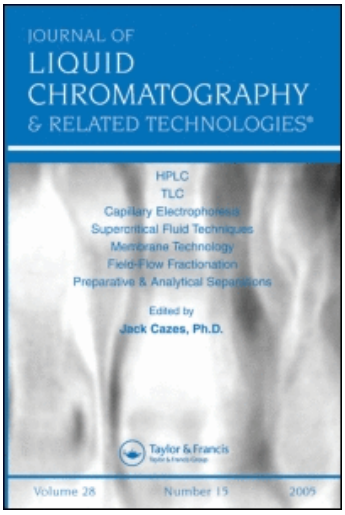

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SENSITIVE ANALYSIS OF ANTIEPILEPTIC DRUGS IN VERY SMALL PORTIONS OF HUMAN BRAIN BY MICROBORE HPLC

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ABSTRACT

A method for the simultaneous determination of phenobarbital (PB), phenytoin (PT), carbamazepine (CBZ), carbamazepine-10,11- epoxide (EPO), and N-desmethyl methsuximide (DM) in very small amounts of human brain (20-100 mg) is described. The weighed tissue is homogenized with a neutral buffer solution containing the internal standard (IS) 5-ethyl-5-(p-tolyl)-barbituric acid (ETB). The antiepileptic drugs (AEDs) are extracted with acetone from the homogenate. After centrifugation the drug solution is transferred onto an Extrelut(R)-III tube (filled with kieselguhr) and an acidic buffer is added. The AEDs are extracted from the matrix by di-chloro methane / propanol-(2) (97:3). The solvents are evaporated off and the residues taken up in methanol/water. The concentrated drug solution is injected into a microbore column which is filled with ODS silica gel (5 um). The mobile phase for the gradient elution is a mixture of a 0.05 M ammonium di-hydrogen phosphate buffer (pH 4.4) and acetonitrile. The column temperature is 65°C and the detection wavelength 207 nm.

INTRODUCTION

An uncomplicated and yet very precise micro-method for the determination of antiepileptic drugs (AEDs) in human brain tissue, which can be used in routine work would be of interest from various points of view. In connection with therapy resistance of some epileptic patients, inspite of adequate serum concentrations, it could be clarified (post mortem) if different areas of the brain, above all the area of an epileptic focus, have different tissue concentrations.

A study of Rapport et al.[1] indicates that the ratio of the PT concentration in the cortex compared with the serum is significantly lower in epileptic patients than in a nonepileptic control group. An explanation may be either a decreased blood flow to the brain area or a decreased penetration of the drug, both secondary to gliosis and scarring [1].

A micro-method would, in addition, be desirable in connection with epilepsy brain surgery. The AED-tissue concentrations in the area of operable tumors, or epileptic foci which can be surgically treated should be determined and related to the AED serum concentrations.

Our aim was to develop a method by which a large number of tissue samples can be routinely analysed. Very low weights of samples (down to ca. 20 mg) should be analysed with sufficient precision and reproducibility. This method should also enable slight differences between the tissue samples to be determined.

The methods published hitherto [2-7] for the analysis of AEDs in tissues by high performance liquid chromatography (HPLC) appeared to be unsuitable for our purposes. The quantities of sample required in the papers were greater than mentioned above (20-100 mg). In the papers cited aliquots of homogenates are analysed. We needed a method for the complete recovery of the AEDs from every single portion of brain.

Apart from this, in only two of the papers [3,4] were human brain samples investigated. We also had to consider the specific problems of the human brain (e.g. differences between the grey and the white matter [4]). In additon we tried to include in our method a sample pretreatment which allows not only the investigation of the relatively smooth tissues of the cerebrum and the cerebellum but also the firm tissue of the peripheral nerves.

MATERIALS

Apparatus

Equipment was obtained from the following firms: automatic pipetter/diluter from Corning/Gilford, Duesseldorf (F.R.G); Ultra-turrax homogenator from IKA, Staufen (F.R.G.); Rotixa/K centrifuge from Hettich, Tuttlingen (F.R.G); vortex mixer/evaporator from Buchler Instruments, Searle Analytic Inc., Fort Lee, NJ (U.S.A); HPLC 1090 and analytical column (20.0 cm x 2.1 mm I.D.) filled with Shandon Hypersil(R) ODS (particle size 5 um) from Hewlett/-

Packard, Boeblingen (F.R.G). Rheodyne HPLC column inlet filters with exchangeable frits (1.5 mm diam., 0.5 um pore size) from ERC GmbH, Alteglofsheim (F.R.G.).

Reagents

Acetonitrile ChromAR(R) was obtained from Promochem, Wesel (F.R.G) and water for HPLC from Baker Chemicals, Deventer (The Netherlands). Extrelut(R)-3 tubes and all other chemicals were of analytical reagent-grade and obtained from Merck, Darmstadt (F.R.G). Albumine (bovine fraction V) was from Paesel, Frankfurt a.M. (F.R.G.).

Extraction buffer pH 6.0: 20.0 g sodium di-hydrogen phosphate 2-hydrate, 4.5 g di-sodium hydrogen phosphate 2-hydrate, and 1.5 g sodium azide are dissolved in 1 l of deionized water (with NaN_3 the solution is stable at room temperature).

Extracting solvent: 970 ml di-chloro methane are mixed with 30 ml propanol-(2).

The mobile phase for the gradient elution consisted of two mixtures of a 0.05 M ammonium di-hydrogen phosphate buffer (pH 4.4) and acetonitrile: mixture A with a ratio of 90:10 and mixture B with a ratio of 40:60 (buffer/acetonitrile). A minumum content of 10% of acetonitrile enhances the stability of buffer solutions.

Standards

We obtained PT, PB, primidone (PR), CBZ, and EPO from Desitin, Hamburg (F.R.G); DM and ETB from Aldrich, Steinheim (F.R.G).

Internal standard (IS) solution: 15 mg ETB were dissolved in 100 ml acetonitril. From this stock solution 10 ml are mixed with 1 l of an ammonium acetate/sodium azide (1%/1%) buffer solution.

Calibration Samples

A stock solution is produced containing 10 mg PR, 20 mg PB, 20 mg DM, 5 mg EPO, 10 mg PT, and 10 mg CBZ in 100 ml acetonitrile. From this stock solution 10 ml are made up with an albumine solution (5% in deionized water) to 100 ml. From this standard solution 100 ul portions are dispensed by an automatic dispenser into 2-ml vials and frozen at -18°C.

METHODS

Pre-treatment of the tissue samples

Small pieces of the brain tissue (20-200 mg) are placed in a centrifuge tube and 1.0 ml of the IS solution added. The sample is homogenized by an Ultra-turrax and the rotor of the apparatus flushed with 1 ml of the extraction buffer. To the sample solution (2 ml) 1 ml of acetone is added and the tube is shaken for 5 min. After centrifugation for 10 min the supernatant fluid is transferred to an Extrelut-3 tube and a further 1 ml portion of the extraction buffer is added. After a delay of 10 min. the AEDs are extracted from the tube with 15 ml of the extracting solvent. The solvent is evaporated off and the

residue is taken up in 50 ul of methanol and then diluted with 50 ul of water. The mixture of methanol/water (1:1) instead of pure methanol, allows injection volumes of 10-25 ul.

Pre-treatment of calibration samples and body fluids

The calibration samples (100 ul) and - if additionally available from the patient - 100 ul of the serum or blood samples (after complete haemolysis of post mortem blood) are mixed with 1.0 ml of the IS solution. These dilutions are transferred to centrifuge tubes, mixed with 1 ml of extraction buffer and treated in the same manner as described for the tissue samples.

From the liquor samples 500 ul are taken, mixed with 500 ul of the IS solution, and 1.0 ml of the extraction buffer is added before the acetone extraction.

Chromatographic Conditions

The column temperature is 65°C. The detection wavelength is 207 nm. The flow rate is 0.3 ml/min. The gradient time programme is as follows: at 0.5 min 15% B, at 9.5 min 45% B, at 10.0 min 45% B, and at 10.5 min 15% B (15% B corresponds to 17.5% acetonitrile and 45% B to 32.5% acetonitrile). The stop time is 12 min and the post run time 1 min. The injection volume is normally 10 ul, but it can be increased by up to 25 ul if necessary (very low sample amounts or AED concentrations). If in-line filters with exchangeable frits (0.5 um) are used to protect the analytical column more than 4,000 injections can be made [9].

RESULTS AND DISCUSSION

In 1986 we investigated brain samples taken after death from 26 epileptic patients. The patients were on PB, PT or CBZ medication. Only one patient was on mesuximide therapy. Our method was developed for the determination of PB, PT, CBZ, EPO, and DM because these AEDs are of the greatest interest in investigating the brain tissues.

Primidone is also added to the calibration samples in order to enable us to measure this drug, if necessary. On the other hand this could be difficult because in the early part of the chromatograms interfering compounds could often be observed (compare with fig. 2).

In the cited publications [3-7] aliquots of tissue homogenates were taken. In our method which was developed for the smallest samples of tissue possible, the total weight of sample taken must be extractable. The IS is already added to the sample before the homogenizing process. Thereafter the volume of the homogenate has no further influence on the result of the analysis, and the AED concentration obtained after HPLC separation can be directly calculated in terms of the tissue concentration.

Since every tissue sample as well as the calibration samples are mixed with 1 ml IS buffer, the result is expressed as ug AED per mg of tissue. This result multiplied by the factor of 1000/mg of the sample weight gives the final result of ug AEDs in 1 g tissue.

The results of different brain samples can thus be well compared with each other, and also with the results

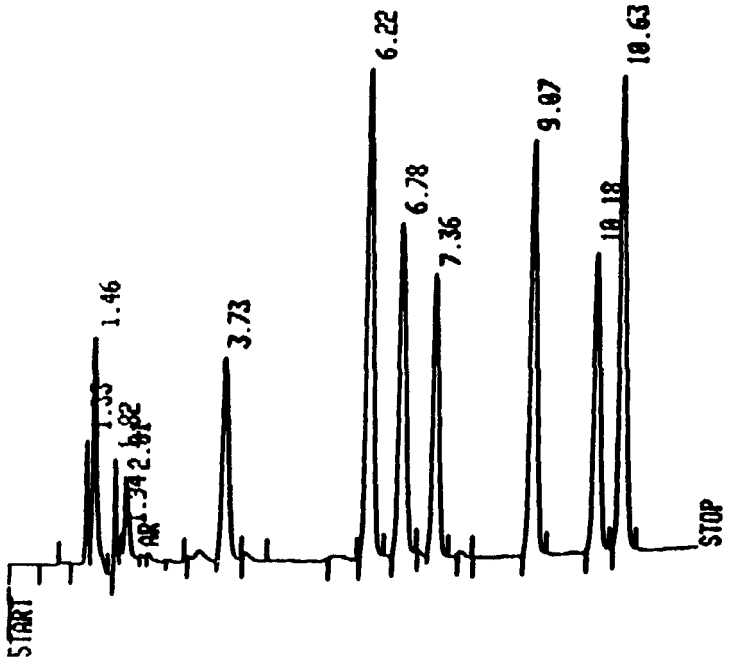

FIGURE 1 Chromatogram of a calibration sample. RT: 3.73 PR, 6.22 PB, 6.78 DM, 7.36 EPO, 9.07 ETB (IS), 10.18 PT, 10.63 CBZ.

from 1 ml liquor and 1 ml serum or blood. Should no serum be obtainable from the post mortem sample then the full blood sample is examined. Published methods [8,9] for the analysis of AEDs in body fluids can be used for examining the blood, serum or liquor samples.

In order to analyse all samples from the same patient in the same series of experiments, the procedure for the tissue samples was chosen (with slight modifications - see exp. part) also for the serum and liquor samples.

For the total extraction of AEDs from the diluted tissue homogenate a solid/liquid extraction procedure

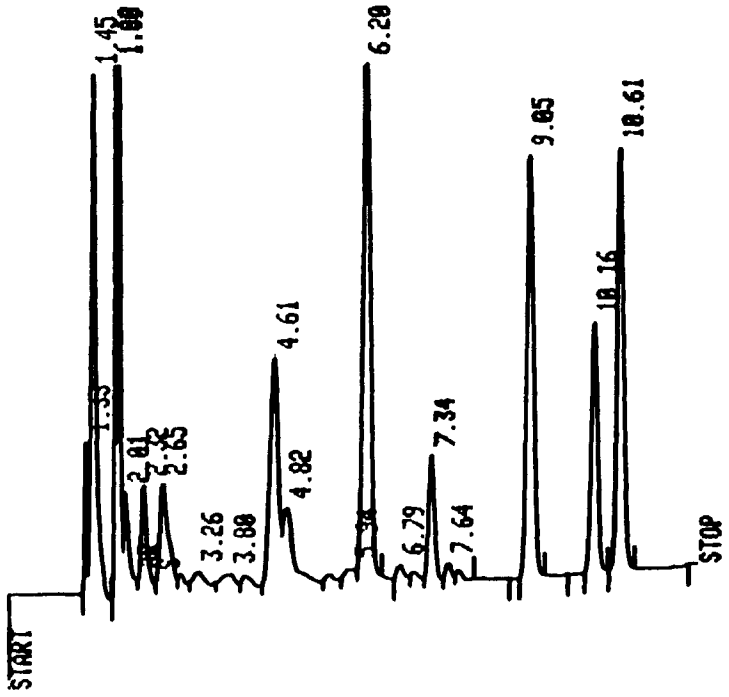

FIGURE 2 Chromatogram of a pooled brain tissue sample. RT/Conc.[ug/ml]: 6.20 PB (2.39), 7.34 EPO (0.21), 9.05 ETB (IS), 10.16 PT (0.82), 10.61 CBZ (0.88).

seemed to be the best choice. Reversed phase silical gel [10,11] as well as Kieselguhr [12,13] were used as the solid matrix for preliminary trials. The brain homogenate, however, led in every instance to a blockage of the extraction tubes.

Hence, the coarse particles must first of all be precipitated from the homogenate. Perchloric acid, as a precipitation agent, was not considered because the recovery rate of the AEDs is thereby lowered [14]. Out of the organic precipitation agents for proteins acetone was

preferred to acetonitrile because it is less toxic, and has been shown to result in a very good yield of AEDs on extraction from serum samples [15-17].

Thus Kieselguhr still remains the only adsorption media for the enrichment step by solid/liquid extraction. This is because after protein precipitation ca. one third of the extract is acetone. The content of organic solvent in the centrifugate would have a through flushing effect in the case of RP materials.

Using Extrelut(R)-3 tubes with a maximum adsorption capacity of 3.9 ml aqueous solution [18], the considerably diluted samples can be distributed over a very large surface area. Thus, a complete extraction of the AEDs into the organic phase can be achieved. The sample is concentrated again by evaporating off the solvents and taking up the residue in only 0.1 ml of methanol/water.

A water content of 50% in the dissolved sample residue enables more than 10 ul to be injected into the microbore column, without reducing the quality of the separation. The enhanced sensitivity of measurement of the microbore HPLC [9] finally provides a necessary additonal advantage in the determination of AEDs in amounts of down to 20-30 mg of human brain.

Recovery and Linearity

For determining the recovery and the linearity of the method described, portions of brain homogenate which are free of the AEDs investigated (brain tissues from patients with valproic acid medication) were spiked with AED solu-

tions. Grey matter of cerebrum (ca. 900 mg) was homogenized with 6 ml of the pH 6 buffer solution (corresponding to ca. 150 mg/ml homogenate). From this homogenate 1 ml portions were spiked with different amounts of an AED stock solution and mixed with 1 ml of the IS buffer. The results of the sample extraction are given in table 1.

As seen from table 1 the recovery of our method is about 100%. Thus, our method is especially suitable for the analysis of very small samples of tissue with low AED concentrations. The apparent loss of recovery in the samples with the highest AED concentrations seems to be caused by a slight non-linearity in detection. The same observation is made if calibration samples with the same AED concentrations are used. If necessary, samples with very high AED contents should be diluted.

Precision

In order to establish the precision of our method, samples of brain tissue were homogenized and pooled. From this pooled tissue homogenate 12 samples of 1.0 ml were taken (corresponding to 100 mg tissue per ml). The results of an "in-day" investigation are summarized in table 2. They show that the precision of the extraction procedure and of the chromatographic analysis is very high if a homogeneous sample is investigated.

Greater deviations are observed if the results of the patient samples are compared. Four different types of tissue are analysed (if available) from each patient.

TABLE 1

Recovery and Linearity tested with spiked Brain Tissue (blank grey Matter, ca. 150 mg per 1 ml Homogenate)

AED	TV	VF	REC	AED	TV	VF	REC
PB	0.50	0.53	106 %	PT	0.25	0.24	96 %
	2.50	2.57	103 %		1.25	1.24	99 %
	5.00	5.04	101 %		2.50	2.49	100 %
	10.00	9.58	96 %		5.00	4.75	95 %
a = - 0.16		b = 1.05		a = - 0.05		b = 1.05	
r = 0.9996				r = 0.9996			
CBZ	0.15	0.16	107 %	EPO	0.05	0.051	102 %
	0.75	0.76	101 %		0.25	0.253	101 %
	1.50	1.50	100 %		0.50	0.503	101 %
	3.00	2.98	99 %		1.00	0.999	100 %
a = - 0.02		b = 1.01		a = - 0.003		b = 1.003	
r = 0.9999				r = 0.9999			

TV = theoretical value, VF = value found, REC = recovery of VF expressed as a percentage of TV, a = intercept, b = slope of regression line, r = coefficient of correlation.

TABLE 2

In-day Precision of the Analyses of a pooled Homogenate from Patient Samples of Brain Tissue (100 mg/1 ml)

AED	Xmin	Xmax	XM	SD	CV(%)
PB	2.36	2.45	2.417	0.029	1.2
PT	0.82	0.84	0.832	0.008	1.0
CBZ	0.87	0.89	0.882	0.006	0.7
EPO	0.21	0.22	0.218	0.004	1.8

Xmin = minimum value of X [ug/ml], Xmax = maximum value of X, XM = mean value of X, SD = standard deviation, CV(%) = coefficient of correlation expressed as a percentage.

Samples were taken from the grey matter and the white matter of the cerebrum, of the cerebellum, and of the peripheral nerves. From each of these four different tissues several samples are weighed. Weights which are higher than 60 mg are classified as "H" and those which are lower than 60 mg as "L" (compare with table 3).

The results are classified according to the special AED, the type of tissue and the sample weight. The conformity of the results from high sample amounts and from low sample amounts is proven and listed in table 3.

The best results in table 3 are shown for PB, certainly because this drug has the highest concentration in

TABLE 3

Correlation of the Results of the different Types of the Patient Samples collected post Mortem

AED	D%"H"	D%"L"	D%HL	AED	D%"H"	D%"L"	D%HL
Cerebrum, grey matter				Cerebrum, white matter			
PB	5.2	5.1	5.1	PB	1.1	4.6	3.2
PT	6.4	7.0	8.7	PT	1.9	4.4	3.9
CBZ	1.1*	7.4	6.1	CBZ	4.3	9.1	10.1
EPO	15.7*	4.2	8.5	EPO	1.2	3.4	9.3
Cerebellum				Nerve tissue			
PB	2.7	2.9	3.0	PB	3.1	3.5	4.2
PT	2.5	3.5	6.4	PT	3.4	2.2*	4.8
CBZ	2.8	9.8	7.9	CBZ	3.5	6.5	7.9
EPO	5.3	2.5*	7.1	EPO	5.6	6.9*	6.4

D% = deviation from the mean value of duplicates expressed as a percentage, "H" = results obtained from the "high" sample weights (see text), "L" = results obtained from the "low" sample weights, HL = comparison of the results from "H" and "L". (*) = value from only 3 duplicates.

the tissue samples. A second reason for this is that the main difficulty in the sample preparation is the separation of the grey and the white matter. Since the concentrations of PT, CBZ, and EPO are higher in the white matter than in the grey matter, remaining white matter adhering to the sample can cause false results in analysing the grey matter. The analysis of PB is not influenced by these difficulties because the concentrations of PB in the grey and the white matters are nearly the same.

A second cause for deviations in the results of the AED determination in the same material is due to water adhering to the tissues. This dilution effect is the greater the smaller the sample amount is and should be kept as low as possible.

CONCLUSION

In about a six month period brain tissues taken at the post mortem of 26 epileptic patients were examined according to the method described.

The sensitivity and precision of the analyses enabled the differentiation between different regions of the human brain which were in close proximity. By this means the differences in the AED concentration between the grey and the white matter of the cerebrum, the cerebellum, and the peripheral nerves could be determined.

In addition it should now be possible in the case of a recognizable focus to differentiate between the AED

concentrations in the focus and in the adjacent tissue. Morphological findings together with liquor concentrations will give a better idea of the distribution of AEDs in the human brain.

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