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Note

Simultaneous determination of Zonisamide and nine other anti-epileptic drugs and metabolites in serum

A comparison of microbore and conventional high-performance liquid chromatography

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Zonisamide* (1,2-benzisoxazole-3-methane-sulfonamide) is a new anti-epileptic drug which has been shown in a pilot study to be effective in the treatment of patients with refractory partial seizures[1]. In this pilot study and in two pharmacokinetic studies[2,3] blood levels of Zonisamide (ZA) were determined by high-performance liquid chromatography (HPLC) under isocratic conditions. Unfortunately, these chromatographic conditions were only suitable for the analyses of samples from patients on ZA mono-medication, and not for the simultaneous determination of other anti-epileptic drugs (AEDs) and their metabolites.

Since September 1984, we have received more than 1000 patient samples for analysis, for a European double-blind and open-label study of the Parke Davis Co. These blood samples contained ZA and additional AEDs with their metabolites.

Thus, we developed an HPLC method for the simultaneous determination of ZA, carbamazepine (CBZ), phenytoin (PT), phenobarbital (PB), primidone (PR), ethosuximide (ET), N-desmethylmethsuximide (DM), carbamazepine-10,11-epoxide (EPO), 10,11-dihydro-10,11-dihydroxycarbamazepine (DIOL), and 2-ethyl-2-phenylmalonediamide (PEMA).

In 1982, we compared the three main methods of sample pretreatment for the determination of AEDs in serum by HPLC: protein precipitation[4–7], liquid–liquid extraction[8–12], and liquid–solid extraction[13–17]. After the analyses of more than 2000 patient samples[18] the most satisfactory method was found to be liquid–liquid extraction with ethyl acetate. This solvent has been increasingly used in recent years for the extraction of AEDs from serum[10–12,19–21]. A six-fold quantity of ethyl acetate, added to a serum sample, gives a complete recovery of all AEDs[18], as well as ZA.

Until last year, we carried out the chromatographic separations by gradient elution, using a conventional column[22]. Last year we developed chromatographic conditions for the use of microbore HPLC columns for routine analyses. The analytical results obtained using both types of columns are compared, and the economical aspects are discussed in this paper.

* AD-810 from Dainippon Pharmaceutical Co., Japan or CI-912 from Warner Lambert Co., U.S.A.

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MATERIALS AND METHODS

Apparatus

The following equipment was used. An automatic pipetter/diluter from Corning/Gilford (Düsseldorf, F.R.G.). A Rotixa/K centrifuge from Hettich (Tuttlingen, F.R.G.). A vortex mixer/evaporator from Buchler Instruments (Searle Analytic, Fort Lee, NJ, U.S.A.). The liquid chromatograph (LC) used with the conventional columns (HP 1084B), the LC used with the microbore columns (HP 1090) and the microbore columns (200 mm × 2.1 mm I.D., packed with Shandon Hypersil(R) ODS, particle size 5 μm) were all from Hewlett-Packard (Waldbronn, F.R.G.). The conventional columns (125 mm × 4.6 mm I.D.) and the directly connected guard columns (40 mm × 4.6 mm I.D.), both packed with the same material as the microbore columns, were obtained from Grom (Ammerbuch, F.R.G.). Rheodyne HPLC column inlet filters with exchangeable frits (1.5 mm diameter, 0.5-μm pore size) were obtained from ERC (Alteglofsheim, F.R.G.).

Chemicals and reagents

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

We obtained ZA from Parke Davis (Munich, F.R.G.); CBZ, PB, PT, PR, and ET from Desitin Werk (Hamburg, F.R.G.); PEMA, DM, 5-ethyl-5-(*p*-tolyl)barbituric acid (ETB) and methyl propylsuccinimide (MPS) = internal standards (I.S.) from Aldrich (Steinheim, F.R.G.); EPO and DIOL from Ciba-Geigy (Basel, Switzerland).

I.S. solution: 5 mg ETB and 15 mg MPS (for the calibration of ET) were dissolved in 5 ml methanol and mixed with 1 l ethyl acetate.

Buffer pH 4 solution: 0.05 *M* sodium dihydrogen phosphate solution was adjusted with phosphoric acid (1%) to pH 4.

Calibration samples and control samples

To 10 ml centrifuge tubes 500 μl calf serum, and 50 μl of a stock solution containing 30 mg ZA, 25 mg PEMA, 100 mg ET, 25 mg PR, 5 mg DIOL, 50 mg PB, 50 mg DM, 5 mg EPO, 25 mg PT, and 15 mg CBZ in 100 ml acetonitrile were added. The tubes were closed with screw caps and frozen at −18°C.

The Merckotest(R) EMIT-AED control (from Merck) was used as control sample for therapeutic AED values and for toxic levels the UTAK control —high range (from DMD, Gailingen, F.R.G.) was used. As control sera for the ZA analyses samples containing different concentrations of ZA were prepared. Some of these pooled samples were fortified with a stock solution containing ZA in order to obtain controls with toxic concentrations.

Extraction procedure

To 500 μl serum 3 ml I.S. solution was added. After mixing for 10 min at 35°C and centrifuging, the supernatant extract was transferred to a clean tube, and the solvent was evaporated under vacuum at 35°C. Ethyl acetate must be removed com-

pletely from the sample, because it interferes with ZA in the chromatographic separations. The dry residue was dissolved in 100 μl of methanol.

Chromatographic parameters

The mobile phase for the gradient elution consisted of two mixtures (A and B) of acetonitrile and phosphate buffer (pH 4). The solvents were pre-mixed for two reasons, namely that degassing can be avoided during gradient mixing, and that mobile phases with a minimum content of 10% acetonitrile are stable for a long time.

Mixture A consisted of phosphate buffer–acetonitrile (90:10, v/v), and mixture B of phosphate buffer–acetonitrile (40:60, v/v). The gradient programme was as follows. From 0 to 0.5 min: 15% B (isocratic); from 0.5 to 9.5 min: 15 to 45% B (linear gradient); from 9.5 to 13 min: 45% B (isocratic); from 13 to 15 min: return to 15% B. Consequently, *ca.* 96 samples can be analysed in 24 h. The column temperature was 60°C. The detection wavelength was 205 nm. A scan of the adsorption curve of ZA in the mobile phase gave three maxima at 202, 242 and 286 nm (scanned by the HP 1084B after stopping the flow). A detection wavelength of 205 nm was chosen in order to get a reasonable sensitivity for ET.

Only two parameters were different for the conventional columns (CC) and the microbore columns (MC). For CC the flow-rate was 1.2 ml/min and the injection volume 5 μl. For MC the flow rate was 0.3 ml/min and the injection volume 2 μl.

The corresponding chromatograms of a calibration and a patient sample are shown in Figs. 1–4.

Lifetime of the columns

With both types of columns used about 4000 samples could be injected under the chromatographic conditions described above. To achieve such a large number of separations, the conventional columns had to be protected by a 4-cm guard column, which was filled with the same material as the analytical column. These guard columns were replaced after *ca.* 1000 injections.

The microbore columns had to be protected from blockage by in-line filter frits, which have a minimal influence upon band-broadening. The frits were changed after *ca.* 500 injections. We tested the first microbore column without such an in-line filter. As a consequence, the column became clogged after *ca.* 350 injections.

RESULTS AND DISCUSSION

Linearity and recovery

First of all different volumes of ZA stock solution (10–150 μl) were mixed with 3 ml I.S. solution. The solvents were evaporated, and the residues taken up in methanol and injected. The sample with 50 μl stock solution (corresponding to 30 μg/ml ZA, see Materials and methods) was taken as the calibration standard. The results obtained after HPLC analysis showed an excellent linearity in the concentration range of 6 to 90 μg/ml ZA.

In order to test the recovery of the serum extraction, the same series of samples as described above was produced in duplicate, and 500 μl of calf serum was added to each tube. The results of the ethyl acetate extraction of the serum samples are listed in Table I. The mean recovery was 100.2% (correlated with the calibration

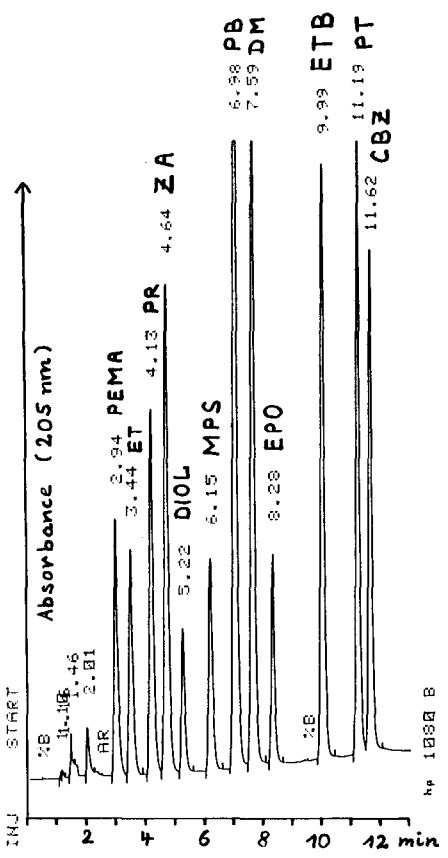

Fig. 1. Chromatogram of a calibration sample obtained from a conventional column.

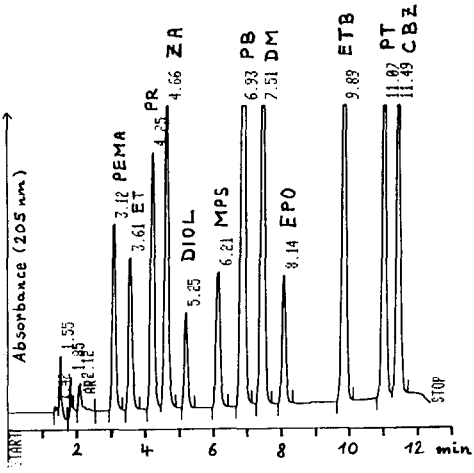

Fig. 2. Chromatogram of a calibration sample obtained from a microbore column.

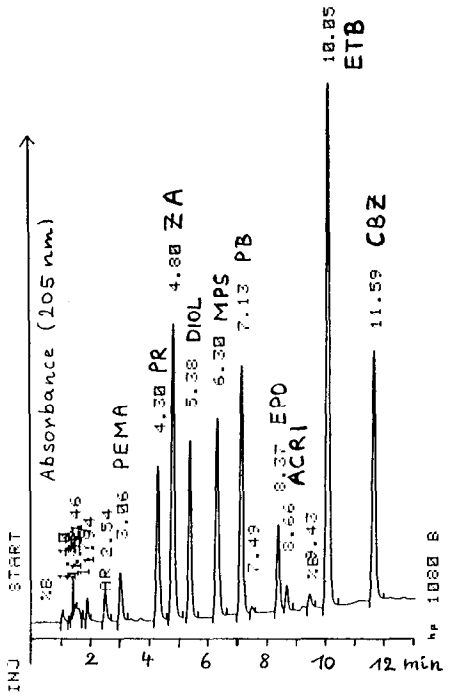

Fig. 3. CC-chromatogram of the serum from patient No. 674/3. (ACRI = 9-hydroxymethyl-10-carbamoyl acridan, a metabolite of CBZ which is a ring-contracted product of EPO in the biotransformation pathways[23].

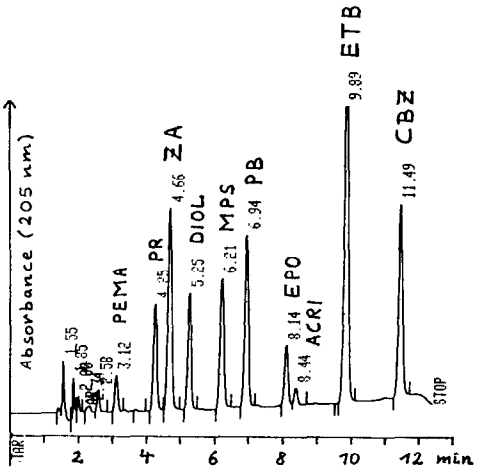

Fig. 4. MC-chromatogram of the serum from patient No. 674/3.

TABLE I

LINEARITY AND RECOVERY OF THE ZONISAMIDE ANALYSIS

$Y = a + bX_m$ (μg/ml). Y = theoretical value, a = intercept, b = slope of regression line, X_m = mean value of the duplicates, r = coefficient of correlation, SL = stock solution (μl). $a = -0.119$, $b = 0.004$, $r = 0.99995$, standard error of estimate = 0.328.

SL	*Y*	X_m	*Recovery (%)*
10	6.0	6.16	102.7
20	12.0	12.22	101.8
30	18.0	18.03	100.2
50	30.0	29.80	99.3
75	45.0	44.88	99.7
100	60.0	59.04	98.4
125	75.0	74.82	99.8
150	90.0	89.79	99.8

sample mentioned above), and the linearity of the results was nearly as good (r = 0.99995) as without serum extraction.

Precision of the results

The comparison of the results obtained using conventional columns or microbore columns was based not only on results of analyses of samples with ZA but also on those of the remaining routine analyses of AEDs (*ca.* 15000 per year).

As a measure of the quality of the results, we checked the day-to-day precision of the analyses of the two commercial control sera and of the pooled ZA samples (including toxic levels, see Materials and methods).

Tables II and III show that the day-to-day precision of the conventional and the microbore HPLC is equally good in the routine analyses of AEDs in serum. The coefficients of variation in the determination of AEDs are, apart from ET, below 5%. The somewhat higher values for ET (C.V.: 4.7–5.4%) are, in this case, certainly less due to the analytical columns than to the method of sample processing, which includes evaporation under vacuum.

Economical aspects

In order to compare the cost of both of the column systems an average column lifetime of 4000 sample injections (*cf.* Materials and methods) was taken as the basis for our calculations.

One analytical column (125 mm × 4.6 mm I.D.) and four guard columns (40 mm × 4.6 mm I.D.) on the average were required for 4000 injections in conventional HPLC. The refill cost was *ca.* US$ 175. Furthermore, 72 l of mobile phase (of which *ca.* 25% was acetonitrile) were needed for a flow-rate of 1.2 ml/min at a cost of *ca.* US$ 540.

In the case of microbore HPLC one analytical column (200 mm × 2.1 mm I.D.) and eight in-line filter frits (0.5 μm) on the average were needed for 4000 samples, at a cost of US$ 380. Only 18 l mobile phase were needed for a flow-rate of 0.3 ml/min at a cost of US$ 135.

TABLE II

DAY-TO-DAY PRECISION OF THE ZONISAMIDE ANALYSES

PS = pool sample, CC = conventional column, MC = microbore column, N = number of days, X_m = mean value of x (μg/ml), S.D. = standard deviation, C.V. = coefficient of variation.

PS	*N*	*Type*	X_m	*S.D.*	*C.V. (%)*
A	46	CC	8.40	0.17	2.1
B	35	CC	18.69	0.43	2.3
C	78	CC	43.27	0.60	1.4
D	60	CC	30.00	0.47	1.6
E*	95	CC	6.26	0.14	2.3
E*	46	MC	6.19	0.11	1.7
F	46	CC	24.22	0.67	2.8
F	69	MC	24.21	0.56	2.3
G	35	CC	59.87	1.34	2.2
G	42	MC	59.43	1.13	1.9
H	15	CC	16.86	0.34	2.0
H	36	MC	16.75	0.39	2.4

* Pool sample E was divided up into 250-μl portions.

TABLE III

DAY-TO-DAY PRECISION OF THE AED ANALYSES

AED	*Type*	X_m	*S.D.*	*C.V. (%)*
Control serum I, N = 98 days				
ET	CC	69.96	3.64	5.2
	MC	72.08	3.35	4.7
PR	CC	11.19	0.27	2.4
	MC	11.37	0.28	2.5
PB	CC	28.83	0.61	2.1
	MC	28.89	0.63	2.2
PT	CC	14.14	0.45	3.2
	MC	14.06	0.41	2.9
CBZ	CC	5.59	0.13	2.3
	MC	5.59	0.14	2.6
Control serum II, N = 50 days				
ET	CC	111.35	5.46	4.9
	MC	109.34	5.85	5.4
PR	CC	12.18	0.31	2.6
	MC	12.11	0.42	3.5
PB	CC	48.17	1.01	2.1
	MC	47.99	0.86	1.8
PT	CC	29.91	0.78	2.6
	MC	29.25	0.92	3.2
CBZ	CC	12.74	0.29	2.3
	MC	12.47	0.48	3.8

The total cost of material for the chromatographic analysis of 4000 samples in our laboratory thus amounts to *ca.* US$ 715 in the case of conventional HPLC, and for microbore HPLC to *ca.* US$ 515. This means that the saving in costs of solvents is of greater significance than the higher price of the microbore columns.

A further aspect is that the quantity of waste solvent, which has to be disposed is considerably reduced in the case of the microbore technique.

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