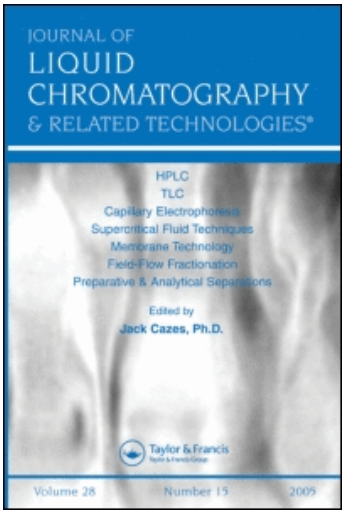

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Liquid Chromatographic Analysis of Antiepileptic Drugs: An Overview

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Review

LIQUID CHROMATOGRAPHIC ANALYSIS OF ANTIEPILEPTIC DRUGS: AN OVERVIEW

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Abstract

Extraction of antiepileptic drugs (AEDs) can be carried out by different procedures which include: (i) protein precipitation, (ii) liquid-liquid and (iii) liquid-solid extraction. The latter yields a cleaner sample and shows efficiency comparable to the other procedures; recovery ranges from 90 to 105% for most AEDs.

Types of column include: (i) conventional, (ii) microbore and (iii) high-speed. Compared with conventional and high-speed columns, microbore columns, which have a diameter of 1-2 mm, allow to achieve the highest sensibility (up to double values) and to reduce solvent consume (up to 70 %). High-speed columns, characterized by a length of 3-10 cm and by a reduced packing particle size (Ø 3µm), make the analysis time more rapid by at least 50%.

Reversed phase chromatography is the most versatile technique as compared to the normal phase and to the

adsorption chromatography. Detection can be performed at low wavelengths (about 200 nm), which are optimal for AEDs and, subsequently, allow the use of small biological samples (about 100 µl).

Reproducibility of LC methods is excellent and the within-day and the day to-day CV show values of <10%.

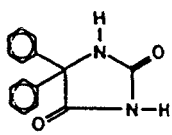
INTRODUCTION

High-performance liquid chromatography (HPLC) is a widely used technique to determine the concentration of many drugs in body fluids. Among these drugs, the antiepileptics (AEDs) are currently monitored in plasma of patients since a good correlation between their blood concentrations and clinical response has been demonstrated. The present paper is devoted to giving a general overview of the LC methods available in the literature for blood determination of AEDs.

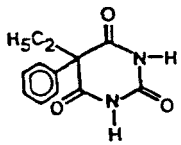
Early published LC methods for AED assay go back to circa 1970. Progress in technology in the last two decades has led to the achievement of high sensibility, precision and accuracy together with reduction of analysis time and cost; these characteristics made LC methods suitable both for routine and research purposes.

PHYSICAL AND CHEMICAL PROPERTIES OF AEDs

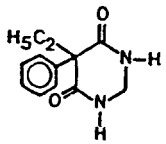
The most currently used AEDs are: Phenytoin (PHT), Phenobarbital (PB), Primidone (PRM), Carbamazepine



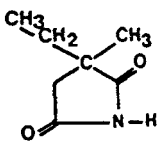
Phenytoin



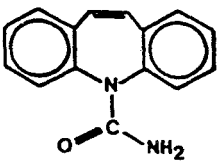
Phenobarbital



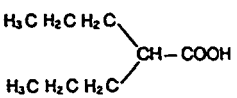
Primidone



Ethosuximide



Carbamazepine



Valproic Acid

Fig. 1 - Chemical structure of AEDs

(CBZ), Ethosuximide (ESM) and Valproic Acid (VPA) (Fig. 1). Except for VPA, which is a stronger acid, all are weak acidic compounds and are easily soluble in organic solvents such as methanol (excluding PHT), ethanol, diethyl ether, chloroform, acetone, dichloromethane, etc.

PB, PHT, CBZ, ESM, and PRM absorb more strongly in the low-UV region (195-210 nm); by contrast, VPA has a poor absorption in UV and needs derivatization.

Table 1 gives a summary of the physico-chemical properties of AEDs.

TABLE 1

Physico-Chemical Properties of AEDs

Drug	M.W.	M.P. °C	Solubility in water	pKa	Partition # coefficient
CBZ	236	193	insoluble	-	94
PHT*	252	298	14 mg/l	8.1	29
PB*	232	176	poorly sol.	7.3	4.2
ESM	141	65	190 g/l	9.3	9.0
PRM	218	287	0.60 mg/l	13.0	0.7
VPA*	144	222	1.27 g/l	4.9	-

partition coefficients have been calculated using chloroform as extraction solvent at pH 3-5.
* solubility of these drugs is referred to the acid form.

AEDs ANALYSIS

Sample requirement

AED determination can be carried out in 0.2-1 ml of serum or plasma, both fluids being suitable. Whole blood can be also used except for PHT; this drug is highly bound to plasma-proteins (about 90%) and its partitioning between serum and the red blood cells is unequal (60-70%). Hence, badly hemolized samples greatly enhance the possibility of erroneously low PHT levels (1).

Test samples, preferably obtained from recently drawn blood, can be collected in standard glass

containers, vacutainers-type tubes or gel barrier tubes (2). Sera may be stored at 4°C for one month and at -20°C for 6-12 months without significant changes in drug concentration (3).

Extraction procedure

Three procedures are basically used to extract AEDs: a) protein precipitation; b) liquid-liquid extraction; c) liquid-solid extraction.

a) **Protein precipitation**

Protein precipitation by acetonitrile is the most commonly used procedure. Serum (or plasma) samples are mixed with equal volumes of acetonitrile and centrifuged (4,5,6). A double acetonitrile volume has been also recommended to ensure a more complete protein precipitation (7,8,9,10). Acetone has the advantage of giving a more complete and faster protein precipitation than acetonitrile; compared with this, however, it is significantly more ultraviolet-absorptive at low wavelength and, consequently, could give solvent front interferences (11,12,13).

Protein precipitation is a very rapid and efficient procedure and shows recovery values of 95-104 % (13). Its obvious disadvantage is that samples are not purified and all the water soluble impurities are

injected onto the column together with the extracted drugs.

b)Liquid-liquid extraction

The most widely used solvent is dichloromethano. It has the highest partition coefficient, especially for CBZ (14), and evaporates at a low temperature (42°C). This minimizes losses of highly volatile drugs, such as VPA and ESM.

Other solvents or mixture of solvents (15,16,17) have been used for AED extraction. Diethyl ether, for example, has a low vapour pressure and is suitable for the assay of VPA and ESM (18,19). In spite of its infrequent use, ethyle acetate has been suggested for the simultaneous extraction of both relatively polar AEDs, such as PRM and ESM, and less polar AEDs, such as CBZ and PHT (14).

Liquid-liquid extraction gives samples almost free from impurities. To eliminate lipids and other components that can appear as early-eluting peaks and may interfere with the assay, ammonium solphate can be advantageously added to plasma (20,21). It adsorbes lipids and facilitates precipitation of protein, which otherwise could contaminate the column.

A more complete precipitation of plasma proteins can be obtained by extracting at low pH (i.e., 3-5) (22).

Recovery values reported with this method range from 55-65 % (23) to 92-100% (20); this variability may be due to different factors including, among others, the type of solvent used, the time of vortex-mixing and the volume of reconstitution.

c) liquid-solid extraction

Liquid-solid extraction is the most recently introduced procedure. At first, it was carried out by using activated charcoal (24) or sized divinylbenzene cross linked polystirene resin (25) as lipophilic sorbants. More recently, tubes with a reversed phase silica gel as sorbent are commercially available (SEP-PAK (R) cartridges; BOND ELUTE (R) tubes) (26,27).

Liquid-solid extraction is a valid alternative to the conventional liquid-liquid extraction and has some advantages over it: the time of execution is more rapid and the sample obtained is cleaner. The recovery for all AEDs at different concentrations is almost complete (92-106%) (27).

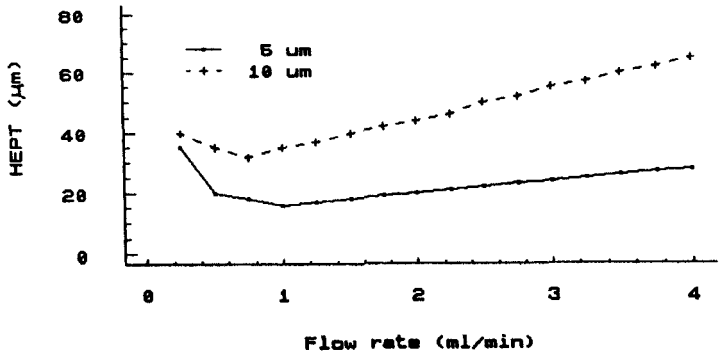


Fig.2 HEPT vs flow for two different packing particle sizes

LIQUID CHROMATOGRAPHIC SYSTEMS

Theoretical notes on column efficiency

Some theoretical aspects on column efficiency are briefly discussed in this section so as to give the essential scientific background for a rational approach to all those factors affecting overall results of the system.

Column resolution (R), which expresses the quality of separation, is equal to:

$$R = \sqrt{N}/4 \cdot \alpha - 1/\alpha \cdot K'/K'+1 \quad (a)$$

where N is the efficiency factor, α the selectivity factor and K' the capacity factor.

To evaluate more directly the influence of each single factor on R keeping constant the remaining two

Fig.3 Effect of alfa on R for constant N and K'

factors, the Kost term may be introduced. Hence, $R = Kost \cdot \sqrt{N}$ (b). N is expressed in terms of number of theoretical plates and, according to the equation $N = L/H$ (c), is directly proportional to the column length (L) and inversely proportional to the Height Equivalent to one Theoretical Plate (H). It derives from (b) and (c) that to improve R by only 40%, the column length needs to be doubled. This, however, implies an abnormal increase in the working pressure with obvious problems for both pump and column. R can be also improved by reducing H. This can be made by optimizing the flow rate and by reducing the packing particle size (Fig.2).High-speed columns (see the section: "column dimensions") have been successfully projected on this basis (28).

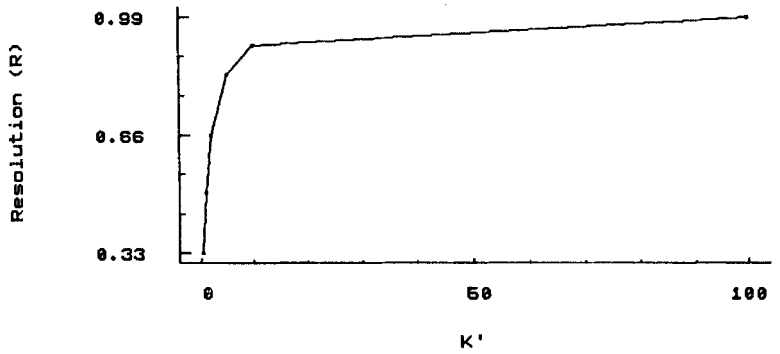


Fig.4 Effect of K' on R for constant N and alfa

The influence of α (which expresses the degree of separation of two peaks) on R is given by:

$$R = Kost \cdot (\alpha-1)/\alpha \quad (d)$$

and is clearly illustrated in the Fig.3.

K' (which expresses the elution speed of a given compound) affects R according to:

$$R = Kost \cdot K'/K'+1 \quad (e)$$

As showed in the Fig.4, K' can affect considerably R only within an optimal range, which has been calculated to be 1 to 10.

Therefore, according to the theory, column resolution can be improved by modifying different parameters which affect the three above examined

Table 2

Parameters affecting the column resolution.

	α	K'	N
Mobile phase			
Elution strength	+	+	0
Polarity	+	+	0
pH (acidic/basic properties)	+	+	+
Viscosity	0	0	+
Linear velocity	0	0	+
Stationary phase			
Retention strength	+	+	0
Polarity	+	+	0
Acidic/basic properties	+	+	+
Particle size	0	0	+
Column temperature	+	+	+
Column dimensions			
Length	0	0	+
Diameter	0	0	0

N = efficiency factor ; α = selectivity factor ; K'= capacity factor.

factors, namely N, α and K' (29,30). A summary of these parameters is given in Table 2.

Column dimensions

LC analysis of AEDs can be carried out by different types of column : (i) conventional, (ii) high-speed and (iii) microbore (Table 3).

Conventional columns allow an isocratic simultaneous separation of the main AEDs (ESM, PRM,

Table 3

Dimensions of chromatographic columns.

Column	Lenght cm	Wide mm	Particles (Ø) µm
Conventional	15-50	2.6-5.0	> 5
High-speed	3-10	2.6-5.0	3
Microbore	25-50	1.0-2.0	5

PB, CBZ, PHT) within 10-20 min, working at a flow rate of 1-3 ml/min. A reduction in the analysis time (at least 50%) can be obtained by using high-speed columns (21,27,32-37). These are packed with particles of 3 µm of diameter and have a length of 3-10 cm (Table 3), these properties allowing a more rapid elution of compounds (28,31) (Table 2; Fig. 2).

Microbore columns, which have a small diameter (1-2 mm) (Table 3) lead to a higher sensibility and to a lesser consumption of mobile phase (28,38). Flow rate can be kept at 200-300 µl/min (vs 1-3 ml/min of the conventional columns) and this, in turn, results in a reduction of the solvent consume (up to 70%) (39,40,41).

Table 4

Operative contions of a method using a conventional column (Kabra et al, 1977)

Column	300 mm x 4 mm
Packing particle size:	5 μm
Stationary phase:	RP C18
Mobile phase	CH_3CN/phosphate buffer (pH 4.4)
Column temperature	50^oC
Detector	195 nm
Flusso	3.0 ml/min
Analysis time	15 min
Consume solvent/analysis	45 ml
Analysis number/hour	4
Separation condition	isocratic
Drugs separated	ESM,PRM,PB,CBZ,PHT
Extraction procedure	protein precipitation by CH_3CN recovery: 95%
Sensitivity	< 1μg/ml
Precision	within-day 5.5%
	day-to-day 5.9%

Although high-speed or microbore columns offer some specific advantages over conventional colums, to minimize extra-column band broadening their use must be supported by modifications of the design of the detector cell-flow and by a reduction of connecting tubing (27,28,42).

Tables 4, 5, and 6 summarize the operative conditions of three different methods using a conventional, high-speed or microbore column, respectively.

In alternative to the above described columns, other tools have been suggested to reduce both the

Table 5

Operative conditions of a method using a high-speed column (Kabra et al, 1983)

Column : 100 mm x 4.6 mm
Packing particle size: 3 μm
Stationary phase: RP C18
Mobile phase :CH_3OH/CH_3CN/phosphate buffer (pH 3.7)
Column temperature : 50°C
Detector : 195 nm
Flusso : 3.0 ml/min
Analysis time : 1.5 min
Consume solvent/analysis : 4.5 ml
Analysis number/hour : 40
Separation condition :isocratic
Drugs separated : ESM,PRM,PB,CBZ,PHT
Extraction procedure : liquid-solid extraction; recovery: 95%
Sensitivity : < 1 μg/ml
Precision : within-day 5.5%
day-to-day 5.9%

Table 6

Operative conditions of a method using a microbore column (Jurgens, 1987)

Column : 200 mm x 2.0 mm
Packing particle size: 5 μm
Stationary phase: RP C18
Mobile phase : $CH_3CN/HClPO_4$ (pH=4)
Column temperature : 65°C
Detector : 207 nm
Flusso : 300 μl/min
Analysis time : 11 min
Consume solvent/analysis : 3.3 ml
Analysis number/hour : 5
Separation condition : gradient
Drugs separated : ESM,PRM,PB,CBZ,PHT,other
Extraction procedure : liquid-liquid extraction; recovery: /
Sensitivity : < 0.5 μg/ml
Precision : day-to-day 4.1%

time and the cost of analysis. The radial compression separation system, for example, applies pressure uniformly to the outside of a flexible-walled cartridge which contains the packing material. This pressure conforms the packing material and prevents channel formation, dead spaces and shifting of the packing. The radial compression cartdiges are less expensive than conventional systems, since they have a longer life and work at lower flow rates of the mobile phase (12,15,25,43).

Automated analysis of AEDs carried out by a column-switching system (44-49) or by the Technicon "Fast-LC System" (50) has been also used with the expected result of an increased number of analyzed samples per day.

Column temperature

AED separation improves by enhancing column temperature up to 50-60 ^{o}C (13,51,52). Additionally, a temperature control avoids variations caused by variability of the room temperature (9,13).

Mobile phase

Selectivity of LC methods can be improved by adding to the mobile phase modifiers such as inorganic acids (sulphoric, phosphoric, etc.,), organic basis (alkil-

amine), buffer (phosphate, acetate, etc.,) (53) or counterions (alkilsulfonate) (54).

pH plays a major role on chromatographic separation. Even slight variations of the pH of the mobile phase in the region of pK of AEDs can result in remarkable changes of the dissociation equilibrium; consequently, retention times can become unrepeatable and peak shape can be abnormally asymmetrical. The optimal pH for AEDs is between 3 and 5 (5,16,55,56); in this range, interferences from endogenous and exogeneous compounds is minimized (27). Since several substances coextracted with drugs can change the pH of the mobile phase, an adequate buffering capacity is necessary to improve reproducibility of the analysis time and resolution (57).

Partitioning chromatography (reverse phase or normal phase) is the most widely used for AED analysis. Reversed phase chromatography, in which the mobile phase is usually constituted by acetonitrile or methanol and water, has some advantages over the normal phase chromatography, in which the mobile phase is constituted by a non polar solvent (exane) mixed with a small amount of a high polar solvent (alcohol). These advantages include: a) the possibility of adjusting pH with consequent

improvement in selectivity, b) transparency of the mobile phase for sensitive low-UV-wavelength, with a consequent reduced sample volume, c) elution of polar metabolites in the early part of the chromatogram, with consequent detection at lower concentrations, d) faster column reconditioning after gradient elution, and e) the aqueous mobile phase is less expensive than the organic phase.

Acetonitrile has a low viscosity and reduces the overall backpressure (13). Additionally, it shows a high elution strength and a lower baseline absorbance which reduces the detector noise at low wavelengths (9,13). Hence, a binary mobile phase, constituted by acetonitrile and water or buffer, gives a higly sensible response for most AEDs and their metabolites, which elute in the early part of the chromatogram (17,58-62). As acetonitrile is relatively toxic and expensive, methanol has also been used (51,63-65). However, since this solvent has a lower elution strength and a higher viscosity than acetonitrile, its use requires adjustment of the column temperature and of the mobile phase pH to optimize the resolving power of the column.

An even better resolution of AEDs can be achieved by optimizing polarity of the mobile phase. This can

be done with a ternary mobile phase, obtained by adding a little amount of methanol or acetone to a binary mobile phase (13,15,66,67).

Stationary phase

Silica is the stationary phase used almost exclusively in adsorption chromatography (39,68). Silica also acts as a solid support to which different groups can be chemically bonded. These include: nitrile, diol or amino groups which are used mainly in normal phase mode and octadecyl (C18) and octil (C8) hydrocarbon groups which are used in reverse phase mode. These groups allow a range of polarity such as to permit adequate matching of the polarity of the stationary phase with the drug polarity.

Nitrile bonded phase can be used either in normal phase or in reverse phase mode (66,68). The latter gave a better resolution of both the parent drugs and their metabolites (68).

C18 and C8 hydrocarbon bonded phases offer an excellent flexibility in the choice of the analytical conditions. The C8 bonded phase is more polar than C18 bonded phase and appears to be of a higher selectivity for both the parent drugs and their metabolites (17,45,52,58,62,70,71).

Detector

Due to its reliability and sensivity for AEDs, the ultraviolet spectrophotometer detector is the most frequently and the most successfully used. It measures the general absorptivity of chromophoric groups on the molecule such as carbonyl, unsaturated bonds or substituted aromatic groups (72).

PHT, CBZ, PRM, ESM and PB show an optmal absorbance at 195-210 nm. PB is optimally detected also at 254 nm.

VPA has a poor absorbance in the ultraviolet range and derivatization is necessary (19,73-75). However, simultaneous determination of VPA and other AEDs is also possible (51,76).

Modern detectors used in HPLC show an excellent linear relationship of output signal to drug concentration over a large part of their detectability range. This overcomes widely the linearity required by the concentration range of AEDs (i.e., approximately 0.5 to 150 µg/ml sample).

Solvent delivery system

There are two types of systems commonly used to drive solvents through the column : a) constant-pressure pump and b) constant-flow pump.

Constant pressure pumps are inexpensive and easy to operate and to maintain. However, the flow rate is not constant during analysis and may cause changes in the eluent/sample dilution ratio with consequent analytical variability.

Constant-flow pumps include reciprocating pumps and sirynge-type pumps. Reciprocating pumps have the advantage of an unlimited solvent reservoir capacity, but the pulsed flow generated may disturb the baseline and limit sensitivity, especially at low flow rates.

Siringe-type pumps have a limited reservoir capacity. They have the advantage of delivering mobile phase smoothly and contributing very little noise to the noise of the total system. This results in a lower limit sensitivity. These characteristics make sirynge-type pumps the most suitable to drive mobile phase through microbore columns, which work at lower flow rates.

PRECISION AND ACCURACY

International quality control schemes for AED assay are available both in the USA and in Europe (77,78).

In a recent study reporting the results of one of these schemes, LC methods have shown excellent

precision and accuracy. These parameters, in fact, resulted close to those of the fluorescence polarization immunoassay technique (FPIA), which was the most precise and accurate among the methods compared (79).

CONCLUDING REMARKS

As reviewed above, progress both in microparticulate packings and in packing techniques has led in recent years to improved column efficiency. This has in turn made possible a decrease in the time of analysis or, even, a reduction of solvent consumption and increasing sensiblity. The improved systems of automation allow determination of a large number of samples per day; thus, LC methods are a technique suitable for routine AED assay. In addition, the characteristics of precision, accuracy and, especially, sensibility make HPLC an invaluable technique in research conditions, where small amounts of drug and/or metabolites have to be detected.

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