Journal of Chromatography, 185 (1979) 505–513

CHROM. 12,036

APPLICATION OF AN AUTOMATED EXTRACTOR/CONCENTRATOR TO THE ANALYSIS OF ANTICONVULSANT DRUGS IN BLOOD SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

REED C. WILLIAMS and JOANNE L. VIOLA
Experimental Station E 334, E. I. DuPont de Nemours, Wilmington, Del. 19898 (U.S.A.)

SUMMARY

An automated extractor/concentrator has been used to extract a variety of anticonvulsant drugs from blood serum in a concentration range from 0.075 to 80 μg/ml. The volume of serum required varies from 0.1 to 2.0 ml, depending on the concentration of drug. The average absolute extraction recovery is 90% larger; recoveries relative to an internal standard are greater than 95%. The serum extracts were analyzed by high-performance liquid chromatography on a reversed-phase column with an acetonitrile and phosphate buffer mobile phase. Comparison of results with gas chromatographic and immunoassay techniques was excellent.

INTRODUCTION

High-performance liquid chromatography (HPLC) is an accurate and precise technique for the analysis of therapeutic drugs in physiological fluids. A number of liquid chromatographic methods have been reported in the literature for the determination of the anticonvulsant drugs in blood serum. Although some of these methods used silica[1,2] or anion-exchange columns[3] for separation of the drugs, most recent methods recommend the reversed-phase chromatographic technique with octyl- or octadecyl-bonded packings[4–8]. The major differences between these methods were usually not in the chromatography but in the serum sample preparation procedure. However, the accuracy and precision of any chromatographic analysis is as dependent on the sample preparation procedure as on the chromatographic separation. Often the sample preparation is the most laborious and least accurate part of the analytical procedure.

Of the various sample preparation methods, liquid–liquid extraction with non-polar, water-insoluble organic solvents has been most widely used[1–5]. This method can extract small concentration of drugs from large volumes of serum into a concentrated clean extract sample that can be injected into a chromatograph. However, liquid–liquid extraction of serum samples can be laborious and is complicated by emulsion formation.

Drug extraction on to a charcoal sorbent has also been used as a sample preparation method[6]. The resultant extracts are clean and can be concentrated, but the

procedure is time-consuming and typically gives extractions efficiencies ranging from only 40 to 75%[6].

More recently, serum protein precipitation techniques have been used for sample preparation for reversed-phase liquid chromatography[7,8]. Although fast and convenient, this method can cause extensive column contamination which may require vigorous column washing procedures with solvents such as acetonitrile and dimethyl sulfoxide[7,8]. This method is not readily adaptable to the analysis of trace amounts of drugs in large volumes of serum because the samples cannot be concentrated.

This paper describes a method for the preparation and analysis of a variety of anticonvulsant drugs and metabolites using the reversed-phase HPLC method. The drugs are extracted from serum with an automated extractor/concentrator; the resulting extracts are clean and dry and can be readily reconstituted into solvent for analysis by HPLC or other analytical techniques.

METHODS AND MATERIALS

Drug standards

Pure drug samples were obtained from the original manufacturer or from chemical suppliers: alphenal (Ganes, New York, N. Y., U.S.A.), nitrazepam and clonazepam (Hoffmann-La Roche, Nutley, N.J., U.S.A.), primidone (Ayerst, New York, N. Y., U.S.A.), carbamazepine and carbamazepin-10,11-epoxide (Ciba-Geigy, Summit, N. J., U.S.A.), phenobarbital (Smith, Kline, & French, Philadelphia, Pa., U.S.A.), N-desmethylmethsuximide (Supelco, Bellefonte, Pa., U.S.A.) and phenytoin (Parke-Davis, Ann Arbor, Mich., U.S.A.). Stock solutions of each drug, 1 mg/ml, were made up in methanol for further use.

Chemicals

Reagent grade KH_2PO_4 and K_2HPO_4 (Fisher Scientific, Pittsburgh, Pa., U.S.A.) were used for preparation of buffer solutions. Spectrograde acetone, acetonitrile, and methanol were obtained from Burdick & Jackson (Muskegon, Mich., U.S.A.).

Biological samples

Fresh serum based solutions for extraction studies were prepared from freeze-dried control serum (Ortho Diagnostics, Raritan, N. J., U.S.A.). Patient serum samples were obtained from Columbia Presbyterian Medical Center (New York, N.Y., U.S.A.) and Wilmington Medical Center (Wilmington, Del., U.S.A.) and were kept frozen until use.

High-performance liquid chromatography

A DuPont Model 850 liquid chromatograph was used with DuPont UV spectrophotometer (195–365 nm) as detector. A Zorbax C_8 column (25 cm × 4.6 mm I.D.) (DuPont, Wilmington, Del., U.S.A.) was used with a mobile phase of acetonitrile–pH 5.6 buffer (mixture of 0.02 *M* KH_2PO_4 and 0.001 *M* K_2HPO_4) (35:65). The mobile phase flow-rate was 2.5 ml/min at a pressure of 2500 p.s.i.; the separations were done at room temperature.

Quantitation

The chromatograms were quantitated by peak height measurements with either a microprocessor-controlled electronic integrator (Supergrator 3 Columbia Scientific, Austin, Texas, U.S.A.) or manually with a ruler. Peak height ratios of drug over internal standard in standard solutions were used to form calibrations; drug concentrations in patient samples were then calculated by comparing the measured peak height ratios with the established calibration. Calibrations were checked daily.

Gas chromatography

Patient serum samples were acidified with 1 *N* HCl and extracted with chloroform. The extracts were derivatized with trimethylphenylammonium hydroxide and analyzed on a Perkin-Elmer 3920 B gas chromatograph equipped with a 3% OV-17 column and an organic nitrogen sensitive detector.

Enzyme immunoassay

Patient serum samples were analyzed directly with commerically available reagents available from the Syva Corp. (Palo Alto, Calif., U.S.A.). The concentration of drug is related to the change in absorbance at 340 nm. Absorbance measurements were made with a Perkin-Elmer KA 150 semiautomated spectrophotometer.

Extraction procedure

All serum samples were prepared for chromatography with a microprocessor-controlled, centrifugally based extractor concentrator (PREP™ 1, DuPont). Extraction cartridges consisting of a resin column, effluent cup, and a sample recovery cup (see Fig.1) are used in this instrument for the processing of the serum samples. The operating sequence for sample preparation with these cartridges and the extractor/concentrator consists of the following steps.

(1) The serum sample is pipetted into the cartridge reservoir. A solution of buffer containing the internal standard is also pipetted into the reservoir. The volume limit of the cartridge is 4 ml.

(2) The cartridge components (resin column, effluent cup, and recovery cup) are placed into the swinging buckets of the unique dual rotor of the extractor/concentrator. A maximum of 12 cartridges can be processed at one time.

(3) A wash solvent and an elution solvent are poured into solvent reservoirs at the front of the instrument. During operation these solvents are dispensed to the hub of the spinning rotor and distributed evenly to the 12 cartridge positions.

(4) The appropriate operating program is selected, and the extractor/concentrator started. Sixteen operating programs ranging from 2 to 30 min are available.

(5) The dual rotor spins in a clockwise direction and the buffered serum passes through the resin column to the effluent cup (see Fig. 2). The drugs are sorbed on to the resin.

(6) The resin columns are then automatically washed with water to elute off serum proteins, etc.

(7) The rotor then reverses direction. The outer ring of the rotor is free to rotate a short lateral distance with respect to the inner ring, so that the resin column now aligns with the sample recovery cup (see Fig. 2).

(8) The extracted sample is eluted from the resin column into the recovery cup with an organic elution solvent.

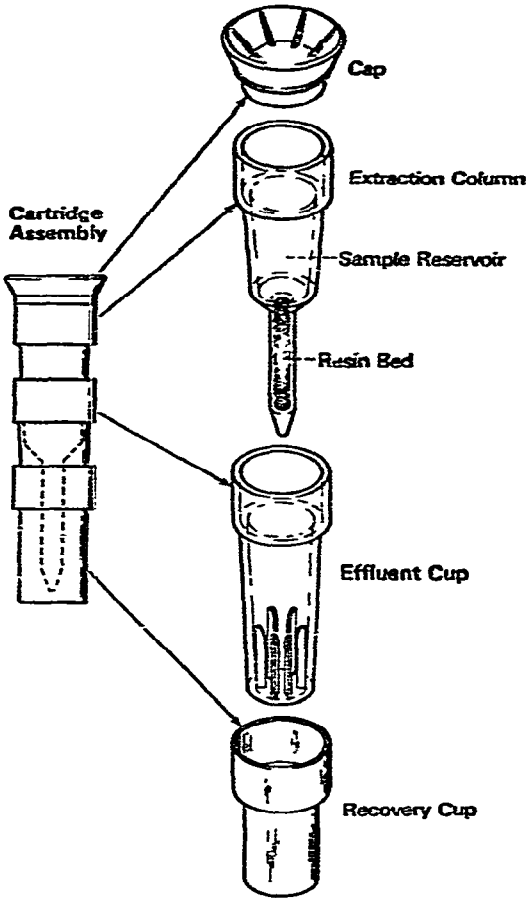

Fig. 1. Extraction cartridge.

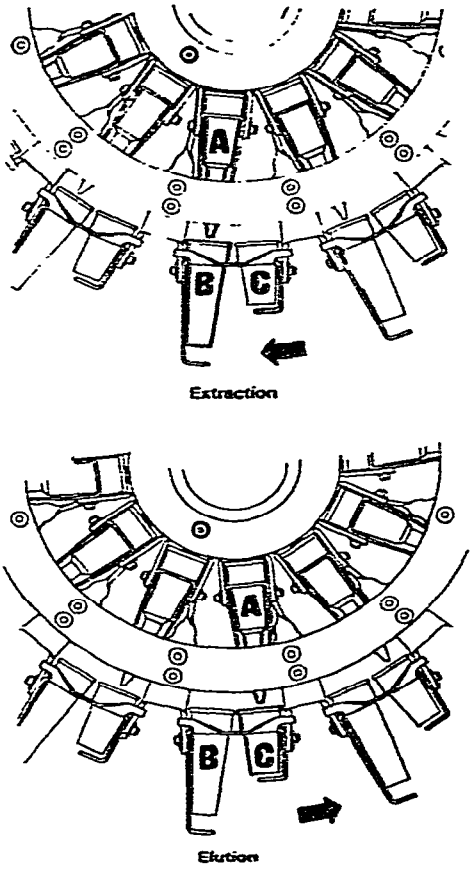

Fig. 2. Rotor. (A) Extraction column. (B) Effluent cup. (C) Recovery cup. Arrow denotes direction of rotor spin.

(9) The organic solvent is then evaporated to dryness by a stream of warm air. Upon completion of the operating sequence the dry extracts are ready for chromatography or other analytical methods.

The resin used in the extraction cartridge may be any suitable sorbant, such as anion-exchange resin. However, the cartridges (Type W) used for this work contained an activated, sized divinylbenzene cross-linked polystyrene resin which acts as a lipophilic sorbant and extracts the less water soluble drugs, metabolites and serum components. A 1-ml solution of 0.3 *M* KH_2PO_4 was used to buffer the serum. Each cartridge was washed with 0.8 ml of water and the extract eluted with 2 m of either acetone or methylene chloride. The extracts were dried in the extractor at 50°. Operating programs of different length and evaporation temperature are available. The program selected for 0.2-ml serum samples was 20 min long, whereas a 28-min program was used with 2-ml serum samples.

The amount of serum required for extraction depends on the concentration of

the drug and the sensitivity of chromatographic detection method. Clonazepam is normally present in such small concentrations (75 ng/ml) that larger volumes of serum, 2 ml, were required in order to extract sufficient drug for reliable detection. All other drugs tested in the extractor studies were present in sufficient concentration to require only small amounts, 0.2 ml or less, of serum for extraction.

RESULTS AND DISCUSSION

Extraction recovery

Small amounts of the anticonvulsant drugs in water–methanol solution were spiked into prepared serum volumes to give samples of known concentration. Appropriate volumes of these spiked serum samples were then aliquoted into the extraction cartridges with the appropriate buffer solution and processed in the extractor/concentrator. The dried extracts were reconstituted in a mobile phase containing a standard compound, methyl primidone (or carbamazepine for clonazepam extraction experiments) and injected into the liquid chromatograph. Peak heights were measured and compared with peak heights from direct injection of pure standards to obtain a percentage value for extraction recovery. The methyl primidone was added during the reconstitution step in order to correct for volumetric errors in sample injection.

Recovery results for a variety of anticonvulsant drugs, metabolites and recommended internal standards are shown in Table I. Some drugs were studied over a concentration range equivalent to their therapeutic range whereas others were studied at just one concentration. A chromatogram of a serum extract containing clonazepam is shown in Fig. 3. All drugs were eluted from the cartridges with acetone, except for N-desmethylmethsuximide and carbamazepin-10,11-epoxide which were eluted with methylene chloride in separate tests. Both solvents make excellent eluting agents for the anticonvulsant drugs.

TABLE I

ABSOLUTE EXTRACTION FROM SERUM

Drug	*Concentration (μg/ml)*	*Sample size (ml)*	*Recovery (%)*	
			Mean	*C.V.***
Phenobarbital	10	0.2	102	3.6
	80	0.2	99	3.4
Phenytoin	5	0.2	96	5.9
	30	0.2	101	6.0
Carbamazepine	2.5	0.2	100	6.7
	10	0.2	102	4.5
Primidone	15	0.2	99	4.2
Alphenal*	20	0.2	100	5.9
Carbamazepin-10,11-epoxide	1	0.2	100	4.6
N-Desmethylmethsuximide	20	0.2	97	4.8
Clonazepam	0.075	2.0	90	4.8
Nitrazepam*	0.075	2.0	95	7.4

* Recommended internal standard.

** Coefficient of variation for 9 to 15 samples.

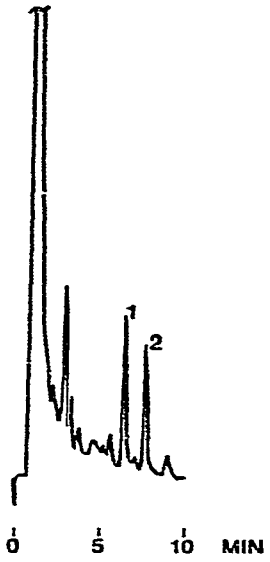

Fig. 3. Chromatogram of extract of 1 ml of serum containing (1) nitrazepam and (2) clonazepam, 75 ng, at a detector wavelength of 254 nm at 0.02 a.u.f.s.

The precision and recovery of drugs relative to an internal standard added to the serum before extraction may be better than the corresponding absolute recovery values. For example, the recovery of clonazepam relative to nitrazepam was 95% with a coefficient of variation of 3.7%, compared with the absolute values of 90% and 4.8% shown in Table I for the same data. The internal standard effectively compensates for any errors in serum pipetting or extraction. For these reasons alphenal was added as an internal standard to all patient serum samples before processing in the extractor/concentrator.

Detection and linearity

The detection wavelengths were chosen to maximize sensitivity while minimizing potential interferences. Clonazepam was monitored at a UV wavelength of 254 nm with a detector sensitivity of 0.02 a.u.f.s. Minimum detection under these conditions is less than 5 nanograms. All other drugs were detected at 220 nm with an attenuation of 0.16 a.u.f.s. Although minimum detection varied from drug to drug, less than 50 ng could be detected for all drugs tested in extraction and correlation studies.

Chromatographic linearity of the method was determined to be well beyond the therapeutic range of the drugs used in these studies. Some of the drugs and their demonstrated linear range include primidone 0–100 μg/ml, phenobarbital 0–160 μg/ml, carbamazepine 0–25 μg/ml and phenytoin 0–100 μg/ml.

Precision

The precision of the method was studied by repeated analysis of serum solutions containing four of the anticonvulsant drugs. The data in Table II shows that the within-day variation (20 trials) of calculated concentration ranged from 1.8 to 4.6%, while day-to-day variation (over 16 days) ranged from 2.5 to 5.8%.

TABLE II

PRECISION OF ASSAYS FOR ANTICONVULSANT DRUGS IN SERUM

	Within day		*Day-to-day*	
	Range (μg/ml ± S.D.)	*C.V. (%)*	*Range (μg/ml ± S.D.)*	*C.V. (%)*
Phenobarbital	15.8 ± 0.4	2.5	15.7 ± 0.4	2.5
Phenytoin	7.1 ± 0.2	2.4	7.2 ± 0.4	5.8
Carbamazepine	4.1 ± 0.1	1.8	4.2 ± 0.2	4.8
Primidone	7.2 ± 0.3	4.6	14.8 ± 0.6	4.2

The day-to-day variation (over 16 days) of retention times ranged from 2.1% for primidone to 3.7% for phenytoin.

Drug retention and interference study

The capacity factors (k') of some anticonvulsant and other common drugs are shown in Table III. No other coadministered or common non-prescription drugs were found to interfere with the analysis of the major anticonvulsant drugs and their metabolites. However, studies have shown that extractable components in some blood collection tubes may interfere with clonazepam analysis.

TABLE III

CAPACITY FACTORS OF SOME ANTICONVULSANT AND COMMON DRUGS

Anticonvulsant drug or metabolite	k'	*Common drugs*	k'
Phenylethylmalonamide	0.9	Acetaminophen	0.3
Primidone	1.1	Amphetamine	14.0
Ethosuximide	1.4	Aspirin	0.3
Carbamazepin-10,11-epoxide	1.8	Barbital	0.9
Phenobarbital	2.0	Caffeine	0.4
Alphenal	2.6	Codeine	2.2
N-Desmethylmethsuximide	2.9	Diazepam	14.0
Phensuximide	3.1	Methaqualone	2.4
Carbamazepine	3.4	Phenacetin	2.4
Phenytoin	4.0	Propoxyphene	0.3
Clonazepam	6.0	Theophylline	0.4
Methsuximide	7.0		

Comparison with other methods

Hospital serum samples were obtained from patients being administered phenobarbital, phenytoin, carbamazepine and primidone. The concentration of the drugs had been determined independently by either gas chromatography or enzyme immunoassay. Small amounts (0.2 ml) of the serum were mixed with buffer and internal standard, processed by the extractor/concentrator, and chromatographed. A typical chromatogram is shown in Fig. 4. The results were compared with those of the gas chromatography and immunoassay techniques through linear regression analysis. The data are shown in Table IV; the agreement between methods is good. A correla-

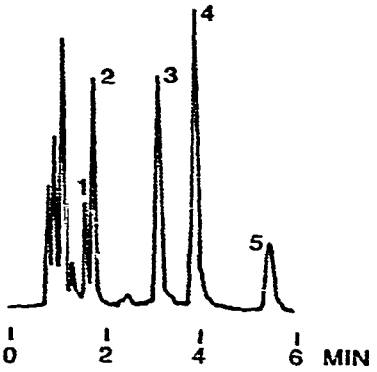

Fig. 4. Chromatogram of extract of 0.1 ml of patient serum containing (1) phenylethylmalonamide, (2) primidone, (3) phenobarbital (4) alphenal and (5) phenytoin at a detector wavelength of 220 nm at 0.16 a.u.f.s.

TABLE IV

COMPARISON OF HPLC RESULTS WITH OTHER ANALYTICAL METHODS

	Samples	*Correlation coefficient*	*Slope*	*y Intercept (μg/ml)*
Spiked samples from quality control program				
Phenobarbital	21	0.997	0.96	−0.48
Phenytoin	21	0.972	0.97	−0.59
Carbamazepine	21	0.997	0.95	0.08
Primidone	21	0.999	0.98	−0.37
Patient samples: GC with liquid–liquid extraction				
Phenobarbital	23	0.990	0.96	−0.05
Phenytoin	22	0.960	0.98	0.04
Primidone	24	0.983	0.87	−0.10
Patient samples: enzyme multiplied immunoassay technique				
Phenobarbital	96	0.993	0.98	−0.03
Phenytoin	103	0.965	1.09	1.01
Carbamazepine	96	0.971	0.98	−0.07
Primidone	76	0.977	0.91	1.37

tion plot comparing the phenobarbital HPLC results with enzyme immunoassay results is shown in Fig. 5.

We were also able to obtain spiked serum samples from a laboratory quality control program. Our results for these samples are compared in Table IV with the mean of the results from five reference laboratories using chromatographic methods. The agreement is very good.

We were not able to include comparisons of the metabolites and less frequently used anticonvulsant drugs as there are no recognized analytical methods for them.

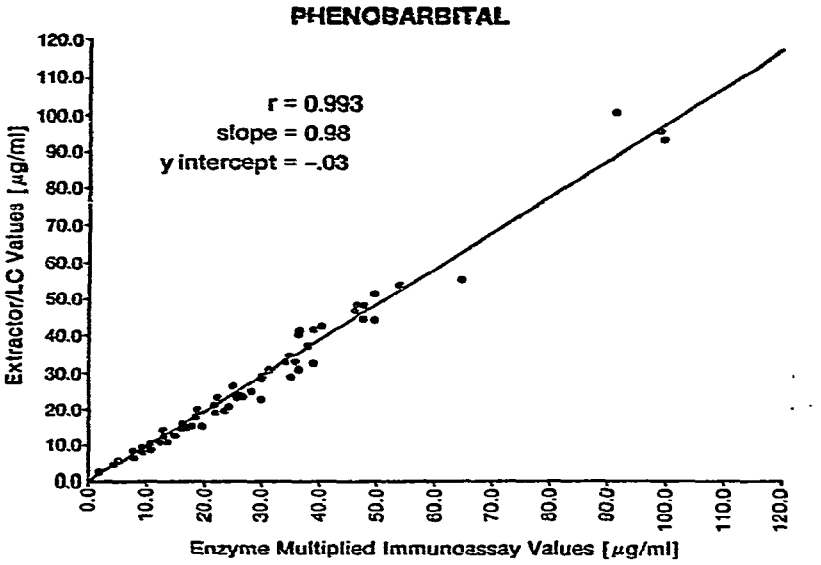

Fig. 5. Comparison of results for patient samples analyzed for phenobarbital by HPLC and enzyme immunoassay.

CONCLUSIONS

(1) The absolute recovery of the anticonvulsant drugs extracted from blood serum with automated extractor/concentrator is 90% or greater.

(2) The relative recovery of these anticonvulsant drugs from blood serum as compared with an internal standard is 95% or greater.

(3) A variety of anticonvulsant drugs can be analyzed by HPLC on a single column with the same mobile phase.

(4) Comparison of results for analysis of patient serum samples with gas chromatography and enzyme immunoassay techniques was excellent with correlation coefficients of 0.96 or larger.

ACKNOWLEDGEMENTS

We thank Dr. C. E Pippinger of the Columbia Presbyterian Medical Center and Dr. E. Richardson of the Wilmington Medical Center for patient samples and the enzyme immunoassay and gas chromatography results.

REFERENCES

1 S. H. Atwater, V. A. Green and W. G. Haney, *J. Pharm. Sci.*, 64 (1975) 806.
2 J. G. Evans, *Anal. Chem.*, 45 (1973) 2428.
3 S. Kitazawa and T. Komura, *Clin. Chim. Acta*, 73 (1976) 31.
4 R. F. Adams, G. J. Schmidt and F. L. Vandemark, *J. Chromatogr.*, 145 (1978) 275.
5 G. W. Miholy, J. A. Phillips, W. J. Louis and F. J. Vajda, *Clin. Chem.*, 23 (1977) 2283.
6 R. F. Adams and F. L. Vandemark, *Clin. Chem.*, 22 (1976) 25.
7 S. J. Soldin and J. G. Hill, *Clin. Chem.*, 22 (1976) 856.
8 P. M. Kabra, B. E. Stafford and L. J. Marton, *Clin. Chem.*, 23 (1977) 1284.