Journal of Chromatography, 371 (1986) 307–312
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 941

Note

High-performance liquid chromatographic determination of antiepileptic drugs by an advanced automated sample processor

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Many different methods for the high-performance liquid chromatographic (HPLC) analysis of antiepileptic drugs (AEDs) have been published. Some of these include sample pretreatment procedures based on liquid–solid extraction[1–12]. Four papers describe methods in which extraction and chromatographic analysis are combined together in an on-line procedure[9–12].

In 1983 Kabra *et al.*[8] published an off-line procedure that uses Bond-Elut columns (with a C_{18} packing) for the adsorption of AEDs from serum samples. After the purification step the AEDs are eluted from the column with 300 μl of methanol. Only 5 μl of the eluate are injected into a short analytical column for "Fast-HPLC" separation.

We tested the suitability of the chromatographic conditions mentioned above[8] for our analytical purposes. Unfortunately, no separation could be obtained between carbamazepine-10,11-epoxide (EPO) and N-desmethylmethsuximide (DM). The latter drug is not found in patient samples in California[13].

A new system for sample pretreatment has recently been developed, called "Advanced Automated Sample Processor", AASP[14]. Instead of single extraction columns (Bond-Elut), cassettes for ten samples are used in sample pretreatment. After purification of the samples the cassettes are placed in the AASP, and the adsorbed drugs are eluted directly into the analytical column by the mobile phase. By this means, remarkable band broadening can arise, such as is mentioned by Uihlein[15] in his chapter dealing with general observations on sample pretreatment techniques.

We have developed a method that enables the simultaneous determination of carbamazepine (CBZ), phenytoin (PT), phenobarbital (PB), primidone (PR), ethosuximide (ET), 2-ethyl-2-phenylmalone diamide (PEMA), 10,11-dihydro-10,11-dihydroxycarbamazepine (DIOL), EPO, and DM in serum using the AASP system for sample pretreatment. A gradient elution circumvents the chromatographic problems mentioned above.

MATERIALS AND METHODS

Apparatus

Equipment was obtained from the following firms: an automatic pipetter/diluter from Corning/Gilford (Düsseldorf, F.R.G.), an AASP and a Vac-Elut chamber

0021-9673/86/$03.50

from Varian (Darmstadt, F.R.G.), a high-vacuum pump from Barkey-Labortechnik (Bielefeld, F.R.G.), a low-pressure gradient-former 250 B, a high-precision pump 300 B, a spectrophotometer SP-4, an Integrator C-R3A, and an analytical column shandon-ODS Hypersil (5 μm, 250 × 4.6 mm I.D.) from Gynkotek (Munich, F.R.G.).

Chemicals and reagents

Acetonitrile (ChromAR grade) was obtained from Promochem (Wesel, F.R.G.) and water for HPLC from Baker (Deventer, The Netherlands). All other chemicals were of analytical-reagent grade, and were obtained from Merck (Darmstadt, F.R.G.).

Phosphate buffer (pH 4)–diluted phosphoric acid (0.01%) was buffered with saturated disodium hydrogen phosphate solution to pH 4. The pH was adjusted using an electronic pH meter.

Standards

We obtained CBZ, PB, PT, PR, and ET from Desitin Werk (Hamburg, F.R.G.); PEMA, DM, and 5-ethyl-5-(*p*-tolyl)barbituric acid (ETB) for use as internal standard (I.S.) from Aldrich (Steinheim, F.R.G.); EPO and DIOL from Ciba-Geigy (Basle, Switzerland).

The I.S. solution was made as follows: 10 mg of ETB were dissolved in 10 ml of methanol and made up to 1000 ml with a buffer solution containing 1% ammonium acetate and 1% sodium azide.

Calibration samples

A 50-μl volume of a stock solution containing 25 mg of PEMA, 100 mg of ET, 25 mg of PR, 5 mg of DIOL, 50 mg of PB, 50 mg of DM, 5 mg of EPO, 25 mg of PT, and 15 mg of CBZ per 100 ml of acetonitrile and 500 μl of ethyl acetate were pipetted into a centrifuge tube with a Gilford diluter. The solvents were evaporated off and the residues were taken up in 500 μl of a 5% bovine albumin solution on shaking for 60 min. The centrifuge tubes were closed with screw caps and frozen at −18°C.

Sample pretreatment

As working with the positive air-pressure manifold of the Vac-Elut system, such as delivered by the manufacturer, was very time-consuming, we preferred the use of a high-vacuum pump for purging the cassettes and for the sample clean-up procedure.

Before the samples were loaded, the AASP cassette was purged with 2 ml of methanol and 2 ml of dilute phosphoric acid (0.01%, v/v). A 100-μl aliquot of each of the patient serum samples, the calibration samples and the commercially available control sera was mixed with 300 μl of the I.S. buffer solution.

From these diluted samples, 100 μl (corresponding to 25 μl of serum) were pipetted into the cassette. The vacuum was switched on, and the samples washed with 0.5 ml of dilute phosphoric acid. The cassette was removed from the Vac-Elut. A further 1.5 ml of phosphoric acid were pumped by the purge pump of the AASP through the cassette packing immediately before every injection. The pre-injection purge was needed because air trapped in the partially dried cassettes, which would

cause spikes in the chromatograms, must be removed. In addition a post-injection purge was needed to remove the remaining mobile phase in the capillaries between the switching valve and the AASP. It must be replaced by non-eluting purge liquid, otherwise the adsorbed drugs would be eluted from the cassette during the pre-injection cycle[14].

In our investigation it was possible to reuse a cassette ten times under the given analytical conditions.

Chromatographic parameters

Column temperature, 70°C; detection wavelength, 205 nm; mobile phase A = phosphate buffer (pH 4)–acetonitrile (9:1), mobile phase B = phosphate buffer (pH 4)–acetonitrile (4:6); flow-rate, 1.5 ml/min. Gradient programme: min 0, 15%B (17.5% acetonitrile); min 1, 15%B; min 8, 50%B (35% acetonitrile); min 10, 50%B; min 10.1, 15%B; min 14, stop.

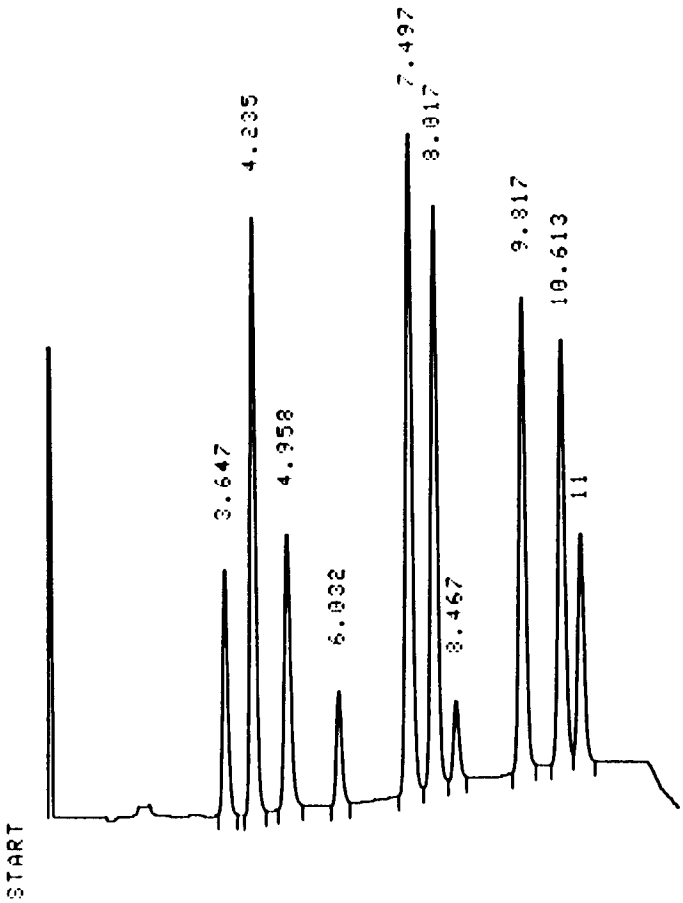

Fig. 1. Chromatogram of a calibration sample. Retention times (min) and AED concentrations (μg/ml): PEMA, 3.647 and 25; ET, 4.235 and 100; PR, 4.958 and 25; DIOL, 6.032 and 5; PB, 7.497 and 50; DM, 8.017 and 50; EPO, 8.467 and 5; ETB (I.S.) 9.817; PT, 10.613 and 25; CBZ, 11.000 and 15.

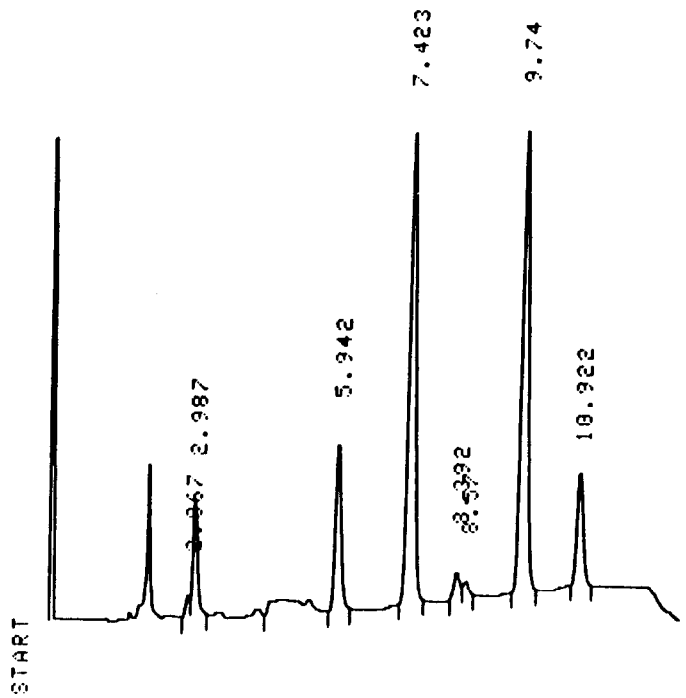

Fig. 2. Chromatogram of a patient sample. Retention times (min) and AED concentrations (μg/ml): DIOL, 5.942 and 7.7; PB, 7.423 and 36.0; EPO, 8.392 and 1.0; ETB (I.S.), 9.740; CBZ, 10.922 and 7.3.

RESULTS AND DISCUSSION

One of the main advantages of the AASP system for sample processing is that the entire amount of purified sample can be transferred to the analytical column. Thus, in principle, considerably smaller amounts of sample can be analysed than by the Bond-Elut system, for example. Using the latter system only aliquots of the last eluate can be injected. Thus, no difficulty is envisaged in determining AEDs from 10 μl of serum, or less, using the sample preparation method described in this paper. On the other hand, volumes of 100 μl, or more, can be pipetted, certainly with greater accuracy. Thus, a suitable compromise was found using the sampling procedure described in the Experimental section.

The advantage of the small amount of sample needed applies to the AASP as well as to the direct injection of serum with a pre-column switching system[10]. But sample clean-up with the AASP cartridges gives cleaner chromatograms (Figs. 1 and 2). The cartridges can, under the given conditions, be reused ten times, but a pre-column in a column-switching system is normally loaded with *ca.* 60 samples in a working day. Contamination is more likely to occur here.

The problem of band broadening of the peaks in the chromatogram, which was mentioned earlier, can be overcome by using gradient elution.

The less polar substances require time to elute from the packing material of the cassette. This is seen in the partial loss of PT and CBZ in the chromatogram if

TABLE I

DAY-TO-DAY PRECISION OF THE AED ANALYSES

Control serum; n = 16 days.

AED	X_{min}	X_{max}	X_m	*S.D.*	*C.V.(%)*
ET	111.7	125.2	120.5	4.57	3.79
PR	11.2	12.6	11.8	0.38	3.19
PB	47.8	51.9	49.8	1.07	2.16
PT	28.1	30.1	29.1	0.60	2.07
CBZ	11.8	12.8	12.3	0.30	2.45

the time of switching the AASP valve is less than 2 min. If the same cassette position is used again in the injection cycle, and the time interval until the valve is switched back is extended, then the remnants of PT and CBZ appear in the ensuing chromatogram.

There is a 100% recovery for all the mentioned AEDs up to double the concentration of the calibration samples, if the samples are diluted prior to pipetting into the cartridges. This applies to C_{18} as well as C_8 cartridges. Should the serum samples not be diluted, a loss in recovery of ET can result with C_8 cartridges. A further advantage of diluting the serum samples, namely improved pipetting, has already been mentioned above.

Finally, by this means the samples can be mixed with an internal standard solution. Even should a leak occur with the Vac-Elut system the results are not invalidated owing to a slight loss of sample.

TABLE II

COMPARISON OF THE RESULTS OF THE LIQUID–SOLID EXTRACTION METHODS (AASP AND PRE-COLUMN SWITCHING) WITH THE RESULTS OF THE ETHYL ACETATE EXTRACTION[16]

$y = b \cdot x + a$; x = AED concentration measured by the ethyl acetate extraction method; y = AED concentration measured by the AASP or the direct injection (DI) method; b = slope of regression line; a = intercept; r = coefficient of correlation; S.E.E. = standard error of estimate; n = number of samples; $M(x)$ = mean value of x (μg/ml), $M(y)$ = mean value of y (μg/ml).

AED	*Method*	*n*	*M(x)*	*M(y)*	*b*	*a*	*r*	*S.E.E.*
ET	AASP	23	50.66	53.84	0.913	6.70	0.989	3.23
ET	DI	23	50.66	51.10	0.883	6.36	0.990	2.88
PR	AASP	43	8.89	9.21	0.977	0.53	0.995	0.58
PR	DI	43	8.89	8.99	0.963	0.43	0.993	0.68
PB	AASP	272	25.83	26.44	1.034	−0.27	0.991	1.58
PB	DI	272	25.83	25.67	1.007	−0.34	0.989	1.65
DM	AASP	31	24.96	25.54	1.016	0.19	0.985	1.90
DM	DI	31	24.96	23.68	0.929	0.49	0.975	2.27
PT	AASP	197	10.79	11.20	1.030	0.09	0.994	0.75
PT	DI	197	10.79	10.88	1.004	0.05	0.991	0.95
CBZ	AASP	275	6.75	6.98	1.011	0.16	0.979	0.63
CBZ	DI	275	6.75	6.38	0.945	0.01	0.949	0.66

The day-to-day precision of the HPLC method described here was tested on sixteen different days using control sera. The results are summarized in Table I.

The results obtained using the AASP method were compared with those obtained from direct injection of serum using pre-column switching[10] and from ethyl acetate extraction[16]. Thus, the patient samples were analysed in turn using all three methods. The results of the routine extraction with ethyl acetate were taken as a reference basis (see Table II).

As seen from Tables I and II, there is good agreement of the results from all three methods. The coefficients of variation for all the AEDs in the control sera are less than 5%. It can be concluded that the HPLC method using the AASP system for sample pretreatment is well suited to routine use.

ACKNOWLEDGEMENTS

The author is especially grateful to Mr. G. S. Macpherson for translating the paper, to Dr. T. May for the development of the statistical programs, to Dr. B. Rambeck for helpful discussions, and to Mrs. G. Kunert and Mrs. M. Berbuesse for technical assistance.

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