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Note

Simultaneous determination of six antiepileptic drugs by capillary gas chromatography

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Valproic acid (VPA), ethosuximide (ET), phenobarbital (PB), primidone (PR), carbamazepine (CZ), and phenytoin (PT) are some of the most frequently used antiepileptic drugs (AEDs). They are prescribed and used in various combinations (polytherapy) and only rarely used singly.

Until now the simultaneous determination of these six AEDs by gas chromatography (GC) has not been reported. Procedures have been established for determination of only a few AEDs [1–5]. Owing to their different levels in blood and high volatility, VPA and/or ET are analysed separately from other AEDs. Most of the reported GC methods use packed columns; capillary columns are used only in a few cases [6,7]. The effective way to isolate AEDs from serum is solid-phase extraction [8–10]. However, the procedures have been developed only for the certain groups of drugs and not for all six together.

For the routine monitoring of levels of AEDs it would be easiest to use only one method for all six drugs, without regard to their number or combination. We tried to develop such a procedure for the simultaneous determination of six underivatized AEDs by capillary GC. Owing to the very different characteristics of the six AEDs (boiling point, thermal stability, pK_a, solubility in

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water, volatility at room temperature) we did not beforehand know we would succeed. This paper describes the results.

EXPERIMENTAL

Chemicals and reagents

Antiepileptic drugs were received as gifts from pharmaceutical firms (SPOFA, Prague, Czechoslovakia, and VEB Arzneimittelwerk, Dresden, G.D.R.). Caprylic acid (CA) was purchased from Serva (Heidelberg, F.R.G.), 5-(4-methylphenyl)-5-phenylhydantoin (MPPH) from Janssen Chimica (Beerse, Belgium). Silipor C_{18} (0.125–0.160 mm), a sorbent for packing extraction columns, was obtained from Lachema (Brno, Czechoslovakia). All other chemicals used were of analytical grade.

A stock solution of the six AEDs in methanol (1 mg/ml) was prepared. This solution was diluted by methanol (test mixtures) or by drug-free horse serum Sevates REPRO (Imuna, Šarišské Michal'any, Czechoslovakia) (serum spiked samples).

Apparatus

An HP-5790A gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) equipped with a split/splitless capillary inlet system and a flame ionization detector was used. A fused-silica capillary column with cross-linked 5% phenylmethylsilicone gum phase HP-5 (Hewlett-Packard), 25 m×0.20 mm I.D., 0.33 μm film thickness, was used. The carrier gas was nitrogen at an inlet pressure of 100 kPa. The oven was operated under temperature-programmed conditions: isothermally at 60°C for 0.5 min after injection, heated at 30°C/min to 200°C, then at 10°C/min to 250°C, and then held at 250°C for 10 min. Injections were made in the split mode at a split ratio of 1:20, and a septum purge rate of 1 ml/min. The temperatures of injector and detector were 240°C and 300°C, respectively. For data-handling the HP 3390A reporting integrator (Hewlett-Packard) was used.

For the solid-phase extraction a laboratory-made ten-place vacuum manifold was used. A dry block thermostat LP-207 (MTA Kutesz, Budapest, Hungary) equipped with a laboratory-made evaporation head was used to evaporate eluates from the extraction columns.

Sample pretreatment

A 500-μl serum sample was mixed with 100 μl of 0.5 *M* phosphate buffer (pH 4.5) or with 100 μl of 0.5 *M* HCl in a 1.5-ml polypropylene microvial, and 10 μl of internal standard solution were added. The contents of the vial were thoroughly mixed using a hand-held rotary mixing device.

Extraction procedure

An extraction column prepared from a common 2-ml disposable polyethylene syringe (9 mm I.D.) packed with 200 mg of Silipor C_{18} was washed with 1 ml of methanol and subsequently with 1 ml of distilled water or 0.5 *M* HCl. Pretreated sample was poured onto the column and allowed to flow through. Then the column was rinsed with two 1-ml portions of water and dried by vacuum. The drugs were eluted with 1 ml of methanol. The collected eluate was preconcentrated by evaporation under nitrogen.

Preconcentration of eluate

A 50-μl volume of KOH solution in methanol (0.02 or 0.05 *M*) was added to 1 ml of eluate, and the solvent was evaporated under nitrogen in a block thermostat at 40°C. The residue was dissolved in 50 μl of HCl in methanol at the same concentration as the solution of KOH. From this solution 2 μl were injected into the gas chromatograph.

RESULTS AND DISCUSSION

Separation of the six AEDs on the capillary column required a dual-ramp temperature programme. Owing to the high volatility of VPA a rather low initial temperature was needed.

A split injection system for the introduction of samples into a capillary column was tested previously [11]. As a result we decided to use an empty liner because any packing material in the liner caused carbamazepine to degrade. The chromatographic run-to-run reproducibility at different concentrations of AEDs is given in Table I. A chromatogram of the test mixture is shown in Fig. 1.

According to the calibration curves of different AEDs, established by linear regression, the response of the flame ionization detector is linear over a wide range of concentrations in serum (0.01–0.10 mg/ml). The least-squares linear equations and S.D. are given in Table II.

Low levels of PB, PR, CZ and PT necessitate the preconcentration of the eluate prior to GC injection. The common procedure of evaporation at high temperature under nitrogen was modified according to Andreolini et al. [12]. This modification is based on the addition of a sufficient amount of KOH to the eluate prior to evaporation to convert acid drugs into their potassium salts. After evaporation a solution of HCl was added to the residue to regenerate the free drugs. Together with HCl, methanol was added to dissolve the drugs. To find the appropriate amount of KOH, a special series of experiments was performed. From a stock solution of VPA, CA and ET (0.5 mg/ml in methanol), 2 μl were injected into the gas chromatograph and the mean of the area counts for each compound was taken as 100% yield. Then 50 μl of the same solution were diluted with 1 ml of methanol and evaporated under nitrogen without and

TABLE I

CHROMATOGRAPHIC RUN-TO-RUN REPRODUCIBILITY FOR VARIOUS CONCENTRATIONS OF AEDs CALCULATED BY THE INTERNAL STANDARD METHOD

$n = 10$. Abbreviations: AED = antiepileptic drug; VPA = valproic acid; ET = ethosuximide; PB = phenobarbital; PR = primidone; CZ = carbamazepine; PT = phenytoin; CA = caprylic acid; HB = hexobarbital; MPPH = 5-(4-methylphenyl)-5-phenylhydantoin.

AED	Internal standard	Relative standard deviation (%)			
		0.01 mg/ml	0.02 mg/ml	0.05 mg/ml	0.1 mg/ml
VPA	CA	1.69	1.09	1.08	1.54
ET	CA	1.01	1.99	1.88	2.23
PB	HB	3.33	3.75	2.43	1.26
PR	HB	3.34	5.25	4.04	1.96
CZ	MPPH	3.00	1.79	1.60	1.78
PT	MPPH	4.81	3.09	0.90	1.26

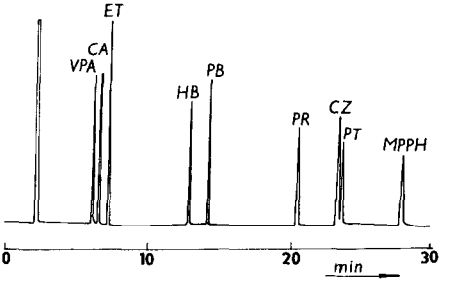

Fig. 1. Typical chromatogram of a test mixture of AEDs and three internal standards in methanol (VPA, CA and ET, 0.5 mg/ml; HB, PB, PR, CZ, PT and MPPH, 0.1 mg/ml). Injected volume, 2 μl.

with different amounts of KOH. The mean of area counts represents the yield under given conditions.

The theoretically calculated amount of KOH that was necessary was 1 μmol (50 μl of 0.02 *M* KOH in methanol). Our experiments showed that this amount was insufficient, therefore we used a higher concentration of KOH (Table III). From this table it is clear that addition of KOH prevents the evaporation of volatile drugs. In further experiments 0.05 *M* KOH was used.

We prepared four series of tests for the evaluation of solid phase extraction of AEDs. Procedures were performed as described in Experimental.

All tests were performed with the spiked serum at one level of AEDs (VPA and ET, 50 μg/ml; PB, PR, CZ and PT, 10 μg/ml). These concentrations are mostly at the low end of the therapeutic range, with exceptions of those of PR

TABLE II

LINEAR EQUATIONS, CORRELATION COEFFICIENTS AND STANDARD DEVIATIONS FOR CALIBRATION CURVES OF TESTED AEDs

Abbreviations as in Table I; $n = 10$.

AED	Linear equation ($y = a + bx$)	Correlation coefficient (r)	Standard deviation		
			a	b	yx
VPA	$y = 1.72 + 382.92x$	0.991	0.50	8.85	0.62
ET	$y = 1.38 + 323.08x$	0.994	0.46	8.14	0.57
PB	$y = 0.47 + 633.54x$	0.998	0.17	3.00	0.21
PR	$y = 0.10 + 729.38x$	0.999	0.11	1.86	0.13
CZ	$y = 0.56 + 1045.01x$	0.998	0.25	4.43	0.31
PT	$y = 0.18 + 857.09x$	0.999	0.11	2.00	0.14

TABLE III

YIELDS OF THREE VOLATILE AEDs AFTER EVAPORATION UNDER NITROGEN AT DIFFERENT CONDITIONS AT 40°C

Abbreviations as in Table I; $n = 10$.

Conditions of evaporation	VPA		CA		ET	
	Area counts ($\times 10^2$)	Yield (%)	Area counts ($\times 10^2$)	Yield (%)	Area counts ($\times 10^2$)	Yield (%)
Test mixture without evaporation	104.9	100	113.6	100	85.0	100
Evaporation in methanol	19.3	18.4	7.0	6.2	14.9	17.6
Evaporation in 0.02 *M* KOH	90.9	68.7	90.8	79.9	72.9	85.8
Evaporation in 0.05 *M* KOH	97.6	93.0	94,3	83.0	75.5	88.8

and CZ, which are in the middle of the recommended range. The aim of this study was to find whether it is possible to extract the volatile AEDs (VPA and ET) together with the non-volatile AEDs. Detailed studies of the solid phase extraction of four AEDs (PB, PR, CZ, and PT) at different concentrations have been published elsewhere [4,8].

Serum samples were modified in the first test with 0.5 *M* HCl, and in the second with 0.5 *M* phosphate buffer (pH 4.5) to suppress ionization of the drugs. Prior to pouring the samples onto extraction columns they were washed with methanol and water.

In the third and fourth tests the serum samples were prepared as in the previous two. The difference was that the extraction columns were washed with methanol and 0.5 *M* HCl.

In all cases an internal standard (CA or MPPH) was added to every sample, at a concentration of 20 μg/ml. Each test was performed ten times. The recovery (Table IV) and within-day reproducibility of these tests (Table V) against the internal standard MPPH were calculated. Calculation against the internal standard CA was not used owing to its high variability. HB was used as an internal standard only in the testing of a chromatographic run: it was not added to serum samples because a rather large peak from the biological matrix was observed close to its retention time.

The main difference in recovery when either 0.5 *M* HCl or buffer was added to serum was observed for the volatile and the more acid drugs (VPA, CA, ET). There was a small effect on the recovery of other AEDs. When serum samples were acidified with HCl, washing the extraction columns with 0.5 *M* HCl had the greatest effect on the reproducibility of the whole procedure.

Tests of the solid-phase extraction of AEDs showed that the addition of 0.5 *M* HCl to the sample, and washing of the extraction columns with 0.5 *M* HCl, gave the best results. The recovery of PT over 100% can be explained by the lower recovery of MPPH. The low recovery of ET is likely caused by its good solubility in water. The term "recovery" means recovery relative to the internal standard, and expresses the loss of other compounds versus internal standard which passes the same way as analysed compounds.

TABLE IV

EXTRACTION RECOVERY OF AEDs FROM SERUM UNDER DIFFERENT CONDITIONS RELATIVE TO INTERNAL STANDARD MPPH

Abbreviations as in Table I. A, extraction column washed with water; B, extraction column washed with 0.5 *M* HCl.

AED	Concentration in serum (μg/ml)	Recovery ($n=10$) (%)			
		A		B	
		Serum + 0.5 *M* HCl	Serum + 0.5 *M* KH_2PO_4	Serum + 0.5 *M* HCl	Serum + 0.5 *M* KH_2PO_4
VPA	50	82.4	29.2	70.2	51.2
CA	20	77.0	35.0	84.5	79.5
ET	50	28.6	26.2	33.4	29.6
PB	10	94.0	94.0	92.0	98.0
PR	10	76.0	71.0	86.0	94.0
CZ	10	87.0	84.0	94.0	78.0
PT	10	104.0	108.0	107.0	101.0

TABLE V

WITHIN-DAY REPRODUCIBILITY OF DETERMINATION OF AEDs UNDER DIFFERENT CONDITIONS OF EXTRACTION RELATIVE TO INTERNAL STANDARD MPPH

Abbreviations as in Table I. A, Extraction column washed with water; B, extraction column washed with 0.5 *M* HCl.

AED	Concentration in serum (μg/ml)	Relative standard deviation ($n=10$) (%)			
		A		B	
		Serum + 0.5 *M* HCl	Serum + 0.5 *M* KH_2PO_4	Serum + 0.5 *M* HCl	Serum + 0.5 *M* KH_2PO_4
VPA	50	7.4	15.2	7.3	19.2
CA	20	7.5	20.4	7.4	18.7
ET	50	8.6	16.1	4.4	8.8
PB	10	9.9	10.8	1.3	15.7
PR	10	8.1	8.1	1.0	14.1
CZ	10	7.2	8.3	1.6	10.9
PT	10	6.5	9.2	2.5	14.3

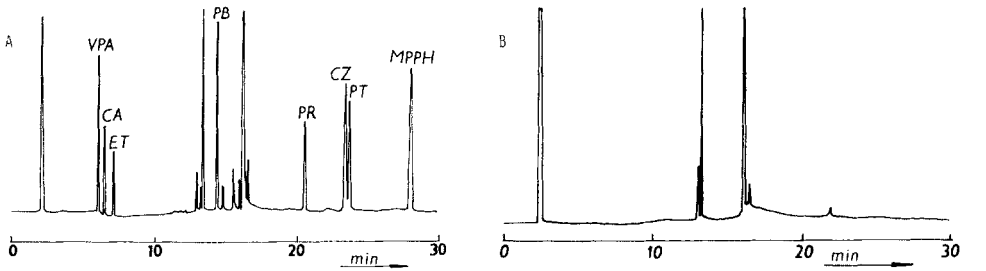

Fig. 2. (A) Chromatogram of a serum sample spiked with 50 μg/ml VPA and ET, and 10 μg/ml PB, PR, CZ and PT, with two internal standards (CA and MPPH) at 20 μg/ml. The serum and column were acidified prior to the extraction with 0.5 *M* HCl. Injected volume, 2 μl of extract. (B) Chromatogram of drug-free serum, treated as in (A). Injected volume, 2 μl of extract.

Capillary gas chromatograms of serum samples spiked with AEDs and with internal standards and of drug-free serum are given in Fig. 2A and B. The practical application of the method is demonstrated by the chromatogram of the serum of the patient treated with PR (Fig. 3); MMPH was used as an internal standard, and PB is an active metabolite.

CONCLUSIONS

We have described the separation conditions for analysis of six underivatized AEDs on a capillary column packed with crosslinked phenylmethylsili-

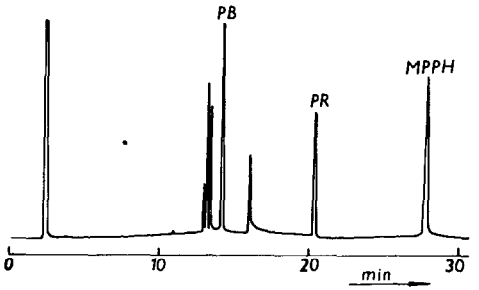

Fig. 3. Chromatogram of the serum of a patient treated with PR, with MPPH as internal standard. PB is an active metabolite. Other conditions as in Fig. 2A.

cone and split injection of samples using an empty glass liner. One chromatographic run of six AEDs plus two internal standards takes ca. 30 min under temperature-programmed conditions.

We have tested various conditions for the isolation of drugs from biological material using solid-phase extraction with a reversed-phase sorbent. The best results were obtained when samples and extraction columns were acidified with 0.5 *M* HCl. Prior to evaporation of eluates from extraction columns a solution of KOH was added to suppress the loss of volatile AEDs during this step.

The results show that the proposed method would be promising for the therapeutic monitoring of AEDs. The method is still under development, and a more detailed study concerning the isolation of various AEDs from serum will be published later.

REFERENCES

1 J.P. Nautsch, L.H. Rolf and G.G. Brune, J. Neurol., 223 (1980) 219.
2 N. Inotsume, A. Higashi, E. Kinoshita, T. Matsuoka and M. Nakano, J. Chromatogr., 383 (1986) 166.
3 M.I. Aranz-Pena, J. Chromatogr., 222 (1981) 486.
4 J. Volmut, 5th Danube Symposium on Chromatography, Yalta, 1985, Abstracts, p. 136.
5 Quantitative Analysis of Underivatized Antiepileptic Drugs, Bulletin 779, Supelco, Bellefonte, PA, 1979.
6 L.L. Plotczyk, J. Chromatogr., 240 (1982) 349.
7 J. Volmut, Biochem. Clin. Bohemoslov., 17 (1988) 153.
8 V. Hudecová, A. Šimonyiová and J. Volmut, Biolab 86, Cheb, 1986, Abstracts, p. 12.
9 M. Werner, R.J. Mohrbacher and J. Riendeau, Clin. Chem., 25 (1979) 2020.
10 Application Notes AN-006, Baker Disposable Extraction Columns for Rapid Extraction of Anticonvulsants from Serum for Analysis by GC, J.T. Baker, Phillipsburg, NJ, 1981.
11 J. Volmut, E. Matisová and Pham Thi Ha, J. High Resolut. Chromatogr., 12 (1989) 760.
12 F. Andreolini, C. Borra, A. Di Corcia and R. Samperi, J. Chromatogr., 310 (1984) 208.