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Evaluation of a Photodiode Array/HPLC-Based System for the Detection and Quantitation of Basic Drugs in Postmortem Blood

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ABSTRACT: A systematic analytical approach has been developed for liquid chromatography determination of a number of basic drugs in postmortem blood. Using selective extraction, that is, back extraction into 0.2*N* sulfuric acid and 6*N* hydrochloric acid after the initial extraction with toluene under basic conditions (from 2 mL of blood), basic and weakly basic drugs, such as propranolol and diazepam, can be simultaneously quantitated and identified with a high degree of confidence. A microcomputer-based photodiode array detector was used to evaluate peak purity and facilitate peak identification. An automatic library search was performed at the end of each analysis using the system software. The method was validated for within-day and between-day precision for ten basic drugs at two concentrations. The coefficient of variation for the between-day precision was less than 8.7%. Accuracy of the assay was tested at four concentrations using linear regression analysis. The coefficients of determination (r^2) for all ten drugs were greater than 0.99, and their slopes were close to unity. The chromatographic conditions developed are suitable for the screening of several basic, acidic, amphoteric, and neutral drugs. Retention data and ultraviolet spectral data for 119 drugs on two reversed-phase columns, using acidic mobile phases, are also presented.

KEYWORDS: toxicology, drug identification, blood, chromatographic analysis, high-performance liquid chromatography, photodiode array detector

Forensic toxicologists are routinely confronted by the difficult problem of detecting and quantitating a wide range of drugs in postmortem blood. The sensitivity and selectivity of gas chromatography using capillary columns and a nitrogen-phosphorous detector allow the screening and quantitation of many basic drugs [*1*]. This approach and the use of gas chromatography/mass spectrometry usually lead to the identification of the drugs of interest. There are many cases, however, in which highly polar compounds, such as temazepam, verapamil, or trazodone have, to be analyzed. High-performance liquid chromatography (HPLC) is particularly well suited for this type of drug. The full potential of HPLC, however, in forensic toxicology has yet to be realized, because of the lack of specific detectors and extraction methods suitable for postmortem blood.

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The conventional HPLC detectors, which are fixed- or variable-wavelength ultraviolet (UV) photometric detectors, yield only a single chromatogram, which may include several other peaks or unresolved, co-eluting peaks. In a relatively short period of time, the potential of absorptiometric multichannel technology based on photodiode array has been realized. This detector allows simultaneous signal and spectral acquisition during the chromatographic analysis and has become firmly established in analytical, clinical, and development laboratories [*2–8*]. The theoretical and practical aspects of the photodiode array detector have been thoroughly reviewed [*9–14*]. While the data obtained are not equivalent to those generated by mass spectrometry, the photodiode array detector provides enough information to solve many analytical problems associated with high-performance liquid chromatography, such as peak purity and the identity of the drug to be analyzed.

In developing an HPLC procedure which could offer the quantitation and identification of a wide range of nonvolatile drugs, the following parameters were considered: (*a*) the choice of column and mobile phase, (*b*) a library of spectra for use in identification of the drug to be analyzed, (*c*) the use of a photodiode array detector for quantitative analysis, and (*d*) the selection of an extraction method suitable for automatic injection.

The method presented in this paper describes the selective extraction, quantitation, and identification of a number of basic drugs encountered in forensic toxicology and forms the foundation of an HPLC screening system. Chromatographic precision and the accuracy of the method are also examined.

Experimental Procedure

Automated Liquid Chromatography System

The LC Star system was purchased from Varian Instruments (Varian Canada Inc., Georgetown, Ontario, Canada) and consisted of the following components: a ternary gradient pump, Model 9010, with flow rates of 0.01 to 5.0 mL/min; an autosampler, Model 9095, with Valco injector, including random sample access, automix, programmable rinsing, and a 105-sample capacity; and the Polychrom 9065 optics module with external scanned array, automatic baseline correction, programed scan rates, and programed detector lamp.

The work station for instrument control and data analysis consisted of a Compaq 386/20e computer with the following hardware configuration: 4 Mbytes of RAM memory, a 100-Mbyte hard disk, a high-resolution VGA monitor, 1.44- and 1.2-Mbyte ($3\frac{1}{2}$- and $5\frac{1}{4}$-in.) floppy disk drives, a mouse, and a GPIB interface board for communication with the Star LC system modules for the Compaq 386/20e. Based on a Microsoft® Windows user interface, the Star 9020 work station software is used for system control and chromatographic data handling and the PolyView™ spectral processing software is used for automated post-run spectral evaluation. An HP LaserJet III printer was used for all chromatograms and spectral plots.

The chromatographic separation was achieved isocratically on two reversed-phase columns using different mobile phases. Column I was an APEX ODS, 5-μm column, 25 cm by 4.6 mm in inside diameter (ID) (Radionics Scientific Inc., Downsview, Ontario, Canada); Column II was a Waters, μPhenyl, 5-μm column, 15 cm by 3.9 mm in ID (Waters, Division of Millipore, Mississauga, Ontario, Canada). The columns were used at ambient temperature (22°C). The mobile phase for Column I was acetonitrile/0.025% phosphoric acid (H_3PO_4) (v/v)/triethylamine buffer, at pH 3.4 (25:10:5, v/v/v), delivered at a flow rate of 0.8 mL/min. The mobile phase for Column II was acetonitrile/0.025%

H_3PO_4 (v/v) (50:50, v/v), and the flow rate was 0.6 mL/min. Both mobile phases were filtered through a Millipore 0.45-μm HA filter (Waters, Division of Millipore, Mississauga, Ontario, Canada) and degassed with helium. After the system was equilibrated (approximately 30 min), the mobile phase was recycled from the detector back to the mobile phase reservoir. Drugs were quantitated at their UV absorbance maxima; the injection volume was 25 μL out of a final volume of 300 μL.

Chemicals and Reagents

All drug standards were obtained in pure form from pharmaceutical manufacturing companies. Stock solutions of each drug were prepared in methanol at a concentration of 0.4 mg/mL of free base. When stored at −20°C, these solutions were stable for several months. The working standard solutions containing the appropriate drug at the desired concentrations were made by diluting the stock solutions with methanol/water (1:1 v/v). Dilution of the stock solution with the mobile phase (1:200) was used to establish retention time. Calibration standards were prepared by spiking outdated Red Cross blood, which was artificially aged at room temperature for approximately three to four weeks; after the addition of 1.0 g of sodium fluoride (NaF)/100 mL, the blood was stored at 5°C for several weeks. The presence of drugs was tested in each batch of aged blood before it was used for the assay.

Deionized water was generated by a Millipore Milli-Q Plus water purification system (Millipore Ltd., Canada). Triethylamine (TEA) buffer at pH 3.4 was prepared by making a 1.0% (v/v) solution of TEA in water and adding concentrated phosphoric acid until pH 3.4 was obtained.

HPLC-grade acetonitrile, methanol, and triethylamine were purchased from Johns Scientific Inc. (Toronto, Ontario, Canada). All other solvents used were of distilled-in-glass quality and the inorganic reagents were of analytical reagent grade.

Extraction Method

To 2.0 mL of blood sample in a 15-mL glass culture tube, 16 by 125 mm, Kimax® with a Teflon® resin faced rubber liner in the screw cap, 7.0 mL of toluene, and 0.1 mL of concentrated ammonium hydroxide were added. The tubes were rotated for 20 min and centrifuged. After centrifugation, the organic phase was back-extracted for 1 min with 2.0 mL 0.2*N* sulfuric acid. Following centrifugation, the toluene layer was transferred to a clean 15-mL screw-cap culture tube (Fraction B). One drop of bromothymol blue indicator was added to the remaining aqueous-acid layer (Fraction A) and made alkaline with 2.5*N* sodium hydroxide (NaOH), and the mixture was extracted for 1 min with 3.0 mL toluene, and then centrifuged. The toluene layer was transferred to a small glass tube containing 0.5 mL of water. This mixture was vortexed for 20 s and then centrifuged. The organic phase was transferred to a small glass culture tube and evaporated in a heating module (Pierce Reacti-Therm III™) at 65°C under a stream of nitrogen. The residue was dissolved in 300 μL of mobile phase.

To the toluene layer (Fraction B), 2.0 mL of 6*N* hydrochloric acid (HCl) was added and the mixture was vortexed for 1 min. After centrifugation, the toluene layer was removed and discarded. The remaining strong acid layer was kept at −20°C for 20 min. After the addition of one drop of indicator, the acid fraction was made alkaline with 5*N* NaOH. During the alkalization step, the tubes were kept in an ice bath. The fraction was then extracted for 1 min with 3.0 mL of toluene and then centrifuged. The toluene layer was washed, evaporated, and redissolved in mobile phase, as described for Fraction A.

Analysis

Quantitation—For calibration, the different drugs were added to drug-free blood in the range corresponding to their therapeutic concentrations, allowed to equilibrate overnight and extracted as described. The volume of drug solution added was <2.5% of the total volume of the sample so that the integrity of the blood was maintained. Standard curves were constructed utilizing duplicate samples at each concentration. Quantitation of drugs was accomplished using peak areas. Although data collected in the work station can be loaded directly into Excel® (Microsoft Corp.) for analysis, a linear regression program was used on a Lotus® 1-2-3 spreadsheet (Lotus Development Corp.). Analysis of an extract of a blank blood was included in each assay for quality assurance.

Automated analysis of spectral data—The library search and unattended spectral processing software employed in this work have been described by Sheehan et al. [*15*]. The PolyView report method was set for double plot and library search with the following parameters: (*a*) For **double plot**, in the top window, the UV absorbance as a function of time at a single wavelength was selected (for example, plot type, chromatogram; wavelength, 229 nm; time range; 0 to 15 min; and scale range, autoscale, for temazepam). In the bottom window, the continuous plot of the purity parameter (between the upslope and downslope inflection point) as a function of time was selected [for example, plot type, purity parameter; wavelength range, 210 to 367 nm (broad range); time range, 0 to 15 min; and scale range, autoscale, for most of the drug analyzed]. (*b*) For **library search**, the search libraries included the two main library data files, consisting of the compound name, retention time, and spectra of 119 drugs (Table 1) and a small library file, created from the appropriate blood standard or standards. The library search was conducted using the following criteria: *data file time range*—from 2.5 min to the end of the run for the two main libraries and a retention time ±1 min for the blood standard library; *wavelength range*—210 to 367 nm; *purity parameter (PuP) interval*—±0.5 nm was set for each library; and *report type*—the short graphic was selected.

Validation Studies

Recovery study—Drug standards prepared in blood at two concentrations were extracted as previously described and the peak areas were compared with those of standards prepared in methanol and diluted with the mobile phase to the appropriate concentrations. This is shown in Table 2, expressed as "% recovery."

Reproducibility study—To 10.0 mL of control blood were added 100 μL of the appropriate working solution of each drug or working solution containing a group of drugs at two concentrations. This was equilibrated overnight, and an aliquot of 2.0 mL of blood sample was analyzed in quadruplicate on the following day. The amount of drug was determined using the daily standard curve obtained in routine analysis. The within-day reproducibilities of analyses for ten selected drugs are listed in Table 2.

The between-day variation of the assay was tested by repeating the within-day experiment on three different days over a period of three months by three different analysts. The between-day precisions of analyses for ten drugs are shown in Table 3.

Accuracy study—Drug standards prepared in blood at four concentrations (not included in the calibration curve) were extracted in quadruplicate, and the amount of drug was quantitated, with the daily standard curve prepared in a different batch of blood and using a separate stock solution. The data were then subjected to linear regression analysis (Table 4).

TABLE 1—*Retention time and UV spectral data for drugs on reversed-phase HPLC columns.*

No.	Compound	Rt[a]	PuP[b]	Rt[c]
1	Acebutolol	4.27	237.35	13.04
2	Albuterol	3.52	223.16	7.53
3	Alprazolam	4.62	224.43	5.08
4	Anileridine	7.41	229.35	dne
5	Atenolol	3.39	222.98	7.01
6	Brotizolam	5.98	231.72	4.66
7	Bupivacaine	7.52	214.35	dne
8	Caffeine	3.19	234.25	1.89
9	Carbamazepine	4.25	233.59	2.82
10	Chlordiazepoxide	5.60	251.96	dne
11	Chlormezanone	4.13	222.79	2.92
12	Chlorpropamide	4.75	229.17	3.41
13	Chlorzoxazone	4.44	227.14	2.91
14	Cimetidine	3.49	219.23	7.12
15	Clomipramine	18.40	225.85	dne
16	Clonazepam	4.58	243.25	3.54
17	Clonidine	5.11	213.89	dne
18	Codeine	3.98	216.07	11.10
19	Cyclizine	10.07	217.67	dne
20	Demoxepam	3.99	243.50	2.67
21	Desalkylflurazepam	5.06	228.11	4.12
22	Desalkylhydroxyflurazepam	4.25	228.67	2.86
23	Desmethylchlordiazepoxide	5.14	243.19	dne
24	Diazepam	6.92	229.86	9.71
25	Dichlorisoproterenol	6.56	216.88	dne
26	Diclofenac	8.01	234.47	6.63
27	Diflunisal	4.65	229.15	4.93
28	Diltiazem	7.92	225.71	dne
29	Diphenidol	7.96	213.83	dne
30	Diphenoxylate	18.53	213.53	dne
31	Disopyramide	6.05	221.86	dne
32	Droperidol	5.56	224.83	dne
33	Encainide	5.98	241.90	dne
34	Ethyldiazepam	8.25	229.62	10.47
35	Ethylmorphine	4.37	216.14	12.51
36	Fenoprofen	6.87	217.50	5.33
37	Fenoterol	3.62	218.90	9.07
38	Fentanyl	7.40	212.29	dne
39	Floctafenine	4.81	251.28	14.15
40	Fluoxetine	10.33	220.78	dne
41	Flurazepam	8.29	228.19	dne
42	Flurbiprofen	7.14	236.59	5.49
43	Fonazine	8.16	247.18	dne
44	Furosemide	3.86	238.70	2.93
45	Glyburide	7.27	222.15	6.92
46	Haloperidol	7.80	228.65	dne
47	Hydrocodone	4.71	215.09	15.14
48	Hydromorphone	3.72	215.95	9.37
49	Hydroxyethylflurazepam	4.60	229.06	3.59
50	Hydroxyzine	8.99	221.71	dne
51	Ibuprofen	9.10	216.94	5.92
52	Indomethacin	7.75	242.16	6.66
53	Ketazolam	6.92	229.83	5.93
54	Ketazolam[d]	7.35	226.20	8.73
55	Ketoprofen	5.36	239.92	3.96
56	Labetalol	4.86	216.09	dne
57	Levorphanol	5.16	223.54	dne
58	Lidocaine	5.64	214.15	dne
59	Lorazepam	4.51	225.94	3.07
60	Mefenamic Acid	11.57	236.66	8.22
61	Mepivacaine	5.54	214.25	dne
62	Metaproterenol	3.45	216.46	7.52
63	Methocarbamol	3.34	224.23	2.05
64	Methotrexate	3.02	272.57	6.16

TABLE 1—*Continued*

No.	Compound	Rt[a]	PuP[b]	Rt[c]
65	Methoxamine	4.41	233.64	12.35
66	Methyldopa	2.72	223.63	4.05
67	Methylphenidate	6.12	212.88	dne
68	Metoclopramide	5.88	249.66	dne
69	Metolazone	4.08	234.82	2.90
70	Metoprolol	4.91	220.92	dne
71	Morphine	3.50	215.52	7.38
72	Nadolol	3.61	217.46	8.34
73	Nalbuphine	4.02	213.05	13.09
74	Naloxone	3.86	215.29	11.17
75	Naproxen	5.47	226.97	3.95
76	Nifedipine	5.65	243.02	4.47
77	Nifedipine[d]	5.99	243.21	5.56
78	Nitrazepam	4.47	249.12	4.49
79	Nordiazepam	5.43	228.56	8.69
80	Norepinephrine	3.21	226.14	5.51
81	Norfluoxetine	9.62	220.95	dne
82	Oxazepam	4.41	229.01	3.07
83	Oxprenolol	5.74	222.80	dne
84	Oxycodone	4.27	215.23	11.81
85	Oxymetazoline	8.80	217.42	dne
86	Pentazocine	6.36	222.57	dne
87	Pentoxifylline	3.34	232.83	2.04
88	Pericyazine	7.10	249.42	dne
89	Phenolphthalein	4.09	224.20	3.19
90	Phenylbutazone	9.71	230.11	dne
91	Pimozide	10.19	215.29	dne
92	Pindolol	4.42	219.62	dne
93	Pipotiazine	6.55	249.92	dne
94	Piroxicam	5.17	288.03	4.66
95	Pramoxine	10.64	227.33	dne
96	Prazepam	10.31	229.38	14.14
97	Prazosin	4.70	249.53	dne
98	Procainamide	4.04	270.94	14.97
99	Propranolol	6.78	221.30	dne
100	Quazepam	14.06	257.82	13.83
101	Scopolamine	4.17	213.01	dne
102	Sotalol	3.79	226.27	8.95
103	Strychnine	5.18	232.01	dne
104	Sulfinpyrazone	4.13	238.52	4.47
105	Sulindac	4.07	266.87	3.21
106	Temazepam	5.41	230.38	3.99
107	Terbutaline	3.56	216.61	8.36
108	Tetracaine	9.97	303.06	dne
109	Thiopental	5.62	279.55	4.12
110	Thymol	8.10	220.77	5.48
111	Timolol	4.53	290.06	dne
112	Tocainide	4.41	213.97	11.16
113	Tolbutamide	5.14	225.91	3.72
114	Tolmetine	4.94	293.08	3.49
115	Trazodone	5.79	222.61	dne
116	Triamterene	4.40	269.57	13.63
117	Triazolam	4.70	221.55	4.07
118	Triiodothyroacetic Acid	6.29	222.88	6.03
119	Verapamil	8.93	229.12	dne
120	Warfarin	6.43	239.31	5.25
121	Zomepirac	5.71	281.35	4.35

[a]APEX Octadecyl (I), particle size 5 μm, 25 cm by 4.6 mm ID.

[b]Broad-range purity parameter (PuP), 210 to 367 nm on Column (I).

[c]Waters μPhenyl (II), particle size 5 μm, 15 cm by 3.9 mm ID. dne = did not elute on this column within 15 min. Rt = retention time.

[d]Secondary peak.

TABLE 2—*Within-day precision for selected basic drugs in blood (values shown the mean ± SD; n = 4).*

No.	Drug	Low Concentration		High Concentration		Recovery, ±5%[a]
		μg/mL	CV%	μg/mL	CV%	
1	Alprazolam	0.235 ± 0.012	5.06	2.19 ± 0.02	0.75	34.1
2	Codeine	0.420 ± 0.010	2.29	4.40 ± 0.06	1.43	78.7
3	Diazepam	0.117 ± 0.003	2.21	1.63 ± 0.07	4.31	83.7
4	Haloperidol	0.414 ± 0.010	2.48	4.49 ± 0.06	1.36	79.5
5	Nordiazepam	0.117 ± 0.005	3.96	1.67 ± 0.01	0.73	89.6
6	Pimozide	0.345 ± 0.012	3.39	3.64 ± 0.09	2.60	58.7
7	Propranolol	0.207 ± 0.007	3.40	2.17 ± 0.05	2.15	82.8
8	Temazepam	0.056 ± 0.002	2.70	0.84 ± 0.02	1.88	90.7
9	Trazodone	0.410 ± 0.018	4.46	4.34 ± 0.05	1.09	81.1
10	Verapamil	0.394 ± 0.015	3.73	4.09 ± 0.10	2.54	60.3

[a]Average of eight determinations, based on the low and high concentration.
SD = standard deviation.
CV% = coefficient of variation.

Results and Discussion

To increase the level of confidence in quantitative analysis, an HPLC retention index database with a UV spectral database was established on two columns using different mobile phases. Table1 lists the retention times on the APEX ODS and μPhenyl columns. Purity parameters in the acidic mobile phase (Column I) are also included. Recycling the mobile phase permitted unattended operation for several weeks, without the need to make up new batches of mobile phase. No increase in background noise has been encountered, even after several hundred samples. The HPLC system was calibrated and verified at the beginning of the analyses using a stock solution containing 12 (14 for the μPhenyl column) selected drugs at a concentration of 0.4 mg/mL (stored at −20°C). For the test injection, 10 μL was added to 2.0 mL of mobile phase and then 25 μL of the mixture was injected onto the column. The two test groups are illustrated in Fig. 1. The system stability and validity of the library database were verified by noting the retention times of codeine, haloperidol, verapamil, and phenylbutazone. Shifts in the retention times of these four drugs are characteristics of changes in the molarity or pH of the mobile phase. Using an isocratic reversed-phase system, retention times have been shown

TABLE 3—*Between-day precision for selected basic drugs in blood (values shown mean ± SD; n = 12).*

No.	Drug	Low Concentration		High Concentration	
		μg/mL	CV%	μg/mL	CV%
1	Alprazolam	0.231 ± 0.010	4.47	2.24 ± 0.06	2.84
2	Codeine	0.438 ± 0.024	5.38	4.29 ± 0.17	4.03
3	Diazepam	0.115 ± 0.002	2.21	1.60 ± 0.06	3.92
4	Haloperidol	0.421 ± 0.018	4.18	4.35 ± 0.18	4.14
5	Nordiazepam	0.112 ± 0.007	6.57	1.54 ± 0.03	1.84
6	Pimozide	0.379 ± 0.033	8.62	3.87 ± 0.27	7.08
7	Propranolol	0.215 ± 0.009	3.95	2.05 ± 0.11	5.17
8	Temazepam	0.055 ± 0.001	2.70	0.80 ± 0.04	4.68
9	Trazodone	0.428 ± 0.025	5.88	4.22 ± 0.12	2.73
10	Verapamil	0.202 ± 0.021	5.24	4.04 ± 0.10	2.41

SD = standard deviation.
CV% = coefficient of variation.

TABLE 4—*Accuracy of determination for selected basic drugs using linear regression data.*[a]

No.	Compound	*n*	Linear Regression, $Y = a + bX \pm$ (SE)	r^{2b}
1	Alprazolam[c]	16	$Y = -0.003 + 1.149X \pm 0.019$	0.9962
2	Codeine[d]	16	$Y = -0.004 + 1.107X \pm 0.010$	0.9988
3	Diazepam[e]	20	$Y = 0.002 + 1.020X \pm 0.005$	0.9995
4	Haloperidol[d]	16	$Y = -0.005 + 1.113X \pm 0.017$	0.9967
5	Nordiazepam[e]	20	$Y = -0.001 + 0.998X \pm 0.009$	0.9985
6	Pimozide[d]	16	$Y = 0.006 + 0.924X \pm 0.013$	0.9971
7	Propranolol[c]	16	$Y = -0.005 + 1.174X \pm 0.018$	0.9967
8	Temazepam[f]	20	$Y = 0.001 + 1.002X \pm 0.003$	0.9980
9	Trazodone[d]	16	$Y = -0.003 + 1.064X \pm 0.009$	0.9990
10	Verapamil[d]	16	$Y = 0.003 + 0.982X \pm 0.008$	0.9989

[a]Abbreviations: Y = drug concentration (found); a = Y intercept; b = slope; X = drug concentration (added); and SE = standard error of coefficient.
[b]r^2 = coefficient of determination.
[c]Blood concentration = 0.2, 0.4, 1.6, and 2.0 μg/mL.
[d]Blood concentration = 0.4, 0.8, 1.6, and 4.0 μg/mL.
[e]Blood concentration = 0.1, 0.2, 0.4, 0.8, and 1.6 μg/mL.
[f]Blood concentration = 0.05, 0.1, 0.2, 0.4, and 0.8 μg/mL. Each concentration was analyzed in quadruplicate.

to be reproducible within the same instrument and on the same column. For retention times, we found a median variation coefficient of 1.37% (range, 0.12 to 3.67%) in a total of 126 measurements, using 18 drugs on Column I (14 on Column II) and extended over a year. No study has yet been undertaken to determine the variation of two columns of the same brand.

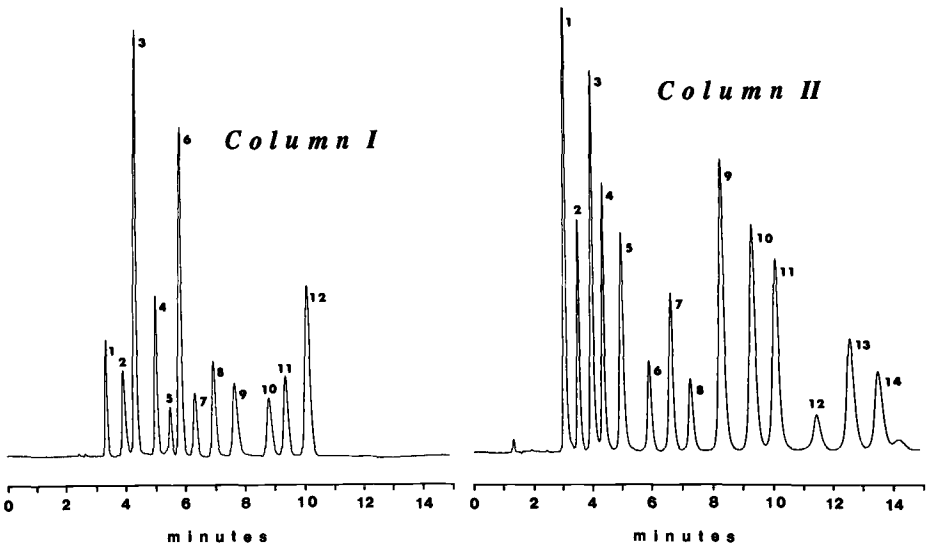

FIG. 1—*Chromatograms obtained with standard solutions containing a mixture of drugs: Column I peaks: (1) atenolol, (2) codeine, (3) oxazepam, (4) tolbutamide, (5) thiopental, (6) brotizolam, (7) warfarin, (8) flurbiprofen, (9) haloperidol, (10) verapamil, (11) phenylbutazone, and (12) prazepam. Column II peaks: (1) oxazepam, (2) clonazepam, (3) temazepam, (4) nitrazepam, (5) alprazolam, (6) methotrexate, (7) indomethacin, (8) salbutamol, (9) nordiazepam, (10) diazepam, (11) ethyldiazepam, (12) oxycodone, (13) acebutolol and (14) floctafenine. Each peak corresponds to 50 ng on Column I and 25 ng on Column II. The peaks were monitored at 229 nm on both columns.*

Numerous sample preparations (single liquid/liquid extraction, solid-phase extraction, on-column injection of filtered plasma and urine samples, and protein precipitation) have been reported for the HPLC analysis of a variety of drugs in serum, plasma, or urine [*16–22*]. The application of these methods, however, failed to give interference-free chromatograms when employed with whole blood. Therefore, a systematic scheme of extraction was developed which eliminated this problem (Fig. 2). Toluene was found to be the most suitable solvent for extraction of basic drugs [*1*]. When this technique is coupled with the double acid back-extraction (0.2*N* H_2SO_4 and 6*N* HCl), a virtually interference-free chromatogram is obtained for both fractions. The additional advantage of this procedure is its increased specificity for certain basic drugs.

The extraction characteristics of eleven drugs are illustrated in Fig. 3. Ethyldiazepam (synthesized in this laboratory) is included in this study as it can be used as an internal standard for quantitative analyses of certain benzodiazepines. Two group standards were used to spike drug-free blood. The concentration of each drug was 2 μg/mL in Group I

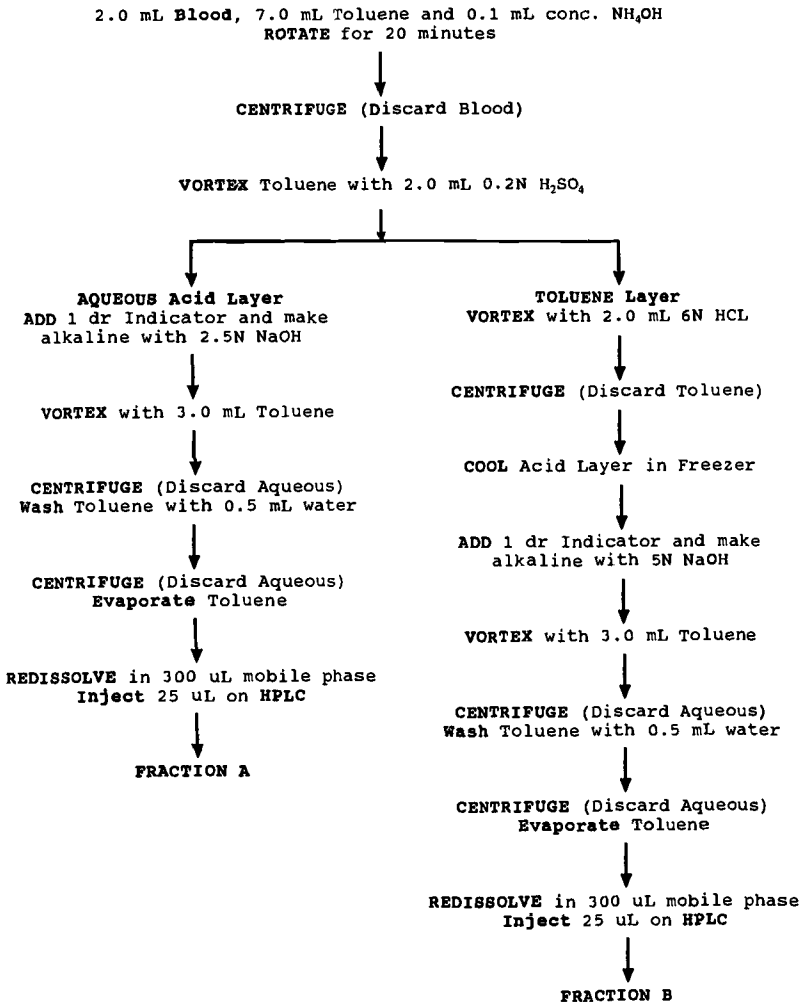

FIG. 2—*Flow diagram of the method for basic drug extraction from blood.*

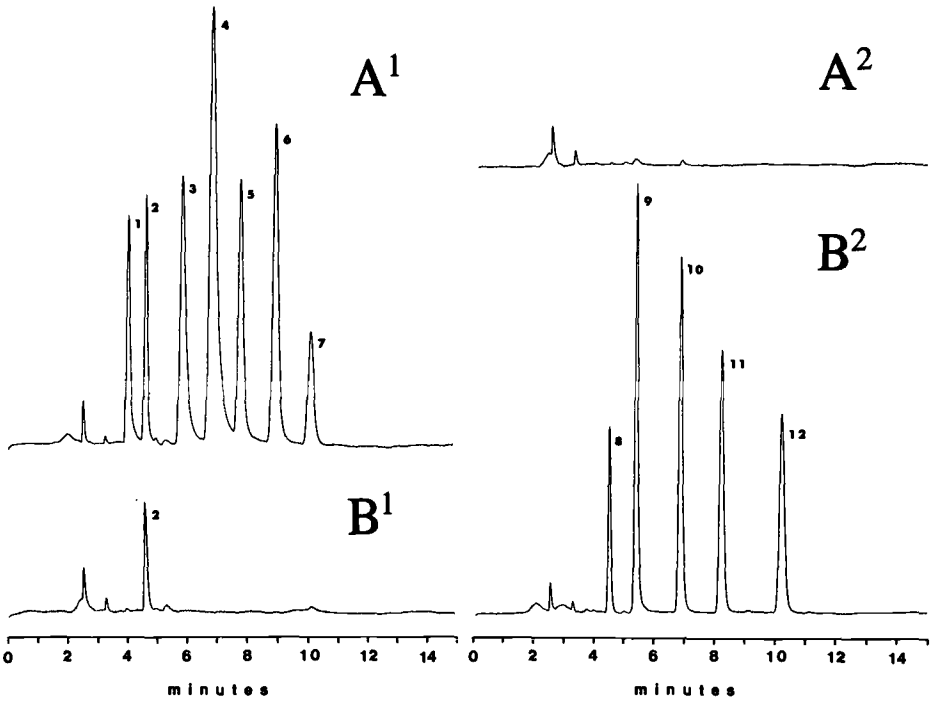

FIG. 3—*Chromatograms obtained from extracts of two spiked blood samples: (A^1) and (A^2), diluted sulfuric acid fractions; (B^1) and (B^2); 6N HCl fractions. Peaks: (1) codeine, (2) alprazolam, (3) trazodone, (4) propranolol, (5) haloperidol, (6) verapamil, (7) pimozide, (8) lorazepam, (9) temazepam, (10) diazepam, (11) ethyldiazepam, and (12) prazepam.*

(chromatograms A^1 and B^1) and 1 μg/mL in Group II (chromatograms A^2 and B^2). Strong bases like codeine are efficiently back-extracted in 0.2*N* H_2SO_4, and the separation of the five benzodiazepines (Group II) was possible by using 6*N* HCl in the subsequent step of the procedure. Alprazolam and nordiazepam are distributed into both fractions; the rations between diluted sulfuric and strong acid were 2.2 to 1 for alprazolam and 1.1 to 1.0 for nordiazepam.

The chromatographic peaks can be identified and their purity can be confirmed with a high level of confidence. Identification of unknown drugs can be carried out concurrently with quantitation of a known drug in the same chromatogram. The multicomponent analysis and increased specificity of the method are illustrated in Figs. 4*a*, 4*b*, and 5. The concentrations of metoprolol and propranolol were determined in this blood sample on the APEX ODS column (Figs. 4*a* and 4*b*). The other drugs were identified using the automatic library search. Initial back-extraction into diluted sulfuric acid excluded the co-extraction of diazepam (Fig. 5), which would have resulted in an error in quantitation and a distortion of the UV spectrum of propranolol (Fig. 6).

To avoid potential drug or metabolite interference when using the APEX column, an alternative phenyl column with different selectivity was required. Figure 7 shows the successful separation of temazepam, nordiazepam, and diazepam. In spite of the use of selective extraction, the quantitation of temazepam cannot be carried out on the APEX ODS column when nordiazepam is present.

The identity of a chromatographic peak was considered definite only when both its retention time and the PuP matched those of a reference compound in the library file.

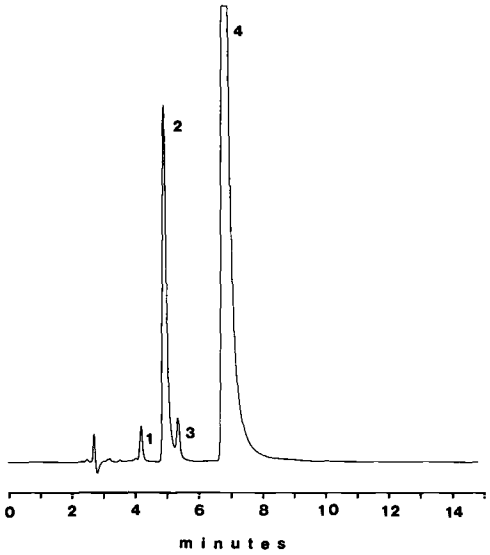

FIG. 4*a*—*Chromatogram obtained from an extract of a forensic blood sample—diluted sulfuric acid fraction: (1) carbamazepine, (2) metoprolol, (3) nordiazepam, and (4) propranolol.*

Using the described method and HPLC system, the purity parameter of a specific drug never varied by more than 0.5 nm. Figure 8 shows the purity parameter statistics report for propranolol. The seven spectra represent pure standard (library spectrum), spiked blood standards, and evidentiary blood samples collected over a period of six months. The concentration of pure standard was 2 μg/mL, and the blood propranolol concentrations ranged between 0.25 and 16 μg/mL. The concentration differences between the target spectrum and the library spectrum did not influence the match quality. The PuP mean and standard deviation, as well as the best and worst similarity and dissimilarity for the seven spectra, were within the established criteria (PuP, ±0.5 nm; similarity range, 1.0 to 0.998; and dissimilarity range, 0.0 to 0.06). Using the extraction characteristics of the drug, together with the library search, enhances the significance of comparing spectral data.

The within-day reproducibility and recovery results for selected drugs are listed in Table 2. The low recovery of alprazolam is probably due to poor extraction efficiency when using toluene as the extraction solvent. For practical purposes, however, it is sufficient in most cases to allow detection and quantitation of low toxic concentrations that have importance in forensic toxicology. With the exception of this drug, the percentages of recovery of the drugs varied from 58.7 to 90.7%. The coefficient of variation for within-day reproducibility was less than 5.1% for low and 4.4% for high concentration.

Excellent precision of measurement was achieved for the between-day variation of the assay. The results for a selected list of commonly quantitated drugs are given in Table 3. The coefficient of variation of three repeated analyses for low and high concentrations was less than 8.7 and 7.1%, respectively. These values take on more significance when one considers that they were obtained over a three-month period by three different analysts.

The linearity (peak area versus concentration) of response for basic drugs was tested in blood. Statistical analysis of the data by linear regression indicated excellent linearity up to 16-fold. The linearity of the overall procedure for four representative drugs is illustrated in Fig. 9.

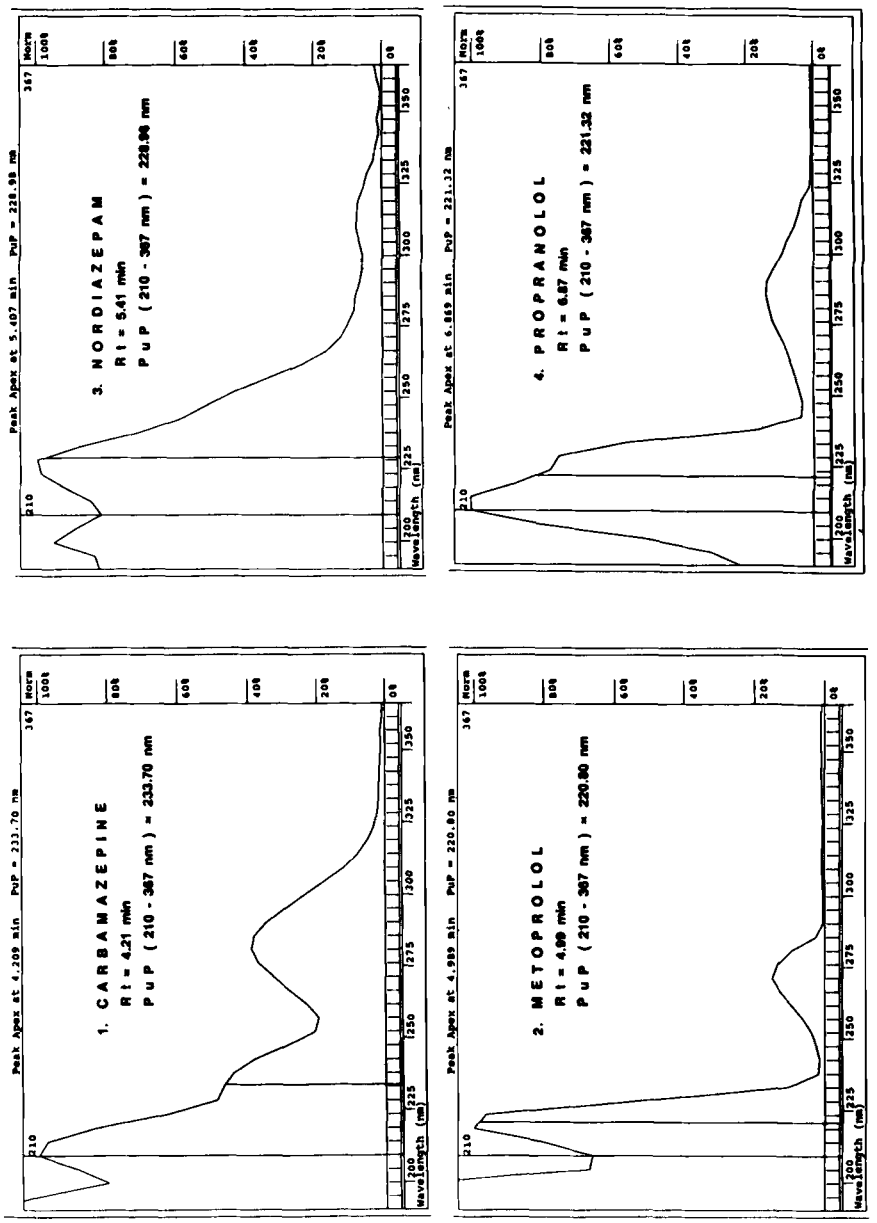

FIG. 4b—*UV spectra of the sample in Fig. 4a.*

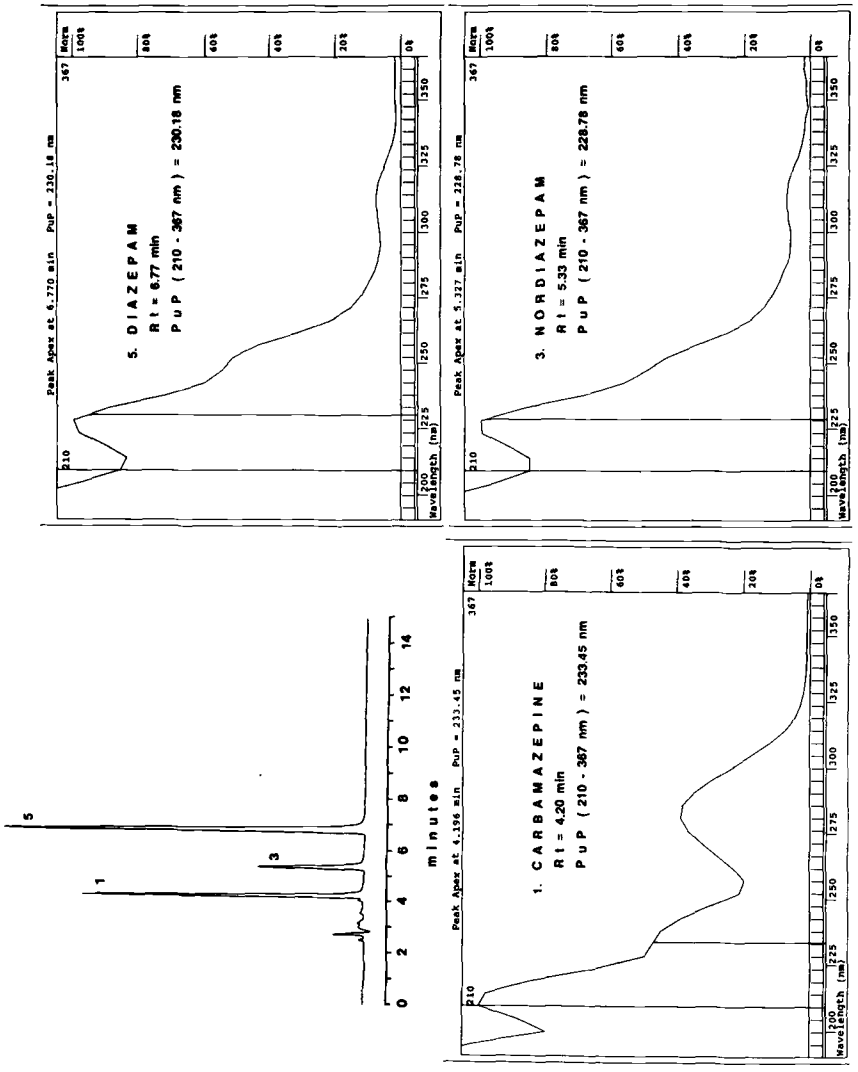

FIG. 5—*Chromatogram and UV spectra obtained from an extract of a forensic case blood sample (the same case blood as in Fig. 4)—6N HCl acid fraction: (1) carbamazepine, (3) nordiazepam and (5) diazepam.*

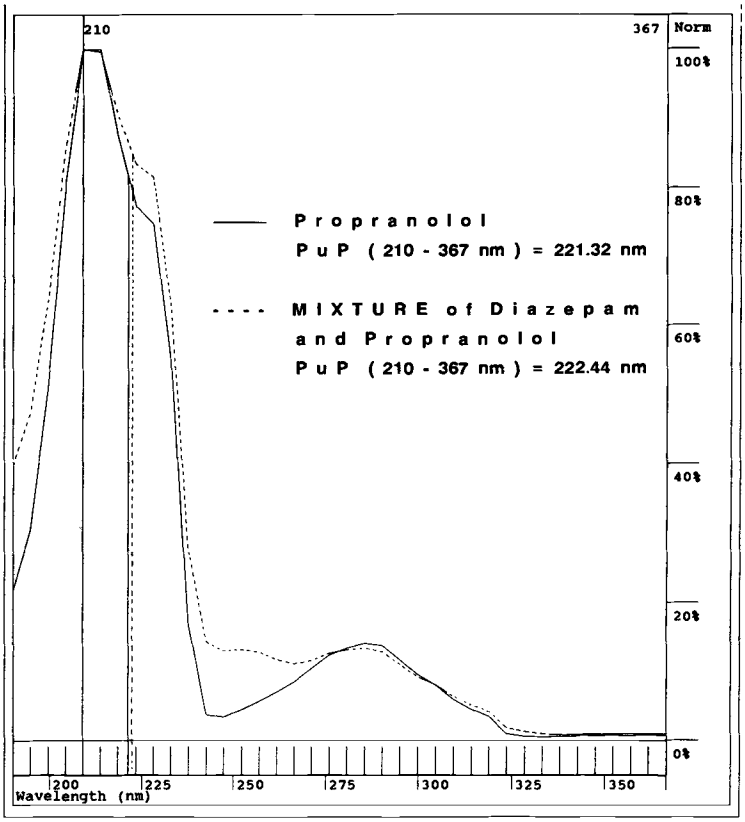

FIG. 6—*Co-elution of propranolol and diazepam.*

Replicate analyses of blood samples to which known amounts of drugs were added demonstrated that the method has acceptable accuracy. Accuracy data for ten drugs are given in Table 4 as actual values determined for four concentrations in quadruplicate and as parameters in the linear regression equation of calculated values versus theoretical concentrations. The coefficients of determination (r^2) for all ten drugs were greater than 0.99 and their slopes were close to unity.

The detection limit varied from drug to drug as a function of the molar extinction coefficient of the drug analyzed. In pure solutions, the detection limit, based on a signal-to-noise ratio of 5, was approximately 2.5 ng/25 μL for most of the drugs studied. However, the limit of quantitation (least concentration of drug giving a distinguishable UV spectrum) of the assay using 2.0 mL of blood was established at about 50 ng/mL. For better sensitivity, up to threefold, the final residue may be dissolved in 100 μL of mobile phase.

Conclusions

An automated HPLC method is described, which enables the determination of several basic drugs in postmortem blood. We have developed a simple isocratic mobile phase to establish a stable and reliable HPLC system for qualitative and quantitative work. The ability of the system to separate individual drugs is not as efficient as that of gradient

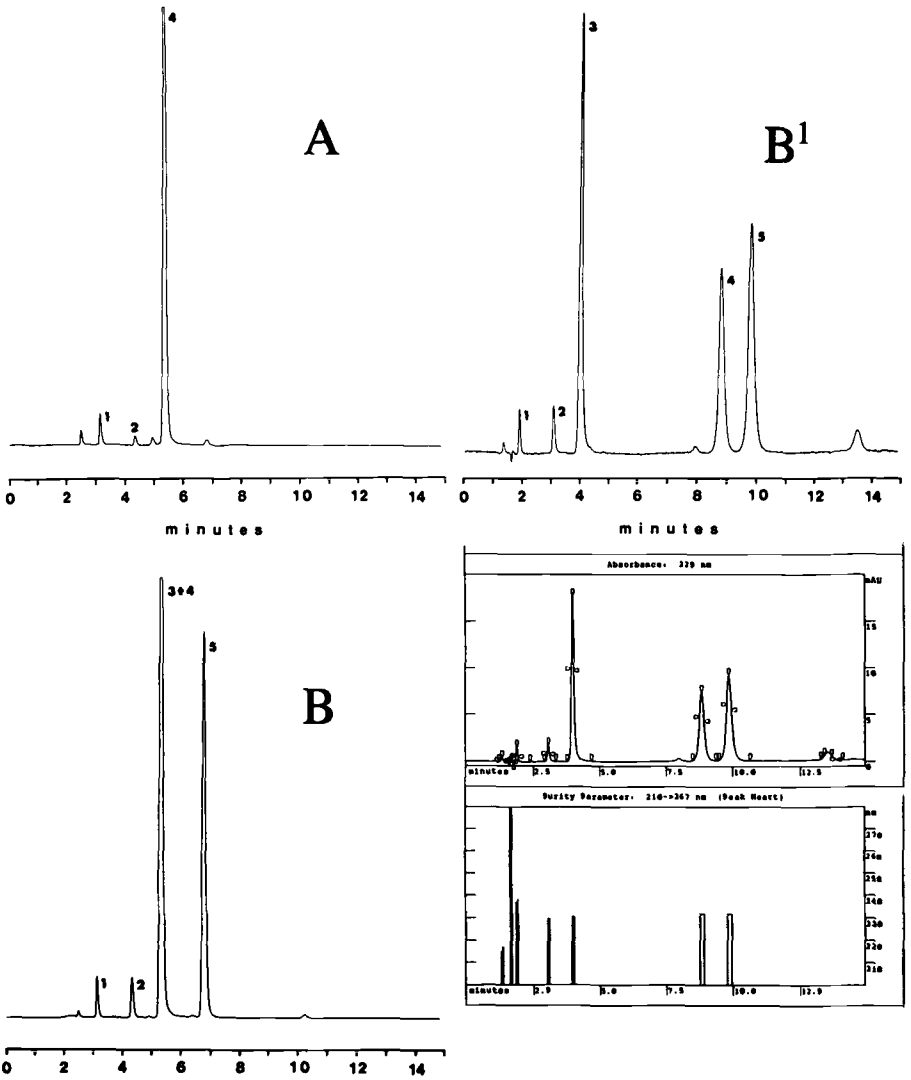

FIG. 7—*Chromatogram and PolyView double report of a case blood extract: (A) diluted sulfuric acid fraction on Column I; (B) and (B¹) 6N HCl acid fraction on Column I and Column II, respectively. Peaks: (1) caffeine, (2) possible metabolite of temazepam, (3) temazepam, (4) nordiazepam, and (5) diazepam.*

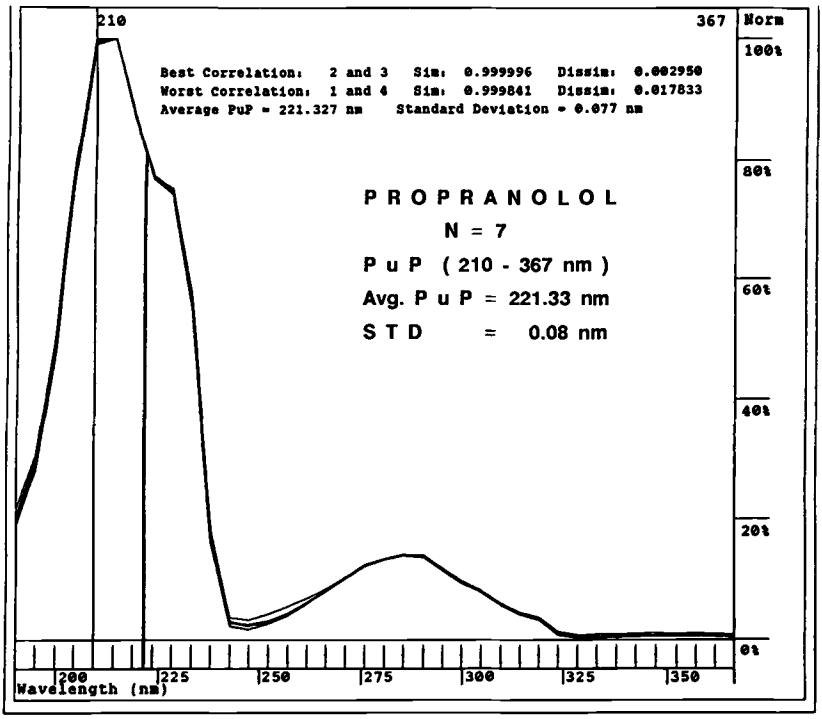

FIG. 8—*Spectral overlay and purity parameter (PuP) statistics for propranolol in methanol and in blood at different concentrations.*

elution, but it is suitable for the detection of over 100 drugs in 15 min. It was demonstrated that the back-extraction procedure resulted in clean extracts so that spectra obtained by photodiode array detection can be used for identification. Using the extraction characteristics of a drug, together with the library search, allows more confidence in determining the identity of a drug.

In conclusion, photodiode array detection, together with the extraction method described, is a very useful technique for qualitative and quantitative determination of multicomponent blood samples in forensic toxicology. Quantitative analysis proved to be precise, accurate, and sensitive, permitting an automated library search and a purity survey. It is our intention to expand the drug list as new drugs and metabolites are received and adapt the method to other basic drugs which are included in the database.

Acknowledgments

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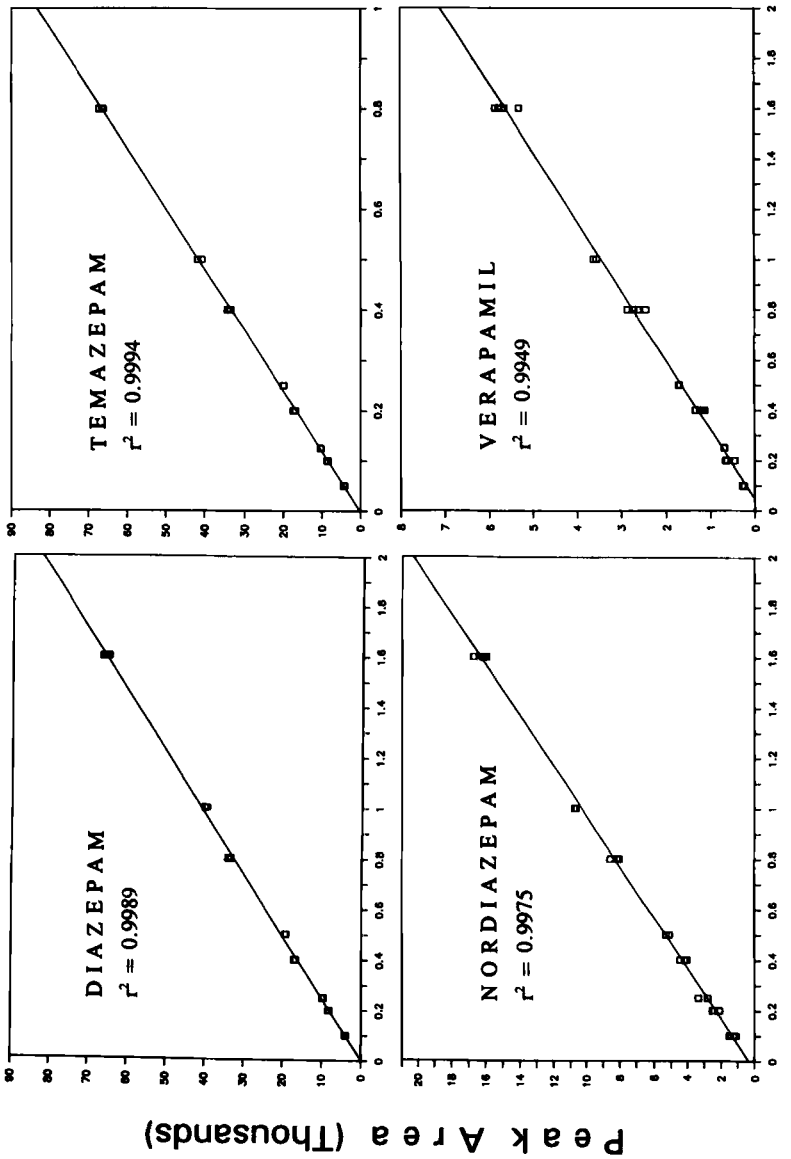

FIG. 9—*Linearity of the overall procedure.*

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