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Measurement of Anticonvulsants in Serum by Reversed-Phase Ion-Pair Liquid Chromatography

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We describe a method for the simultaneous liquid-chromatographic determination of ethosuximide, ethylphenacemide, primidone, phenobarbital, carbamazepine, and phenytoin in serum. The drugs, together with an internal standard, are extracted into ethyl acetate at pH 7.0. The extract is analyzed isocratically at ambient temperature on a reversed-phase column of "SAS Hypersil" with a mobile phase of acetonitrile/tetrabutyl ammonium phosphate solution (2/8 by volume). The eluted drugs are detected by their absorption at 200 nm, and quantitated from their peak heights as compared with those of extracted standards. The day-to-day CV of the method varied between 5.1 and 9.6% for concentrations ranging from less than therapeutic to toxic. The results, when compared with those by gas chromatography, gave correlation coefficients of 0.936 for phenytoin, 0.977 for phenobarbital, and 0.939 for primidone. No drug interference was found except that amobarbital and ethylphenacemide co-chromatographed.

Liquid chromatography is a useful method for the measurement of anticonvulsant drugs in serum (*1–4*). The advantages are that all the commonly used drugs can be separated on one column from a simple extraction without any need for derivatization. The methods published involve reversed-phase C_{18} columns with a mobile phase of acetonitrile/water or phosphate buffer. To obtain sufficient resolution between components, these methods require the system to be run at warm temperatures, up to 50 °C (*3*). We describe a system that will run at ambient temperature (20–25 °C) on a reversed-phase column. The mobile phase contains a counter ion of tetrabutyl ammonium phosphate.

Materials and Methods

Apparatus

The high-pressure chromatograph consisted of a Varian 8500 pump and injection head (Varian Associates Ltd., Walton-on-Thames, KT12 3PJ, U.K.) with a Cecil CE212 variable-wavelength ultraviolet detector (Cecil Instruments Ltd., Cambridge CB4 1TG, U.K.). The stainless steel column was 125 × 4.5 (i.d.) mm, packed with SAS Hypersil (5 μm av particle size; Shandon Southern Products Ltd., Runcorn, WA7 1PR, U.K.). A stainless-steel guard column 50 × 2.1 (i.d.) mm was dry-packed with Co:Pell ODS (Whatman Lab Sales Ltd., Maidstone, ME14 2LE, U.K.).

Reagents

Drug standards: Stock solutions of the drugs and internal standard of heptabarbital were made up in methanol. The working standards were prepared in horse serum no. 5 (Wellcome Reagents Ltd., Beckenham, BR3 3BS, U.K.).

Sodium phosphate buffer, 0.4 mol/L, pH 7.0, was prepared in glass-distilled water.

Tetrabutyl ammonium phosphate solution, 5 mmol/L, was prepared by adjusting the pH of the tetrabutyl ammonium hydroxide solution (BDH Chemicals Ltd., Poole, Dorset, BH12 4NN, U.K.) with phosphoric acid to pH 7.5 and diluting with glass-distilled water to 5 mmol/L. The *mobile phase* was a mixture of 200 mL of acetonitrile (Rathburn Chemicals, Walkerburn, EH43 6AU, U.K.) with 800 mL of 5 mmol/L tetrabutyl ammonium phosphate solution.

Procedure

Extract a mixture of 500 μL of plasma or standard, 100 μL of internal standard, and 500 μL of 0.4 mol/L phosphate buffer (pH 7.0) with 10 mL of redistilled ethyl acetate. Evaporate the extract at not more than 50 °C, redissolve in 20 μL of methanol, and inject a 3-μL aliquot onto the column. Chromatographic flow rate was 1.16 mL/min; pressure was 1200 psi; temperature was ambient; detector wavelength was 200 nm.

Results

Typical retention times of the pure drugs are (minutes): ethosuximide 3.4, primidone 4.2, phenobarbital 6.6, heptabarbital (internal standard) 9.8, ethylphenacemide (Pheneturide) 13.6, carbamazepine 16.4, and phenytoin 18.6. The drug concentrations in sera from patients were quantitated by peak-height measurements. There was a linear relationship between peak height and concentration over the range of the working standards.

Precision: The precision of the method was assessed by repeated analyses of plasma specimens containing low, normal, and above-normal concentrations of the drugs being investigated. The results are shown in Table 1. The values are acceptable and compare well with those obtained with previously reported methods.

Recovery: The analytical recovery of drugs was measured by extracting serum containing known amounts of drug but without internal standard. The extracts were reconstituted in methanol with internal standard. The solutions were chromatographed and the peak heights compared with those obtained from unextracted solutions of pure methanol standards. The results are given in Table 2. The average analytical recovery of ethosuximide, primidone, phenobarbital, ethylphenacemide, carbamazepine, and phenytoin was greater than 80%.

Interference from lipemic and icteric specimens was also measured. Known weights of drugs were added to drug-free sera that were visibly lipemic or icteric. The results are given in Table 2. Background interference from 21 drug-free sera was obtained by chromatographing extracts and measuring absorbance at the retention time of each drug. The greatest interference (0–9.2 μmol/L) was found at the retention time

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Table 1. Precision for Measurement of Anticonvulsant Drugs in Plasma

Concn, μmol/L	Within-day CV,% (n = 20)	Between-day n	Between-day CV,%
	Ethosuximide		
200	8.6	30	9.5
400	6.5	34	7.8
800	5.8	24	8.1
	Primidone		
20	5.4	30	7.8
35	7.4	32	9.0
75	4.6	26	8.5
	Phenobarbital		
60	4.2	29	6.5
100	3.9	31	7.5
200	1.6	29	7.5
	Ethylphenacemide		
20	7.5	33	8.2
50	5.0	28	7.2
100	2.1	24	5.1
	Carbamazepine		
15	4.3	33	5.9
30	4.8	35	6.2
50	3.9	30	7.4
	Phenytoin		
40	4.3	33	7.8
60	4.8	37	9.6
100	3.9	31	6.7

Table 2. Recovery of Drugs Added to Plasma

Concn, μmol/L	Absolute recovery, mean % (n = 5)	Relative recovery, mean % (n = 5) Icteric	Lipemic
Ethosuximide, 400	83	108	110
Primidone, 40	85	100	100
Phenobarbital, 80	81	101	100
Ethylphenacemide, 40	81	102	100
Carbamazepine, 20	82	104	101
Phenytoin, 80	83	104	102

of ethosuximide. This was, however, less than 0.3% of the lower therapeutic concentration of the drug. Background interference (μmol/L) for the other drugs tested was: primidone, 0–0.6; phenobarbital, 0–1.0; ethylphenacemide, 0–1.2; carbamazepine, 0–1.7; and phenytoin, 0–0.7.

Comparison with Gas Chromatography

Concentrations of phenytoin, phenobarbital, and primidone in sera were measured by the gas-chromatographic method in routine use in this laboratory (*5*). The gas-chromatographic results (x) were compared with the results obtained by the liquid-chromatograph method presented here (y). The results for phenytoin gave a correlation coefficient (r) of 0.936, slope (m) of 0.923, and y-intercept (b) = 2.6 for n = 100; for primidone: r = 0.939, m = 0.809, b = 7.0, n = 72; for phenobarbital: r = 0.977, m = 0.92, b = 4.6, n = 149. There were insufficient specimens from patients on other drugs to make valid comparisons.

We measured the retention times of several ultraviolet-absorbing drugs and metabolites of anticonvulsants that have been shown to interfere in other, similar methods. The results, relative to the internal standard, are given in Table 3. The principal interference was the co-elution of amobarbital with ethylphenacemide. In our experience this combination of drugs is unlikely to be administered in clinical practice. There was no interference from the *p*-hydroxylated metabolite of phenytoin or from the phenylethylmalonamide metabolite of primidone.

Table 3. Retention Time of Drugs Relative to Heptabarbital

Caffeine	solvent front	Heptabarbital	1.000
Theophylline	solvent front	Pentobarbital	1.235
Phenylethylmalonamide	0.313	Methsuximide	1.255
Ethosuximide	0.341	Cyclobarbital	1.284
Barbital	0.351	Ethylphenacemide	1.402
Primidone	0.421	Amobarbital	1.402
Sulthiame	0.459	Glutethimide	1.604
Sulfamethoxazole	0.459	Carbamazepine	1.663
Phenobarbital	0.673	Secobarbital	1.688
Ethotoin	0.681	Phenytoin	1.916
Butobarbital	0.779	5-(*p*-Methylphenyl)-5-phenylhydantoin	3.255
5-(*p*-Hydroxyphenyl)-5-phenylhydantoin	0.878	Benzodiazepines	did not elute

Table 4. Serum Drug Concentrations Giving a Recorder Response of 5% Full-Scale Deflection

	Concn, μmol/L, at 194 nm	200 nm	210 nm
Ethosuximide	6.5	16.0	29.0
Primidone	2.2	2.5	2.0
Phenobarbital	2.2	2.5	2.3
Ethylphenacemide	6.4	6.9	5.6
Carbamazepine	4.4	3.8	2.2
Phenytoin	4.2	5.2	4.5

The concentrations of drug in serum that give a recorder deflection of 5% of full-scale for detector wavelengths at 194 nm, 200 nm, and 210 nm are given in Table 4. This table presents for each drug both the sensitivity of the method and the relative response at the different wavelengths.

Discussion

The results show that the method has adequate precision and accuracy for measuring anticonvulsant drugs in serum. The sensitivity of the method is suitable for the measurement of all the drugs at the therapeutic concentrations found in serum. In this method, 500 μL of serum is extracted but only the equivalent of 75 μL is injected on the column. If this volume of serum is not available, the extraction volumes can be adjusted and more extract injected. Unlike gas chromatography, increasing the injection volume has minimal effects on the efficiency of separation.

The detector wavelength has an appreciable effect on the sensitivity of detection of the different drugs. We chose 200 nm instead of 194 nm, because the detector response at this wavelength for each of the drugs is more closely related to the mean concentration of drug found in serum (Table 4). Measurement at this wavelength also reduced the nonspecific in-

terference with ethosuximide, which was in some sera. The measurement of sulthiame is not recommended by this method, because nonspecific interference is found in some sera.

Comparison of results obtained by liquid chromatography and gas chromatography on the same samples is further evidence for the specificity of the method. The greatest discrepancy was found with primidone. The slope of the correlation line was only 0.809, which suggests a systematic error between the liquid- and gas-chromatographic methods. A similar observation was made by Soldin and Hill (*2*), who speculated that the latter method was at fault. Our experience would support this suggestion.

One of the effects of the tetrabutyl ammonium phosphate is to reverse the elution order of phenytoin and carbamazepine. With a new column, about half a liter of running solvent must be passed through the column before the retention times of phenytoin and carbamazepine are constant. In contrast, improved resolution of the other drugs is obtained without preconditioning of the column with running solvent. These observations support the concept that several mechanisms are responsible for the separation of compounds on a reversed-phase ion-pair system (*6*). The change in retention times of phenytoin and carbamazepine may be related to the ion-pair reagent's modifying the stationary phase.

The guard column was advantageous for routine analysis; it doubled the life of the analytical column, but had minimal effect on the separation efficiency of the system. With continuous daily use, we repacked the guard column every three months and the analytical column every six months, to retain the separation efficiency of the system.

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Semiautomated Homogeneous Enzyme Immunoassay of Total Serum Thyroxine with a Kinetic Analyzer

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A new semiautomatic procedure for determination of total serum thyroxine was adapted to a kinetic analyzer, LKB 2086 Mark II. The ABA thyroxine assay kit was used, and although the assay procedure was modified, the advantage of small reagent volumes, short measuring times, and automation were retained. The results were analyzed off-line with a programmable desk-top calculator. The method has a precision of 7% (CV) and a sensitivity of 8 nmol/L. Values in sera from 64 patients analyzed by enzyme immunoassay and by radioimmunoassay correlated well ($r = 0.975$). One kit contains enough reagents for 500 assays, and an operator could do 25 samples (i.e., 64 assays) in about 4 h.

Additional Keyphrases: *thyroid status · methods for the small laboratory · radioimmunoassay*

Until 1975 radioimmunoassay was the only practical method available for quantitative thyroxine estimation; it still is the most used. However, the development of homogeneous enzyme immunoassay or enzyme-multiplied immunoassay techniques (*1, 2*), with almost the same sensitivity as radioimmunoassay, presents a promising alternative. I report adaptation of this enzymic technique to a semiautomatic procedure on the LKB 2086 Mark II kinetic analyzer. This method should be useful in small laboratories that have a limited supply of automatic equipment.

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Materials and Methods

Reagents

The "EMIT" ABA thyroxine assay kit was obtained from Syva Corporation, Palo Alto, CA 94304, L-malic acid from Merck, 6100 Darmstadt, F.R.G., and glycine from Sigma Chemical Co., St. Louis, MO 63178. The reagents in the kit were prepared accurately and stored according to the manufacturer's instructions. A solution of enzyme reagent, diluted fourfold with buffer, was freshly prepared for each run (its activity decreased by about 2%/h at 25 °C). Additional buffer used was 0.14 mol/L L-malic acid in 0.3 mol/L glycine at pH 9.5.

Apparatus

I used a 2086 Mark II kinetic analyzer, 2077 multimixer, 2082 kinetic data processor (i.e., Hewlett Packard 9815 A), and 2082-075 software (all from LKB-Product AB, S-161 25 Bromma, Sweden). The settings for the kinetic analyzer were as follows: temperature, 37 °C; wavelength, 340 nm; light setting, operate (OA); injection nozzle, red (25–50 μL); dispensing volume, 125 μL; preinjection position, 6; measuring range, 0.05 *A*; feed-in mode, timer; cuvette size, 7 mm; normal-delay mode, delay.