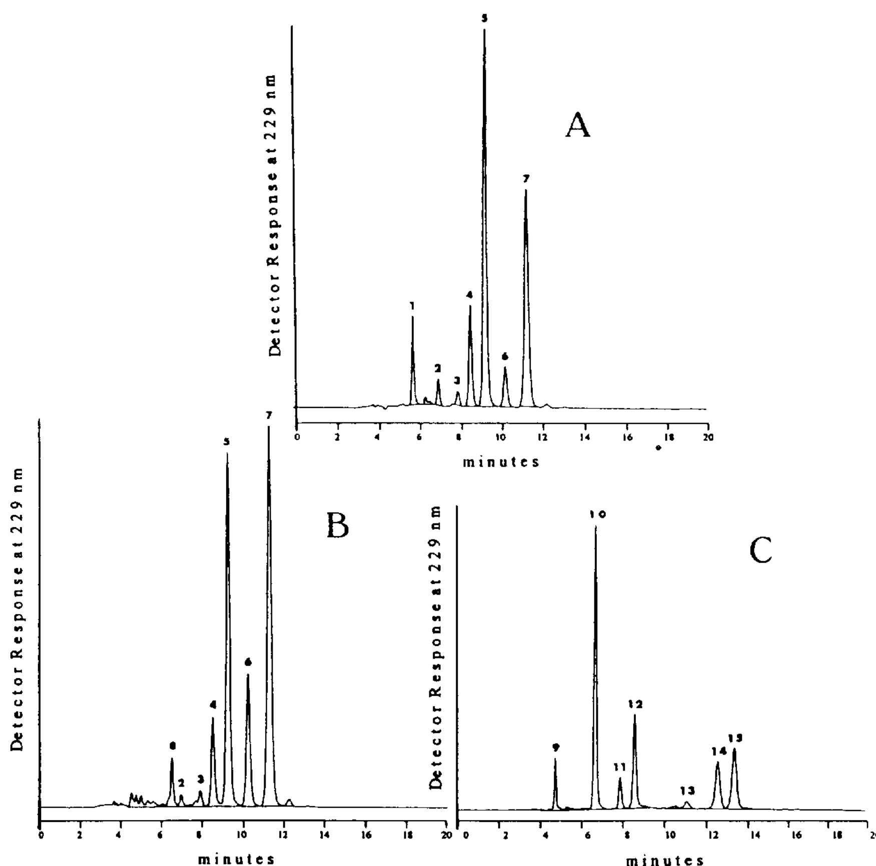


Fig. 8. Chromatograms obtained from the basic extracts of three forensic blood samples (A, B and C). Peaks: 1 = bromazepam; 2–4, 6 = diltiazem metabolites; 5 = desacetyldiltiazem; 7 = diltiazem; 8 = acebutolol; 9 = caffeine; 10 = trimethoprim; 11–14 = verapamil metabolites; 15 = verapamil.

and the peak area obtained was compared with the peak area of the equivalent standard in a previous bromazepam assay; it was within 10%. Using an average factor for the recovery of bromazepam from blood (based on long-term statistical data), the concentration of bromazepam was calculated.

Chlorpromazine, trifluoperazine and their metabolites were identified in another case blood (Fig. 9A). Manual inspection of peak number 4 indicated the presence of a benzodiazepine and phenothiazine. Using the qualitative MCA method, the peak was identified as bromazepam and a phenothiazine metabolite. In spite of the presence of several drugs and their metabolites, the concentration of temazepam was determined accurately using selective extraction (Fig. 9B).

Fig. 10 shows a chromatogram obtained from the XAD-2 extract of a forensic case blood sample in which naproxen was expected. In addition to naproxen, ibuprofen and ketorolac were found. Due to the negligible absorption of ketorolac at 229 nm, there was no observable peak in the chromatogram at $t_R = 6.2$ min. The automatic library search, however, provided a positive identification of ketorolac. Two extraneous peaks occurred at retention times of 4.03 and 5.35 min. The similarity of spectral properties of peak number 2 and ibuprofen indicated that the peak at $t_R = 5.35$ min is the metabolite of ibuprofen. Peak number 1 was not identified.

The screening of two gastric content samples in forensic cases is illustrated in Fig. 11A and B. Zopiclone, mesoridazine, perphenazine and

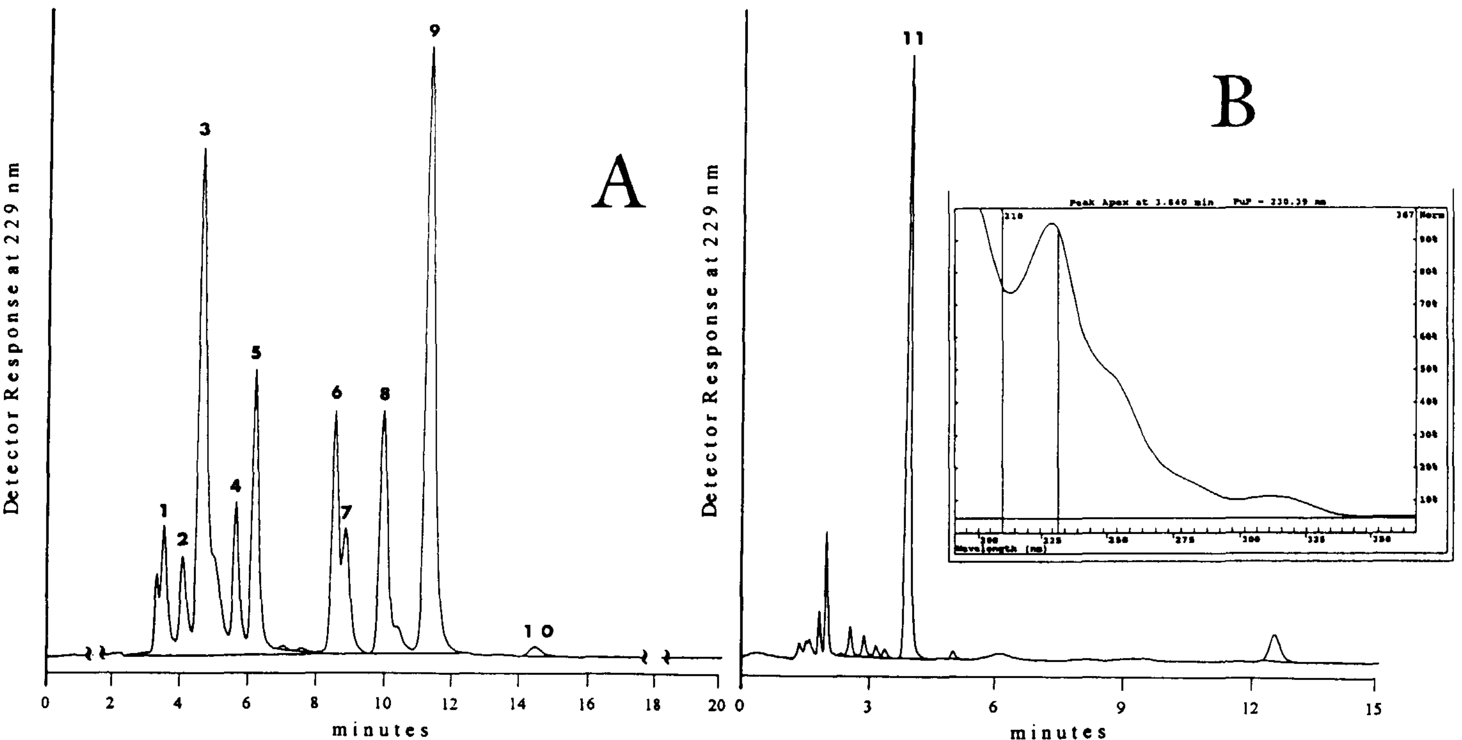


Fig. 9. Chromatograms obtained from the basic extracts of a forensic blood sample: (A) 0.1 M H_2SO_4 acid fraction and (B) 6 M HCl acid fraction. Peaks: 1 = caffeine; 2 = chlorpromazine sulfoxide; 3 = trifluoperazine sulfoxide; 4 = coelution of bromazepam and phenothiazine metabolite; 5, 7, 8 = phenothiazine metabolites; 6 = not identified; 9 = chlorpromazine; 10 = trifluoperazine; 11 = temazepam.

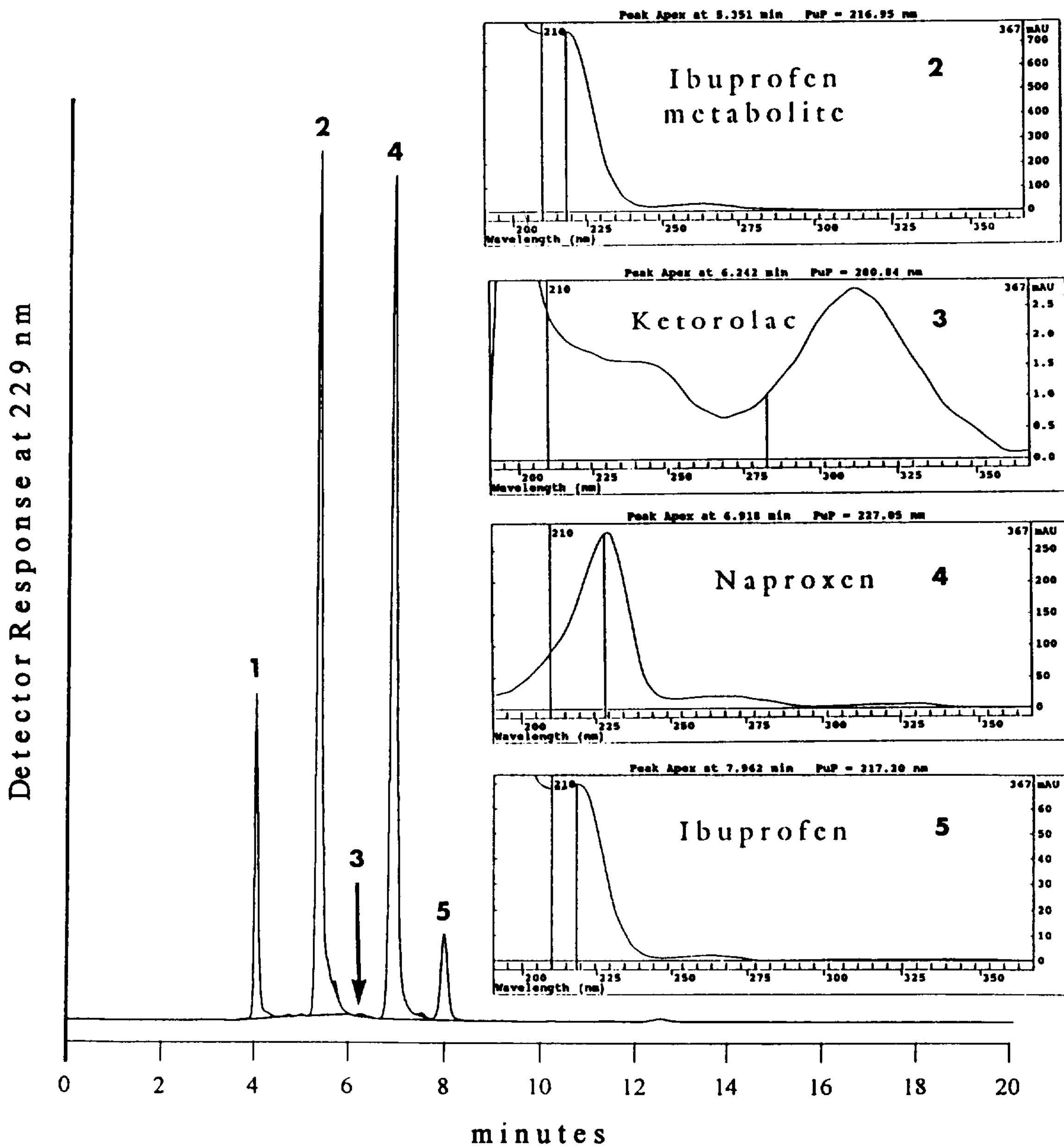


Fig. 10. Chromatogram obtained from the XAD-2 extract of a forensic blood sample and UV spectra of peaks 2, 3, 4 and 5. Peaks: 1 = not identified; 2 = ibuprofen metabolite; 3 = ketorolac; 4 = naproxen; 5 = ibuprofen.

thioridazine were identified in case 1. It should be noted that the peak eluted at about 9.9 min represents an enormous amount of thioridazine. Over 150 gastric content samples were screened in the last year and no carryover was observed during analysis. The mobile phase was re-routed to the waste during the assay to prevent contamination of the mobile phase in the reservoir due to the high concentration of drugs in the gastric contents. By contrast with case 1, the chromatogram of the extract of gastric content in case 2 revealed only one chromatographic peak. No

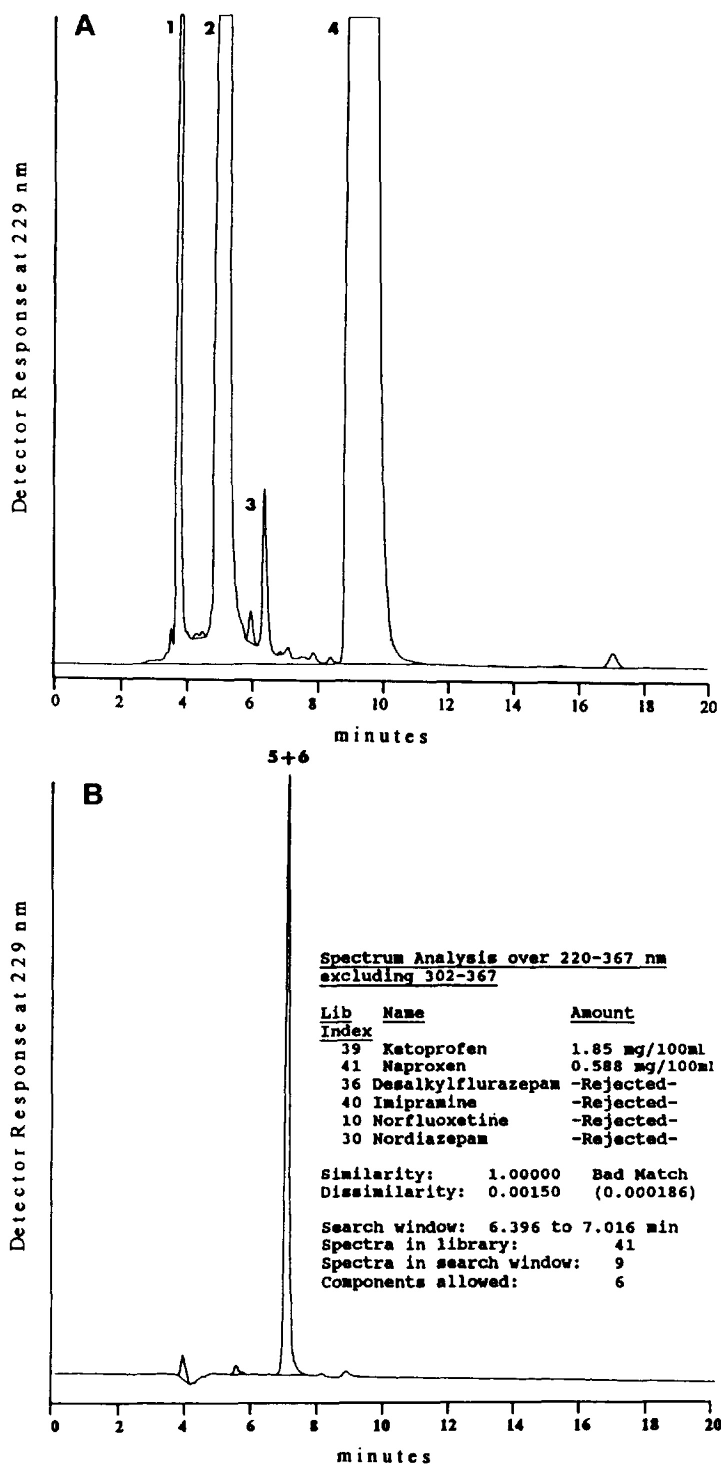


Fig. 11. Chromatograms obtained from extracts of two gastric contents (A and B). Peaks: 1 = zopiclone; 2 = mesoridazine; 3 = perphenazine; 4 = thioridazine; 5, 6 = co-elution of ketoprofen and naproxen.

match was made with the library search but the PUP indicated that it was not a homogeneous peak. Using MCA, the presence of ketoprofen and naproxen was confirmed.

4. Conclusions

The suitability of an isocratic HPLC–DAD system with computer-aided drug identification for toxicological screening and quantitation has been described. Extracts of postmortem blood samples as clean as possible were prepared, thus preventing co-elution of endogenous compounds with any drugs that may be present. It has been demonstrated that the back-extraction procedure resulted in clean extracts allowing identification of drugs based on spectra obtained by DAD. The use of the extraction characteristics of drugs together with the library search, allows more confidence in determining the identity of a drug. MCA presents to HPLC a major advance in the identification and quantitation of co-eluting chromatographic peaks. The results of this work have successfully demonstrated the application of PolyView-MCA software to the quantitation of 76 drugs. It was concluded that DAD together with MCA, offers a valuable tool for the HPLC analysis of forensic case samples involving multiple drugs. Using a library with candidate spectra, MCA can be employed to determine which drugs are present in a fused chromatographic peak.

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