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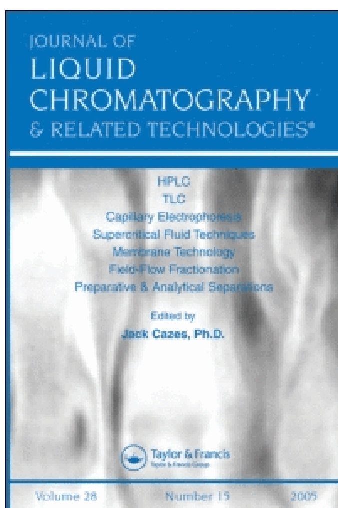

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HPLC Analysis of Antiepileptic Drugs in Blood Samples: Microbore Separation of Fourteen Compounds

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HPLC ANALYSIS OF ANTIEPILEPTIC DRUGS IN BLOOD SAMPLES: MICROBORE SEPARATION OF FOURTEEN COMPOUNDS

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I REVIEW OF HPLC METHODS FOR ANTIEPILEPTIC DRUG ANALYSES

A. INTRODUCTION

The regular drug monitoring of antiepileptic drugs (AEDs) in the blood of patients has become, for nearly ten years now, an integral part of treatment in the Epilepsy Centre "Bethel". In the last year (1985) ca. 15,000 blood samples containing AEDs were analysed in our laboratory. Only a few patients in Bethel (ca. 10%) are on monotherapy. About 20% of the patients take 2 AEDs, about 40% take 3 AEDs, and about 30% are on polytherapy with 4 or more AEDs.

Six AEDs, which Soldin [1] refers to as the principal antiepileptic agents, namely phenytoin (PT), phenobarbital

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(PB), primidone (PR), carbamazepine (CBZ), ethosuximide (ET), and valproic acid (VPA) are the main drugs prescribed in Bethel. Apart from these there are still a number of patients who receive methsuximide, which is almost completely metabolised in the liver to its demethylated form, N-desmethylmethsuximide (DM) [106].

Furthermore the serum concentrations of the clinically relevant metabolites (MBs) of PR, 2-ethyl-2-phenyl-malonediamide (PEMA), and of CBZ, carbamazepine-10,11-epoxide (EPO) and 10,11-dihydro-10,11-dihydroxy carbamazepine (DIOL) are routinely determined in our laboratory.

We have reprints of 104 publications (76 original papers and 28 short communications) which describe the high-performance liquid chromatographic (HPLC) analysis of the 6 main AEDs (PT, PB, PR, CBZ, ET, and VPA) in blood, plasma, or serum [2-105]. Papers about comparisons of methods and those about clinical applications which refer to methods already published are not included.

The HPLC determination of VPA is normally carried out separately from the determination of the remaining AEDs because its slight UV absorption makes derivatization necessary [25,31,32,56,58,70,71,76,85]. A simultaneous determination of VPA and four other AEDs is described in a paper which has just recently been published [103]. VPA is thereby eluted between PB and PT. The sensitivity of the detector is temporarily increased from 0.04 to 0.005 a.u.f.s. (absorbance unit full scale). For routine analyses it would be necessary that the detector is switched

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automatically by a time controller. In our laboratory, the determination of VPA is carried out by a gas chromatographic (GC) method [107] because the sample pretreatment is faster using this GC method than using an HPLC method with a derivatisation step.

As a minimal requirement, in our opinion, a paper giving a routine method for the analysis of AEDs by HPLC (VPA papers excluded) should describe the simultaneous determination of the 5 common AEDs mentioned above (PT, PB, PR, CBZ, and ET). This minimal requirement is, however fullfilled in only 36 of our reprints [12-14,16,18,26,27, 35,38,39,44-48,52-54,59,62,64,66-68,72,77,79,82,86,87,89, 91,93,95,97,104]. Many publications only describe the analytical procedure of a single AED. This applies especially to CBZ which is frequently the only drug, mostly together with its main metabolite EPO, determined. In the remaining publications the determinations of only 1-4 of the 5 AEDs, mentioned above, are described. These AEDs are sometimes determined together with substances which are not antiepileptic agents.

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B. SAMPLE PRETREATMENT

The various methods for sample processing in the HPLC analyses of drugs in blood, plasma or serum samples can be divided into three basic procedures:

1) Protein precipitation using a solvent which is miscible with water.

2) Liquid-liquid extraction with a solvent which is immiscible with water.

3) Adsorption onto a solid material from which the drugs can be eluted after cleaning the sample.

Silica gel columns were exclusively used in chromatographic separations of AEDs, as described in the earlier papers (from 1973 to 1976). The sample pretreatment in these papers were according to procedure 2 (extraction with phase separation).

Only after the introduction of the reversed phase columns (from 1976 on) protein precipitation was made possible as the single step in the processing of the samples. In the papers with sample pretreatment according to the basic procedure 1 the acetonitrile precipitation of proteins is the favorite first step (about a quarter of all papers).

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Liquid/liquid extraction of the samples including a phase separation from the water layer did not, however, loose its significance. The advantage of protein precipitation is the speed of operation, but its disadvantage is, however, that all water soluble impurities of the samples are injected together with the extracted drugs onto the column. On the contrary the chromatographic separations following liquid/liquid extraction are almost free of sample background.

A liquid/liquid extraction was carried out in about the half of the papers cited above. In these a chlorinated solvent was used most frequently (24 papers), although there are two disadvantages in its use. First chlorinated solvents show the highest partitition coefficient espe-

cially for CBZ, but not for the other AEDs [108]. Secondly the organic solution is in the lower phase after centrifuging.

Extraction with diethyl ether or with solvent mixtures come next in the order of frequency of use. Surprisingly enough, only 4 papers had been published up until 1983 which mentioned the use of ethyl acetate as an extraction agent [38,39,53,61], although Meijer [108] showed, as early as 1975, that just this solvent is especially suitable for the simultaneous extraction of relatively polar and non-polar AEDs.

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From 1982 to 1983 we compared the three basic procedures for sample pretreatment for the HPLC determination of AEDs in serum analysing more than 2,000 patient samples. From the results we decided, for technological and economical reasons, to use ethyl acetate extraction for the routine analytical processing of AED samples in our laboratory [109]. Ethyl acetate has in the last two years also been used by some other laboratories for the extraction of AEDs from serum or plasma samples [86,88, 95,97,105].

The adsorption of the drugs onto a solid material has been quoted as basic procedure 3 of the sample processing methods for AED analysis. After separation from the sample matrix the AEDs are then again eluted from the adsorbent by an appropriate solvent. Ever since the mid-70s activated charcoal was used for this purpose [12,13, 69,73].

A fully automated method was described in 1979 in which "an activated, sized divinylbenzene cross-linked polystyrene resin which acts as a lipophilic sorbant" was used [41]. Unfortunately this procedure did not include the determination of ET.

The first use of reversed phase (RP) silica gel as sorbent for AEDs from serum (SEP-PAK(R) cartridges) was described in 1981 [66]. The next off-line procedure using RP material (BOND ELUTE(R) tubes) for sample pretreatment was described by Kabra et al.[82] in 1983.

A fully automated column switching system using pre-columns filled with RP material for an on-line cleaning of biological samples was described in 1981 [110]. Following on this publication, the column switching method appeared to us to be also suitable for the determination of AEDs in serum samples. We described in 1983 the principle abilities of such a system for the analysis of AEDs in a preliminary study [77]. At first, we included protein precipitation with acetonitrile followed by a dilution of the solution with buffer because a major problem with serum direct injection was the blockage of the pre-columns.

A method for the direct injection of untreated serum samples was submitted by us in October 1983 for publication [89]. In February 1984 Nazareth et al.[90] likewise submitted a paper on the same subject. ET was, however, not determined in this paper. At the same time a further paper dealing with the AED determination by column switching with serum direct injection was published by Kuhnz et al.[91].

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TABLE 1

Distribution of Sample Pretreatment Techniques in the Determination of AEDs in Blood Samples

Procedure 1		Procedure 2		Procedure 3	
Aceto-nitrile	28	Dichloromethane Chloroform	24	Reversed phase silica gel	5
Acetone	3	Solvent mixtures	12	Activated charcoal	4
Methanol Ethanol	2	Methylbutyl ether Diethyl ether	11	Kieselguhr	1
Perchloric acid	1	Ethyl acetate	9	Divinyl-benzene	1
		Petroleum ether Pentane	2		

In paper [3] direct injection of plasma samples onto an ion exchange column was made

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The use of kieselguhr for the adsorption of AEDs from biological samples [28,109] is a technique which is a hybrid between procedure 2 and 3. First of all the aqueous sample is adsorbed onto the kieselguhr, but the following elution by a solvent which is not miscible with water is a liquid/liquid extraction between the organic solvent and the bound aqueous phase. In respect of the technical processing in the laboratory the use of kieselguhr should, however, be classed with the third basic procedure of sample pretreatment (adsorption on solid material).

C. LIQUID CHROMATOGRAPHIC CONDITIONS

With two exceptions, in which ion exchange columns were used [3,8], silica gel columns were used in the

methods first published for the analysis of AEDs in blood samples by HPLC. From 1976 on silica gel columns were nearly entirely replaced by columns of the reversed phase type. Two of the advantages of this column material are that the separation is independent of the water content of the sample and that a gradient elution is possible.

The gradient elution was, however, for a long time not used at all and even after 1982 only sparingly [77,86, 89,97,102,104], although modern HPLC systems are able to produce gradient profiles with excellent reproducibility. Gradient elutions shorten the separation times and enhance the sensitivity of detection of those substances which are eluted later on (especially PT and CBZ).

Some of the columns used for chromatographic analyses had in the first papers lengths of 25 cm [10,11.13] and internal diameters of 2 mm [4,5] or 9 mm [3]. Soon, however, two standard sizes were used, namely 25 cm x 4.6 mm and 30 cm x 3.9 mm. The latter size applies to the " µ-Bondapak"(R) column, which was until recently the most frequently used RP column.

In the first years of AED analyses by HPLC the columns were filled with a material with a particle size of 10 µm, or greater. The first separations using a material with a diameter of 5 µm were described in 1978 [24,28]. Due to a reduction in the particle size of the packing materials the separation efficiency of the columns was increased. Consequently, there is a tendency to reduce the length of the columns and consequently the time for a chromatographic run.

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Hitherto only two reports of really genuine "high-speed" separations of AEDs (elution of the substances within 2 min) have, however, been published [73,82]. For these applications relatively short (10 cm) columns were filled with 3 µm packing materials. "High-speed" separations were achieved by using a relatively high flow rate of 3 ml/min. We tested the chromatographic conditions of Kabra et al.[82] but did not find sufficient resolution of PE, ET and PR as well as of DM and EPO.

Another direction of HPLC development has been a saving of eluents and an increase of sensitivity by reducing the internal diameter (I.D.) of the columns. A few of the earlier papers describe the use of silica gel columns with 2.0-2.1 mm I.D. [2,4,5,8,10]. The flow rates, in some of these papers, were also relatively low [3,5,8]. But the separations were not comparable with todays "microbore" HPLC because the HPLC equipment was so constructed that a considerable band spreading occurred. Only the modern generation of HPLC systems enable the advantages of microbore columns to be used.

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II DETERMINATION OF ANTIEPILEPTIC DRUGS BY MICROBORE HPLC

A. INTRODUCTION

One of the main advantages of the modern microbore technique in HPLC is a saving in expensive solvents. As more than 15,000 patient samples are analysed in our

laboratory per year, we have partly switched over to microbore HPLC in the routine determination of AED concentrations in serum samples. The prerequisite for this was the installation of an HPLC system which in respect of the injector, capillaries, and the detector complies with the requirements of microbore HPLC (see Materials).

In 1984 our laboratory was commissioned by the firm Parke Davis & Co. (U.S.A.) to measure serum concentrations of the new antiepileptic drug "Zonisamide" (CI-912 or AD-810) for a European study. The samples were taken from patients suffering from complex focal seizures. Former studies with zonisamide (ZA) were carried out in Japan and in the U.S.A. with patients on ZA mono-medication. Consequently, the HPLC determinations of ZA in the blood samples could be carried out isocratically with short separation times [111,112].

The patients in the European study (which is still in progress) normally take in addition to ZA some of the 6 common AEDs mentioned in the first part, namely PT, PB, PR, CBZ, ET, and VPA. Apart from these some patients take further AEDs, namely oxcarbazepine [88,97,104,105,113-115] and mephenytoin [13,26,27,86,97,104,116]. Only trace concentrations of oxcarbazepine (OXC) were found in the blood of patients because it is nearly completely metabolised to 10,11-dihydro-10-hydroxy carbamazepine (OHC) and DIOL [113, 114]. The same applies to mephenytoin (MEPT) which is almost entirely demethylated to nirvanol (NIR) [116].

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The isocratic conditions for the determination of ZA, described in [111,112], were not suitable for our purposes because CBZ (with an elution time of ca. 22 min) would interfere with ZA in the following chromatograms. Consequently, we had to develop new chromatographic conditions, using at first a conventional HPLC column (length 12.5 cm plus 4 cm guard column) filled with an ODS silica gel with a particel size of 5 µm. Using these conditions ZA, PT, PB, PR, CBZ, ET, and DM, as well as the metabolites PEMA, EPO and DIOL could be simultaneously analysed, without disturbing the separation by OXC, OHC, MEPT, or NIR [117].

The conditions for extraction were the same as those for our hitherto routine analyses of AEDs [109]. In the last year we then developed a base line separation of all 14 of the above mentioned compounds using a microbore column (20 cm x 2.1 mm).

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B. MATERIALS and METHODS

Apparatus

Equipment was obtained from the following firms: automatic pipetter/diluter from Corning/Gilford, Duesseldorf (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 µm) from Hewlett /Packard, Boeblingen (F.R.G). Rheodyne HPLC column inlet filters with exchangeable frits (1.5 mm diam., 0.5 µm pore size) from ERC GmbH, Alteglofsheim (F.R.G.).

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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 obtained from Merck, Darmstadt (F.R.G).

The mobile phase for the gradient elution consisted of two different mixtures of acetonitrile and diluted perchloric acid (HPLC water was adjusted to a pH 4 with diluted $HClO_4$). Mixture A: $HClO_4/CH_3CN$ (9:1 v/v), mixture B: $HClO_4/CH_3CN$ (4:6 v/v). The solvents were pre-mixed for two reasons. First degassing during the gradient mixing can be avoided, and secondly mobile phases with a minimum content of 10% acetonitrile are stable for a long time.

Standards

We obtained PT, PB, PR, CBZ, and ET from Desitin, Hamburg (F.R.G); PEMA, DM, and 5-ethyl-5-(p-tolyl)-barbituric acid (ETB) from Aldrich, Steinheim (F.R.G); EPO, DIOL, OXC, and OHC from Ciba-Geigy, Basle (Switzerland); MEPT and NIR from Sandoz, Nuernberg (F.R.G); ZA from Parke Davis & Co., Munich (F.R.G.).

The internal standard (ISTD) solution consisted of 15 mg ETB in 1000 ml ethyl acetate.

Calibration Samples

To 10-ml centrifuge tubes 0.5 ml calf serum and 50 µl from a stock solution containing 25 mg PEMA, 100 mg ET, 25 mg PR, 30 mg ZA, 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 freezed at -18°C.

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Extraction Procedure

To 10-ml centrifuge tubes 0.5 ml serum, 1.0 ml ISTD solution (pipetter/diluter), and 2.0 ml ethyl acetate were added. The tubes were closed with screw caps and mixed with a vortex-mixer for 10 min at 30°C. After centrifuging for 10 min, the supernatant fluid is transferred to clean tubes and evaporated to dryness (trace amounts of ethyl acetate can interfere with ZA in the chromatogram). The residue is dissolved in 100 ul methanol and 5 ul are injected.

Chromatographic Parameters

The flow was 0.3 ml/min, the column temperature was 65°C, the detection wavelength was 207 nm, and the injection volume was 1.5 µl.
The time program is as follows:

time [min]	0	0.5	9.5	10.0	12.0	15.0
	start	15%B	45%B	15%B	stop	end of run.

The chromatographic separation of 96 samples can be carried out in 24 hours by this gradient program (including the re-equilibration time).

C. RESULTS

The use of a microbore column for the analysis of AEDs in serum samples in our laboratory reduced the costs of solvents by 70%. This is because the flow rate was

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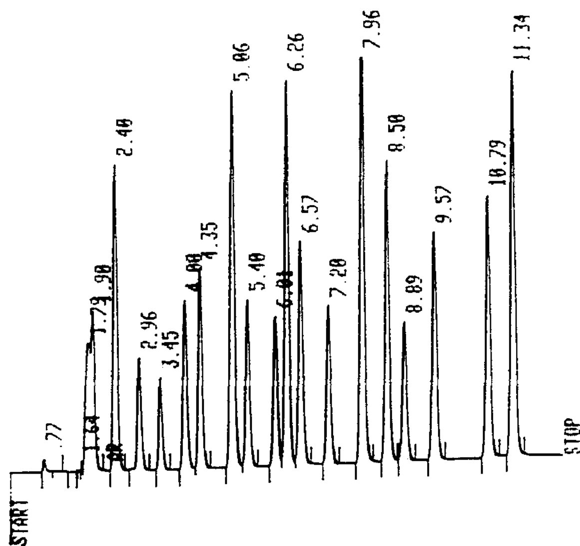

FIGURE 1 Chromatogram of a test solution; RT: 2.40 Caffeine, 2.96 PEMA, 3.45 ET, 4.00 PR, 4.35 ZA, 5.06 DIOL, 5.40 ISTD used in [111,112], 6.01 NIR, 6.26 OHC, 6.57 PB, 7.20 DM, 7.96 EPO, 8.50 OXC, 8.89 MEPT, 9.57 ETB (ISTD), 10.79 PT, 11.34 CBZ.

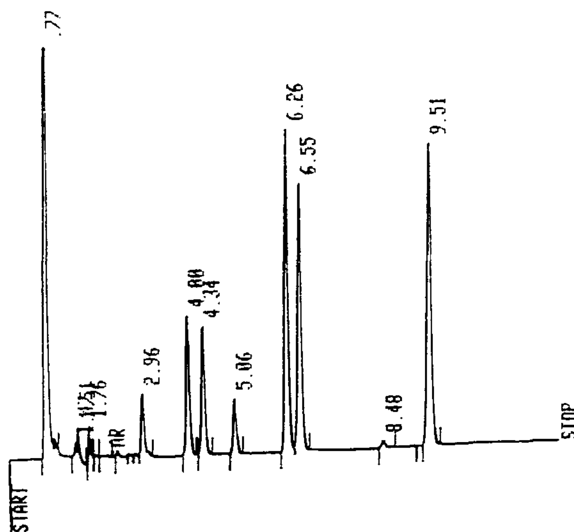

FIGURE 2 Chromatogram of a serum from a patient on ZA, PR, and OXC medication; RT: 2.96 PEMA, 4.00 PR, 4.34 ZA, 5.06 DIOL, 6.26 OHC, 6.55 PB, 8.48 OXC, 9.51 ETB.

reduced from 1.0 to 0.3 ml/min. The price of the microbore column (which is 3-4 times higher than the price for a refill of our conventional column) was thus justified.

The use of a suitable in-line filter between the injector and the column is decisive for a long life of a microbore column. Without such a filter our first microbore column became unusable, due to blockage, after about 350 injections of sample extracts. If, however, exchangeable in-line filter discs with 0.5 µm frits were used (see Materials) we could inject about 4,000 samples onto the microbore column without loss in resolution.

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TABLE 2

Day-to-Day Precision of the Microbore Method measured by Control Sera with different Concentrations of AEDs

COS	AED	N	Xm	Range	S.D.	C.V.(%)
I	ET	31	70.17	± 4.45	2.20	3.14
	PR	31	11.24	± 0.35	0.19	1.70
	PB	31	28.43	± 1.25	0.48	1.68
	PT	31	13.88	± 0.75	0.29	2.11
	CBZ	31	5.51	± 0.25	0.11	1.92
II	ET	14	59.84	± 4.55	2.47	4.13
	PR	14	10.52	± 0.55	0.28	2.67
	PB	14	15.90	± 0.90	0.42	2.61
	PT	14	10.34	± 0.45	0.29	2.78
	CBZ	14	5.12	± 0.20	0.13	2.44
III	ET	14	155.31	± 8.75	5.32	3.43
	PR	14	26.10	± 0.85	0.46	1.76
	PB	14	45.35	± 1.20	0.77	1.69
	PT	14	49.99	± 1.65	1.13	2.26
	CBZ	14	15.21	± 0.35	0.24	1.56

COS = control serum, N = number of days, Xm = mean value of concentrations [µg/ml], S.D. = standard deviation [µg/ml], C.V.(%) = coefficient of variation expressed as a percentage.

The requirement for this was an exchange of the filter discs after 500-600 injections when the pressure increased over 200 bars.

The precision of the analysis made with microbore HPLC is, as can be seen from table 2, just as good as using conventional HPLC.

The larger coefficients of variation for ET, as opposed to those for the remaining AEDs are certainly not due to the separation by the microbore column, but obviously related to the evaporation step of the extraction method [104]. But, as shown by table 2, all coefficients of variation come within the "desirable limits" of 5% or less, defined by Szabo et al.[72].

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