

# Analysis of Basic Drugs in Postmortem Blood by HPLC with Diode Array Detection<sup>1</sup>

# LC

## Varian Application Note

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### Introduction

Forensic toxicologists are routinely confronted with the difficult problem of detecting and quantitating a wide range of drugs in postmortem blood. Gas chromatography/ mass spectrometry (GC/MS) may be used for the analysis of many basic drugs, but there are some highly polar drugs, such as temazepam, verapamil, or trazodone, which are not suited to this technique. For such compounds, high performance liquid chromatography (HPLC) together with diode array detection is an excellent alternative.

### Experimental

**Instrumentation:** A Varian LC Star System was used: Model 9010 ternary gradient pump; Model 9095 AutoSampler; Polychrom® 9065 Diode Array Detector; Star HPLC Workstation with software for system control and quantitative reports; and PolyView™ spectral processing software for automated post run spectral evaluation. Chromatography was carried out isocratically at ambient temperature on one of two reversed-phase columns using different filtered, degassed mobile phases.

**Column I:** 5 mm, APEX ODS 25 cm x 4.6 mm ID

**Mobile phase (Column I):** 0.8 mL/min flow rate; acetonitrile/0.025% H<sub>3</sub>PO<sub>4</sub> (v/v)/triethylamine buffer (1% v/v in water) at pH 3.4 (25:10:5 v/v/v).

**Column II:** 5 mm, m-Phenyl, 15 cm x 3.9 mm ID

**Mobile phase (Column II):** Acetonitrile/0.025% H<sub>3</sub>PO<sub>4</sub> (v/v) (50:50 v/v) at 0.6 mL/min.

**Extraction Method:** 2.0 mL of blood sample was placed in a 15-mL glass culture tube and extracted following the procedure outlined in Figure 1.

**Analysis:** For quantitative calibration, drugs were added to drug-free blood in the range corresponding to their therapeutic concentrations. Standard curves were constructed from duplicate samples at each concentration. Spectral libraries were created which contained compound names, Purity Parameters (PuP), retention times, and spectra of 119 drugs.

**Validation Studies:** Studies were performed to determine the linearity, recovery, accuracy, precision, and reproducibility of this analysis [refer to Reference (1)]. All validation studies were satisfactory.

### Results

To increase the level of confidence in quantitative analysis, an HPLC retention index database with a UV spectral database was established on two different columns using different mobile phases. Table 1 lists this data for 15 drugs. Retention times were reproducible, producing a median variation coefficient of 1.37% (0.12% to 3.67% range) in a total of 126 measurements for 18 drugs on column I (14 on column II) and extended over a year. The double-back acid extraction technique described in Figure 1 produced virtually interference free chromatograms for both fractions, while increasing the specificity of the analytical method itself. Figure 2 shows chromatograms obtained from (A) sulfuric acid extracts and (B) hydrochloric acid extracts from two spiked blood samples, using column I.

**Table 1. HPLC retention time and UV spectral purity parameters for some drugs<sup>2</sup>**

Abstracted from Reference (1) with permission.

Compound	Rt <sup>a</sup>	PuP <sup>b</sup>	Rt <sup>c</sup>
Alprazolam	4.62	224.43	5.08
Carbamazepine	4.25	233.59	2.82
Codeine	3.98	216.07	11.10

Diazepam	6.92	229.86	9.71
Ethylidiazepam	8.25	229.62	10.47
Haloperidol	7.8	228.65	dne
Lorazepam	4.51	225.94	3.07
Metoprolol	4.91	220.92	dne
Nordiazepam	5.43	228.56	8.69
Pimozide	10.19	215.29	dne
Prazepam	10.31	229.38	14.14
Propranolol	6.78	221.30	dne
Temazepam	5.41	230.38	3.99
Trazodone	5.79	222.61	dne
Verapamil	8.93	229.12	dne

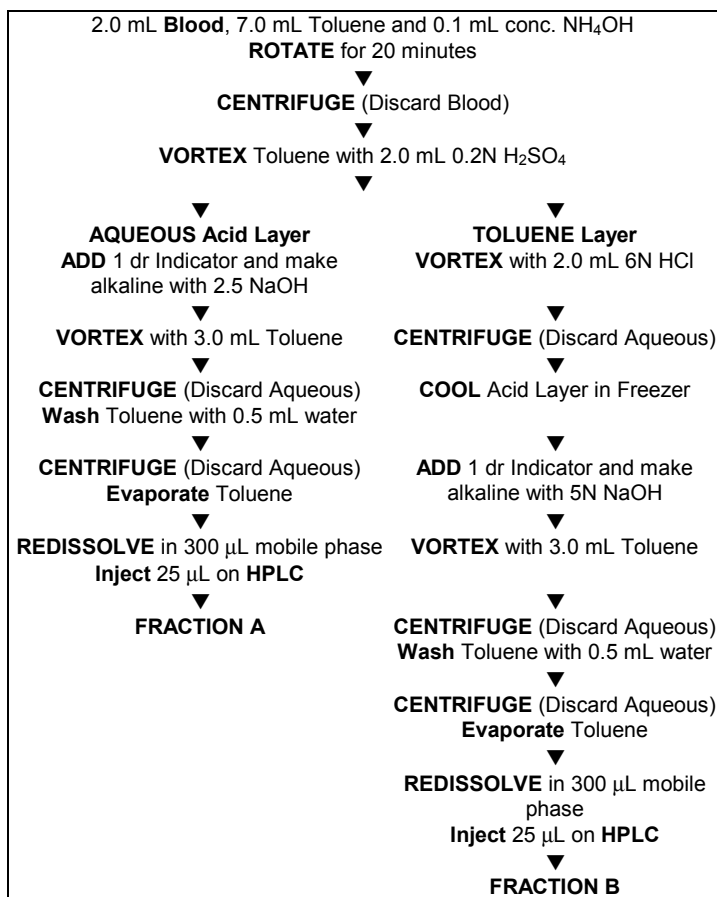
<sup>a</sup> Column I

<sup>b</sup> Broad range purity parameter (PuP). 210 to 367 nm on column I

<sup>c</sup> Column II

dne = did not elute from this column within 15 min

Rt = retention time in minutes



**Figure 1. Flow diagram of the method for basic drug extraction from blood<sup>3</sup>**

Using this technique, chromatographic peaks can be identified and their purity can be confirmed with a high level of confidence. A sample of postmortem blood was

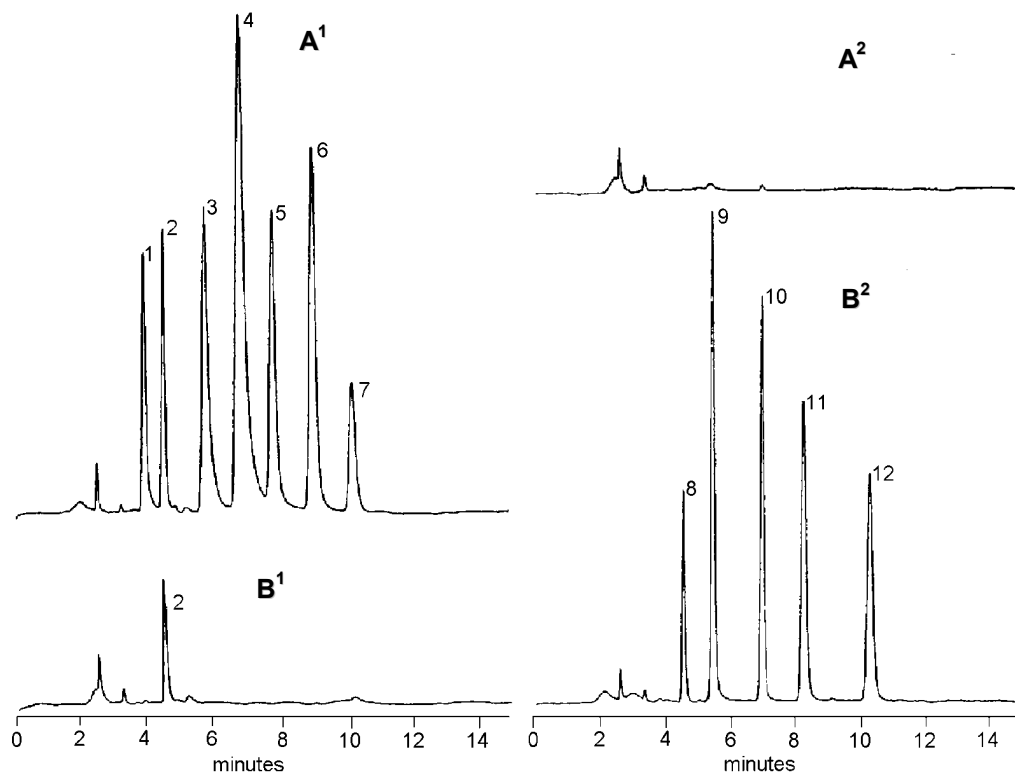
extracted using the method described in Figure 1, and the two fractions were analyzed by HPLC using column I. Figure 3 is the chromatogram of the sulfuric acid extract, and Figure 4 is the chromatogram of the hydrochloric acid extract. Note that diazepam and propranolol eluted at similar retention times and would have co-eluted had they not been separated into two different fractions during the extraction procedure. Co-elution of diazepam would have resulted in an error in quantitation and would have yielded UV spectrum consisting of both propranolol and diazepam spectra (Figure 5). The drugs in this blood sample were identified by automatic spectral library search and multicomponent analysis (MCA). For some samples, the m-Phenyl column was also required to avoid potential drug or metabolite interference with the APEX column.

The identity of a chromatographic peak was only considered definite when both its retention time and its spectral purity parameter (PuP) matched that of a reference compound in the library file. Figure 6 shows the PuP statistics report for propranolol. The seven spectra represent pure standards, spiked blood standards, and evidentiary blood samples collected over a six month period. Concentrations ranged from 0.25-16 mg/mL. The PuP mean and standard deviation, as well as the similarity and dissimilarity values for all seven spectra were well within the established limits (PuP  $\pm 0.5$  nm; similarity 1.000-0.998; dissimilarity 0.00-0.06).

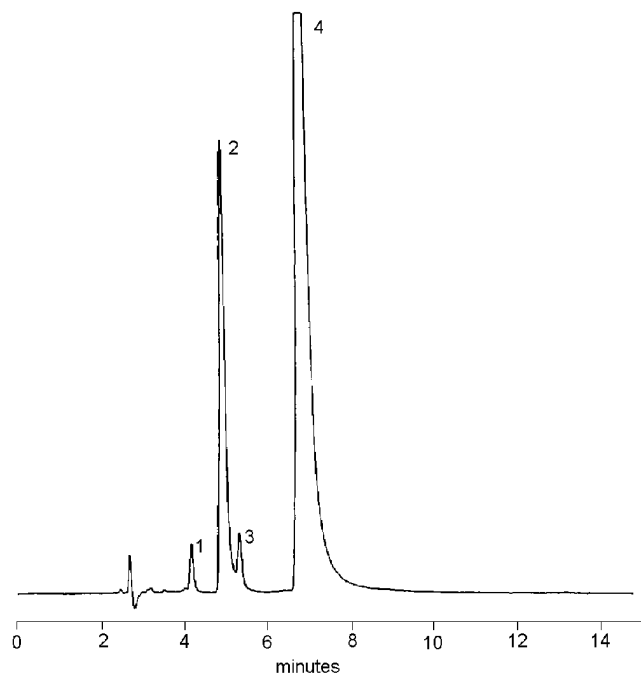
The within-day reproducibility and recovery results for selected drugs are listed in Table 2. With the exception of alprazolam, the recovery percentages varied from 58.7-90.7% and the coefficient of variation was less than 5.1% for low and 4.4% for high concentrations. A 16-fold linearity of response was achieved for basic drugs in blood using this method. The detection limit, determined at a signal-to-noise of 5, varied from drug to drug. In pure solutions, it was approximately 2.5 ng/25 mL injection for most of the drugs studied, while in blood, the detection level was 50 ng/mL using a final residue volume of 300 mL. For better sensitivity, the final residue may be dissolved in 100 mL of mobile phase.

<sup>2</sup>Abstracted from Table 1 in Reference (1) where this data is given for 121 drugs.

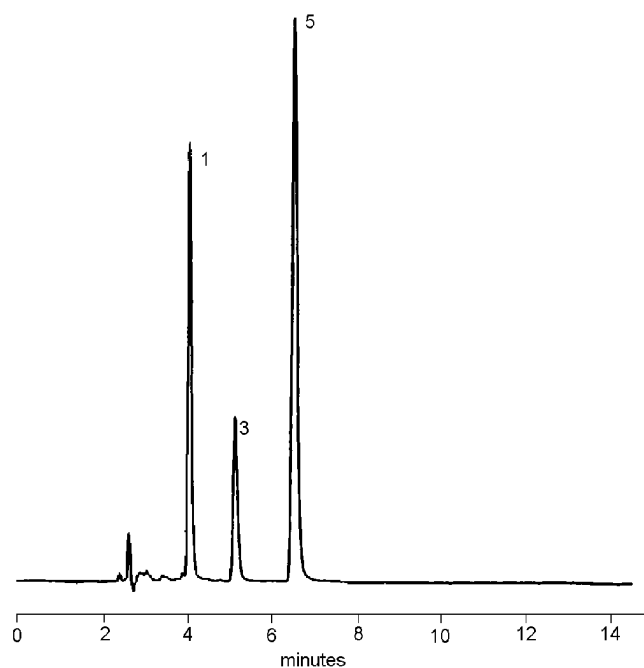
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**Figure 2. Chromatograms obtained from extracts of two spiked blood samples: (A<sup>1</sup>) and (A<sup>2</sup>), diluted sulfuric acid fractions; (B<sup>1</sup>) and (B<sup>2</sup>); 6N HCl fractions. Peaks: (1) codeine, (2) alprazolam, (3) trazodone,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



**Figure 3. Chromatogram obtained from an extract of forensic blood sample; diluted sulfuric acid fraction: (1) carbamazepine, (2) metoprolol, (3) nordiazepam, and (4) propranolol<sup>3</sup>**



**Figure 4. Chromatogram obtained from an extract of a forensic case blood sample (the same blood as in Figure 3); 6N HCl acid fraction: (1) carbamazepine, (3) nordiazepam, and (5) diazepam<sup>3</sup>**

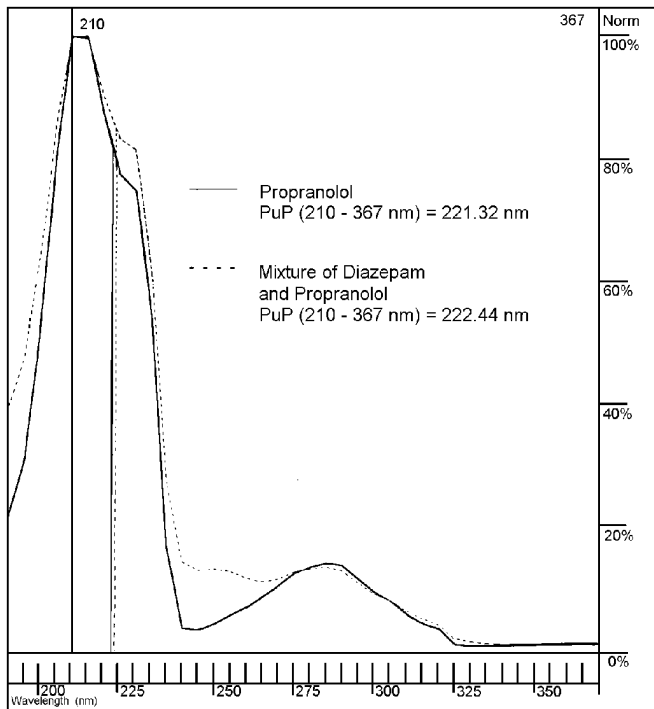


Figure 5. Co-elution of propranolol and diazepam<sup>3</sup>

Table 2. Within-day precision for selected basic drugs in blood (values show the mean  $\pm$ SD; n=4)<sup>3</sup>

Drug	Low Concentration		High Concentration		Recovery $\pm$ 5% <sup>a</sup>
	$\mu$ g/mL	CV%	mg/mL	CV%	
Alprazolam	0.235 $\pm$ 0.012	5.06	2.19 $\pm$ 0.02	0.75	34.1
Codeine	0.420 $\pm$ 0.010	2.29	4.40 $\pm$ 0.06	1.43	78.7
Diazepam	0.117 $\pm$ 0.003	2.21	1.63 $\pm$ 0.07	4.31	83.7
Haloperidol	0.414 $\pm$ 0.010	2.48	4.49 $\pm$ 0.06	1.36	79.5
Nordiazepam	0.117 $\pm$ 0.005	3.96	1.67 $\pm$ 0.01	0.73	89.6
Pimozide	0.345 $\pm$ 0.012	3.39	3.64 $\pm$ 0.09	2.60	58.7
Propranolol	0.207 $\pm$ 0.007	3.40	2.17 $\pm$ 0.05	2.15	82.8
Temazepam	0.056 $\pm$ 0.002	2.70	0.84 $\pm$ 0.02	1.88	90.7
Trazodone	0.410 $\pm$ 0.018	4.46	4.34 $\pm$ 0.05	1.09	81.1
Verapamil	0.394 $\pm$ 0.015	3.73	4.09 $\pm$ 0.10	2.54	60.3

<sup>a</sup> Average of 8 determinations, based on the low and high concentration  
SD = standard deviation  
CV% = coefficient of variation

## Conclusions

An automated HPLC system with diode array detection has been described for the determination of several basic drugs in postmortem blood. The simple isocratic mobile phase produced 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 elution, but it is suitable for the detection of over 100 drugs in 15 minutes. It was demonstrated that the back-extraction procedure resulted in clean extracts, so that the spectra obtained by photo diode array detection can be used for identification. The extraction characteristics of a drug together with its library search, allows more confidence in determining the identity of each drug.

## Reference

1. E. M. Koves and J. Wells, J. of Forensic Sciences, JFSCA, Vol. 37, No. 1 (1992)

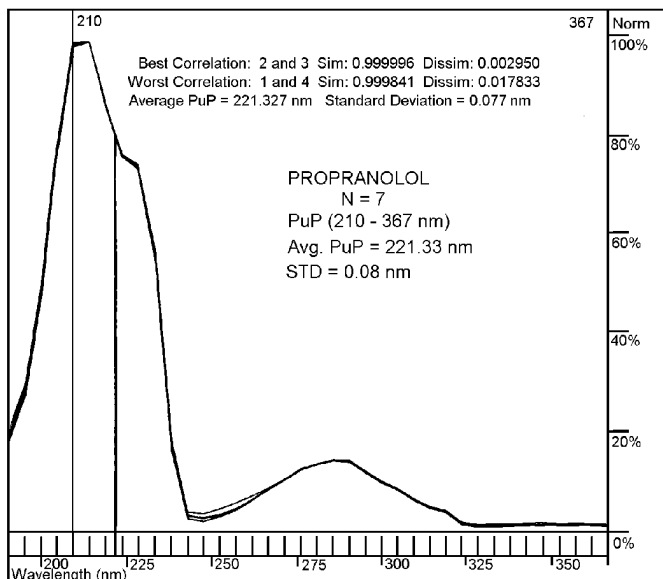


Figure 6. Spectral overlay and purity parameter (PuP) statistics for propranolol in methanol and in blood at different concentrations<sup>3</sup>

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