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REVIEW

DETERMINATION OF ANTIEPILEPTIC DRUGS

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1. INTRODUCTION

Clinical pharmacokinetics, which hinges on the relationship between plasma levels and clinical effect as well as on the concept of a therapeutic interval between subtherapeutic and toxic ranges, is best exemplified by anticonvulsants. The therapeutic window of these drugs is well defined and drug monitoring has been a cornerstone in the pharmacological treatment of epilepsy [1—4].

Table 1 indicates the effective concentrations usually recommended for the most common antiepileptic (anticonvulsant) drugs. It is obvious from this table that the sensitivity requirement for clonazepan with therapeutic levels in the ng/ml range is more stringent than for other drugs with steady-state concentrations well over 1 μg/ml. However, sensitive methods are always needed for paediatric samples as well as for pharmacokinetic studies.

Anticonvulsants have been determined by most analytical techniques: spectrophotometry, spectrofluorometry, chromatography [thin-layer chromatography (TLC), gas—liquid chromatography (GLC) and high-performance liquid chromatography (HPLC)] as well as immunoassays [radioimmunoassay (RIA), enzymeimmunoassay, fluoroimmunoassay and nepheloimmunoassay] [5,6]. Apart from spectrophotometry and spectrofluorometry, which lack specificity and are no longer in favour, analytical methods currently used for the determination of anticonvulsants can be classified as either chromatographic methods

TABLE 1

THERAPEUTIC CONCENTRATIONS OF MAJOR ANTIEPILEPTIC DRUGS

Data taken in part from ref. 4.

Drug	Abbreviation	Active metabolite	Therapeutic range (μg/ml)	Unbound fraction in plasma (%)	Saliva/plasma* (%)
Phenobarbital	PB	–	20—40	49	30
Primidone	PRM	+	8—12	81	100
Phenytoin	PHT	–	10—20	11	11
Mephenytoin	MHT	+	5—16	65	
Ethosuximide	ESM	–	40—100	100	100
Carbamazepine	CBZ	+	6—10	18	18
Valproic acid	VPA	–	50—100	17	< 6
Clonazepam	CZP	–	0.003—0.06	53	
Progabide		+		5	

*Saliva/plasma concentration ratio (expressed in per cent).

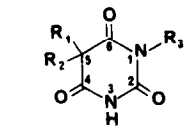
or as procedures relying on antigen—antibody interactions.

The very nature of the immunological process produces very rapid and sensitive analytical methods; nevertheless, they only permit analysis of one compound at a time. Chromatographic procedures are more tedious, but the power of chromatographic separations makes them more amenable to the analysis of multicomponents. This advantage is particularly important for the determination of anticonvulsants since the pharmacological treatment of epilepsy usually requires the administration of two or more drugs. Furthermore, a few of these drugs are metabolized into active species which should also be monitored in order to assess the relationship between the dose and the clinical effect.

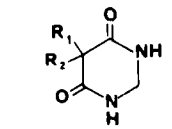
A literature survey shows that hundreds of publications dealing with the qualitative and quantitative analysis of anticonvulsants by chromatography have been published. Some of them are very similar if not outright identical. Although quantitative TLC has a proven potential, this technique is not widely used for monitoring antiepileptic drug levels; thus this paper will only examine GLC and HPLC.

Our review is not aimed at listing exhaustively all the methods ever published on the determination of anticonvulsants but rather at categorizing the assays into different types of procedures. Methods adapted to clinical monitoring will be emphasized, although recent progress in the separation of anticonvulsants and their metabolites and in the resolution of enantiomers of anticonvulsants will also be mentioned.

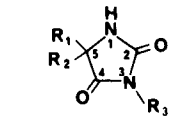
The problems specific to each class of anticonvulsants (barbiturates, hydantoins, succinimides, etc.) will be dealt with first, then the separation and quantification of several coadministered anticonvulsants will be treated. Various approaches to sample preparation will be discussed in a separate section. Finally, we will compare GLC and HPLC along with other techniques used for the routine assay of antiepileptic drugs. In particular, chromatographic methods will be compared to the enzyme-multiplied immunoassay technique (EMIT).



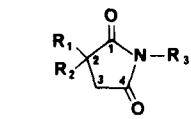
	R_1	R_2	R_3
Phenobarbital	C_6H_5	C_2H_5	H
Mephobarbital	C_6H_5	C_2H_5	CH_3



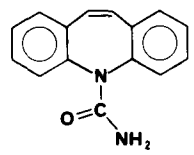
Primidone	C_6H_5	C_2H_5	—



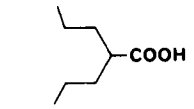
Phenytoin	C_6H_5	C_6H_5	H
Mephenytoin	C_6H_5	C_2H_5	CH_3



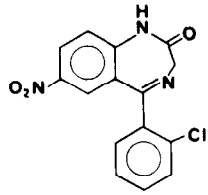
Ethosuximide	CH_3	C_2H_5	H
Methsuximide	CH_3	C_6H_5	CH_3
Phensuximide	H	C_6H_5	CH_3



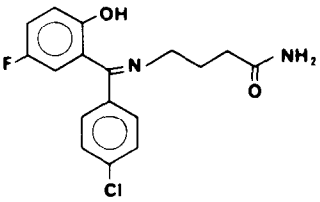
Carbamazepine



Valproic acid



Clonazepam



Progabide

Fig. 1. Chemical structures of the antiepileptic drugs.

2. ANALYSIS OF INDIVIDUAL CLASSES OF ANTIEPILEPTICS

Anticonvulsants have a weak acid function or an amide group capable of forming hydrogen bonds, as well as a hydrophobic moiety consisting of alkyl or aryl groups (Fig. 1). As shown in Table 1, the solubility in water may vary

from freely soluble ethosuximide to nearly insoluble carbamazepine. The difference in partition coefficient and in acidity between primidone and phenytoin indicates that a slight modification of the structure can have a dramatic influence upon the physical properties of these compounds, and hence on the extraction procedure for their determination. These differences will be described briefly in this section, which discusses methods peculiar to each class of anticonvulsants.

2.1. Barbiturates

Barbiturates are derivatives of malonylurea with various groups on the carbon at the 5-position. Although many barbiturates have been shown to possess anticonvulsant activity, only phenobarbital and a few related to it are prescribed. Other barbiturates are too sedative, and consequently cannot be used.

Phenobarbital is a weak acid (pK_a 7.3), and hydrogens on both nitrogens N-1 and N-3 can easily be replaced by an alkyl group. It is therefore amenable to gas chromatographic (GC) determination either underivatized or as an alkyl derivative. Furthermore, its UV absorption permits analysis by liquid chromatography (LC) with UV detection.

Phenobarbital is metabolized by hydroxylation, mainly in the *para*-position. Although *p*-hydroxyphenobarbital is devoid of antiepileptic activity, its determination is important when studying phenobarbital elimination.

2.1.1. Gas chromatography

A few years ago Pillai and Dilli [7] published a comprehensive survey of the analysis of barbiturates by GC. In that review, the authors emphasized historical developments and one should consult their work for a glimpse at older procedures and references.

Many of the techniques described in this section are not specific to the barbiturates, but may also be applied to other anticonvulsants, as will be described in the following sections. Furthermore, not all of these methods offer the same degree of practicality for drug monitoring in the clinical setting. Analysis of the underivatized drug and on-column alkylation are by far the most often utilized procedures for routine practice.

2.1.1.1. Analysis of free barbiturates. As stated above, phenobarbital has two imide functions responsible for its acidity and its potential hydrogen bonding interactions. It is obvious that interactions with basic sites and with groups capable of forming hydrogen bonds should be minimized in order to achieve a high-quality chromatographic analysis.

This can be accomplished by adding a volatile acid like formic acid to the carrier gas. Formic acid neutralizes basic sites and adsorbs onto the support through hydrogen bonds with Si—OH and Si—O—Si groups, thereby preventing adsorption of barbiturates. This was applied early on by Welton [8] and more recently by Woo and Lindsay [9], who observed symmetrical peaks with nanogram quantities on an Apiezon column. Silanization prevents interactions with Si—OH but not Si—O—Si groups; nonetheless, the silanization procedure is widely used. It has also been claimed that the sodium salts of barbiturates may be directly injected [10]. Although this is possible, one should expect a short column life from such a practice.

Many stationary phases have been proposed for the separation of underivatized barbiturates on packed columns [11—15], and more recently their separation has been described on wall- or support-coated capillary columns [16,17]. The development of these newer bonded phases minimizes the interactions with the column and increases the stability of the stationary phase [18]. Furthermore, the handling of the capillary columns has been made simpler by the introduction of flexible fused-silica capillary columns which circumvent the breakage problem of the older-type glass capillary columns.

Analysis of free barbiturates in biological material may, however, be subject to serious limitations because of the accumulation of underivatized endogenous compounds on the column. Introduction of a clean-up step into the sample preparation procedure can help alleviate this problem.

2.1.1.2. On-column alkylation. Although barbiturates may be analysed directly, their chromatographic behaviour shows tailing, the importance of which depends upon column conditions. In order to improve the peak shape, many methods include a derivatization step prior to the chromatographic separation. Among such procedures, on-column alkylation (also called "flash alkylation") predominates for routine drug level monitoring, and much has been published on this method.

Methylation is the most common alkylation reaction for barbiturates. With this technique, the acidic imide is methylated in the injector port with a quaternary ammonium salt in a Hoffman degradation reaction, liberating a tertiary amine:

$$R{-}\overset{+}{N}(CH_3)_3 + \geq N^- \rightarrow > N{-}CH_3 + R{-}N(CH_3)_2$$

Robb and Westbrook [19] described the methylation of acidic compounds with tetramethylammonium hydroxide (R = CH_3); and as early as 1966, Stevenson [20] reported separating eighteen compounds following methylation with this reagent. Other authors have described variations of this method [21—23]. In particular, Brochmann-Hanssen and Oke [24] found that R = phenyl was a better leaving group than R = methyl, thus allowing the reaction to take place at a lower temperature. The reagent trimethylphenylammonium hydroxide (2*M* in methanol), which is commercially available, may be the most widely used methylating agent.

The procedure, although rapid, is not devoid of problems. In many instances, a peak eluted before the N,N′ derivative of phenobarbital is observed. This "early phenobarbital" peak, thought to be 2-ethyl-2-phenylmalondiamide, was identified as N-methyl-2-phenylbutyramide by Wu [25] and Osiewicz et al. [26]. The importance of this degradation product depends upon the concentration, the nature of the reagent and upon the chromatographic conditions. By decreasing both the concentration of the quaternary ammonium hydroxide and the temperature of the injector, it is possible to obtain a single peak with phenobarbital [27]. Also, since N,N′-dimethylphenobarbital is not stable in basic medium, even at room temperature [28], another way of minimizing this degradation is to adjust to neutral pH prior to injection into the gas chromatograph [24,29,30].

If the "early phenobarbital" peak cannot be avoided, the responses due to

N,N′-dimethylphenobarbital and N-methyl-2-phenylbutyramide may be summed in order to measure the concentration of phenobarbital in plasma. This stresses the need for an internal standard that behaves similarly to phenobarbital during the derivatization process. Reliable determinations are obtained with 5-phenyl-5-tolylbarbituric acid as the internal standard [31].

All the acidic groups may be alkylated by on-column derivatization. With tetramethylammonium hydroxide, the trimethyl derivative of *p*-hydroxyphenobarbital is formed. This reaction has, therefore, also been proposed for the quantitation of both phenobarbital and its hydroxylated metabolite [32].

Mephobarbital differs from phenobarbital in that it has a methyl group on the N′-position (see Fig. 1), and consequently flash results in the same derivative for both compounds. Furthermore, phenobarbital is an important circulating metabolite of mephobarbital. MacGee [33] reported that tetraethylammonium hydroxide ethylates these two compounds thereby permitting their chromatographic separation.

2.1.1.3. Precolumn derivatization. Alkylation reactions are numerous and most of them have been applied to the derivatization of barbiturates. This topic was developed in great detail by Hulshoff and Förch [34] a few years ago. Their review, which concerned the alkylation of acidic pharmaceuticals, gives many examples of anticonvulsants and of barbiturates in particular.

Alkyl halides react with barbiturates in basic medium to give alkyl derivatives. All alkyl iodides from methyl to *n*-heptyl have been used, but methyl iodide is the reagent of the first choice. The reaction should be done under S_{N2} conditions, which implies a polar aprotic solvent. Acetone is a convenient volatile solvent; however, higher boiling ketones such as methyl ethyl ketone, acetonitrile and dimethylformamide may be used [35]. Wu and Pearson [36] reported that the addition of methanol in acetone increases the reaction rate of methylation. The reaction was complete in 10 min at 60°C with the mixture acetone—methanol—methyl iodide (1:1:1).

A base is necessary for the reaction in order to convert the acid into the anion. Sodium hydroxide may be used in solutions of different strengths. Solid anhydrous potassium carbonate in acetone is very convenient, since it is almost insoluble in organic solvents, and can be present in large excess. Dünges [37] reported the alkylation of barbiturates on a microscale using such a system.

Just over ten years ago, Greeley [38] proposed a method by which alkylation takes place in a polar aprotic solvent (usually dimethylacetamide) with tetramethylammonium hydroxide as the base. The procedure is rapid: 5—10 min are needed for a complete reaction. All *n*-alkyl iodides, from C_1 to C_7 have been applied [34]. Kapetanovic and Kupferberg [39,40] applied this derivatization technique to the determination of phenobarbital and *p*-hydroxyphenobarbital as their ethyl or propyl derivatives.

Another way of derivatization is extractive alkylation in which the barbiturates are transferred as an ion pair from the aqueous phase into the organic phase where they react with the alkyl halide [41,42]. In order to achieve good efficiency in the extraction of the ion pair, the counter-ion, tetraalkylammonium, must contain alkyl groups with at least four carbon atoms. Tetrabutyl-, tetrapentyl- and tetrahexylammonium counter-ions are mostly employed. The reaction mixture requires shaking for various times depending upon the substrate.

Barbiturates may be methylated with other reagents in addition to those described above. Diazomethane rapidly methylates these compounds to form N,N′-dimethyl derivatives as the major products along with N,O′ and O,O′ isomers [43]; this procedure allows complete removal of the reagent for gas chromatographic—mass spectrometric (GC—MS) studies. Dimethylformamide dimethylacetal also methylates acidic groups [44] and this technique has been applied to barbiturates by Venturella et al. [45].

Silylation may likewise be used; however, N-trimethylsilyl derivatives of barbiturates are relatively unstable and should only be used with caution [24, 46]. Silylation is nonetheless recommended for derivatizing metabolites containing hydroxyl groups following alkylation of the acid groups.

2.1.2. Gas chromatography—mass spectrometry

Bonnichsen et al. [47] and Gilbert et al. [48] reported the identification of barbiturates by mass spectrometry in 1970. In the early seventies, the mass spectrometer was operated in the electron-impact (EI) mode. Unfortunately, under these conditions barbiturates tend to give only fragment ions. Chemical ionization (CI), which produces intense molecular ions, is more suited for the quantification of barbiturates.

The introduction of computers led to the rapid analysis of barbiturates with stable isotopes as internal standards. Methods for the identification and quantification of barbiturates by GC—MS were developed by Horning et al. [49]. They derivatized barbiturates with diazomethane, and then detected them by selected-ion monitoring (SIM) under CI conditions with a ^{13}C-labelled internal standard.

The power of GC—MS is most evident in metabolic studies requiring the determination of metabolites along with the parent drug. The disposition of most barbiturates, and of phenobarbital in particular, has been investigated using GC—MS techniques [40,50,51]. Horning et al. [52] have used this technique to show that barbiturates are present in the breast milk of nursing mothers for a long period after the administration of the drug.

The cost of drug quantification by GC—MS may seem a trifle high when compared to standard drug monitoring techniques. Although this may be true, GC—MS analysis should be considered the reference procedure by which all others should be compared [53].

2.1.3. Liquid column chromatography

Barbiturates are weak acids and as such can be separated by ion-exchange chromatography. Early methods described the analysis of barbiturates by this technique [54,55]; however, they are seldom used for routine application. Fransson et al. [56] reported the feasibility of ion-pair chromatography for the determination of barbiturates [56].

Phenobarbital may be assayed under normal- or reversed-phase conditions; the latter predominates. As phenobarbital is often prescribed with other anti-epileptics, many procedures have been developed for their separation. This will be dealt with in Section 3.

For drug monitoring, phenobarbital is detected by UV. Its absorbance, which is pH-dependent, increases by seven-fold when the molecule is ionized in

alkaline pH. Sensitivity could therefore be increased if needed by increasing the pH between the column and the detector.

2.2. *Primidone*

Primidone is very similar in structure to phenobarbital (see Fig. 1). However, as primidone lacks the carbonyl group between the two nitrogen atoms, this compound is much less acidic than phenobarbital. Primidone has a pK_a of 13, and therefore, unlike phenobarbital, it is not ionized at physiological pH. The amide nitrogen groups in primidone are also more difficult to alkylate than the acidic imide nitrogens in phenobarbital. Furthermore, primidone has a lower partition coefficient than phenobarbital and the recovery of primidone following organic extraction is generally poor.

The metabolism of primidone yields two major products, phenobarbital and phenylethylmalonamide (Fig. 2). Even though phenylethylmalonamide has intrinsic activity, there is usually no need to quantify this compound during routine drug monitoring. Phenobarbital, however, has a longer half-life than primidone and this compound should be monitored during chronic treatment with primidone. Schaefer [57] has recently reviewed methods for the determination of primidone and its metabolites.

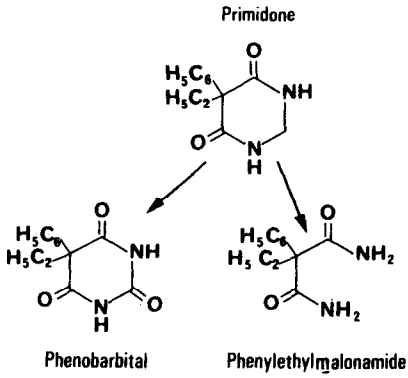


Fig. 2. Clinically important routes of primidone metabolism.

2.2.1. *Gas chromatography*

The procedures described for phenobarbital are usually amenable for the determination of primidone. Hence, primidone may be assayed underivatized on packed columns with the same stationary phases used for phenobarbital (SP-2510-DA, SP-2110—SP-2510-DA) [15,58,59] or with capillary columns [60].

On-column methylation converts primidone and phenylethylmalonamide to their N,N′-dimethyl derivatives. Although primidone is more stable than phenobarbital in these conditions, decomposition may occur and, in order to overcome this problem, *p*-methylprimidone should be included as an internal standard.

Important differences with phenobarbital can be noted with certain pre-column derivatization techniques. For instance, primidone cannot be alkylated

with methyl iodide and potassium carbonate since the latter is too weak a base [61,62]. However, with a stronger base the dialkyl derivative can be prepared. It should also be mentioned that dimethylformide dimethylacetal reacts with phenylethylmalonamide to give the dimethylaminoethylene derivative, but not with primidone [63].

For drug monitoring, underivatized and alkylated primidone is usually detected by flame ionization or with a nitrogen-specific detector. If higher sensitivity is required, the perfluoroacyl derivative can be detected by electron capture [64,65]. This reagent does not produce stable derivatives with phenobarbital.

2.2.2. Gas chromatography—mass spectrometry

As for phenobarbital, primidone yields low-molecular-weight ions under EI. For SIM, the MH^+ ions can be obtained by CI. Horning et al. [66] separated primidone, phenylethylmalonamide and phenobarbital following methylation of phenobarbital and trimethylsilylation of the other functional groups. In that experiment, [^{13}C]phenobarbital was added as an internal standard. In another procedure deuterium-labelled primidone was used as the internal standard [67] to study the placental transfer of primidone and its excretion into milk [68].

2.2.3. Liquid column chromatography

Primidone and its metabolites absorb in the UV spectrum, and therapeutic levels are high enough so they may be measured by HPLC with UV detection. As primidone and phenylethylmalonamide are very polar, they tend to be eluted near the solvent front under reversed-phase conditions, and may not be separated from each other. By decreasing the content of the organic solvent in the mobile phase, separation of primidone and phenylethylmalonamide may be achieved on octadecyl columns [69]. Primidone is usually prescribed with other antiepileptics, and the HPLC separation of primidone, phenytoin, phenobarbital and carbamazepine will be described in more detail in section 3.

2.3. Phenytoin and mephenytoin

Two of the major anticonvulsants, phenytoin and mephenytoin, and a lesser one, ethotoin, are hydantoin derivatives with various substituents at the 5-position (Fig. 1). Mephenytoin differs from phenytoin by the replacement of a phenyl by an ethyl radical, and by methylation of the nitrogen at the 3-position.

At variance with barbiturates, the two nitrogens of phenytoin are not equivalent. The amide (N-1) is less acidic than the imide (N-3), and by virtue of the latter phenytoin is a weak acid with a pK_a of 8.06 [70]. The solubility of phenytoin in water at room temperature is around 20 μg/ml and it increases to 75 μg/ml in plasma at 37°C. This is due in part to its binding to plasma proteins [71]. Both phenytoin and mephenytoin have a high partition coefficient, and their extraction from acidified plasma can be essentially complete.

Phenytoin is metabolized by hydroxylation in the *para*-position of one of the phenyl rings. This *p*-hydroxylated compound (*p*-HPPH) accounts for around 70% of the dose. Other metabolites (dihydrodiol, catechol and O-

methylcatechol) have also been identified. None of these metabolites seem to have anticonvulsant activity. In a few patients, phenytoin accumulates due to a genetic defect of hydroxylation. Therefore, even though *p*-HPPH is not active, it may be necessary in those instances to measure its urinary excretion. Phenytoin is a prochiral drug since upon hydroxylation on one phenyl ring it becomes a chiral molecule. In man, *p*-HPPH is laevorotatory [72]; Poupaert et al. [73] established its absolute configuration as (*S*)-(−)-*p*-HPPH.

Mephenytoin is extensively demethylated to 5-ethyl-5-phenylhydantoin (nirvanol), an active compound. Nirvanol like phenytoin is a weak acid with a pK_a of 8.5 [74] and is less soluble in organic solvents than mephenytoin. Apart from being N-demethylated, mephenytoin is also hydroxylated on the phenyl ring. This hydroxylation occurs preferentially on the *S*-(+)-enantiomer, whereas N-demethylation occurs preferentially on the *R*-(−)-enantiomer (Fig. 3) [75, 76]. The hydroxylated metabolite has no activity.

Recently, the chromatographic determination of phenytoin, mephenytoin and ethotoin has been reviewed by Glazko [71] and by Kupferberg [74].

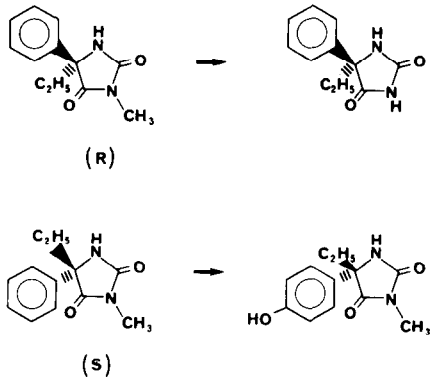


Fig. 3. Principal routes of metabolism of the *R*- and *S*-isomers of mephenytoin.

2.3.1. Gas chromatography

Procedures for the determination of underivatized phenytoin [71,77—85] mephenytoin and ethotoin [74] have been reported, and unfortunately they suffer from the same drawbacks as for barbiturates. Tailing peaks are likely to occur unless great care is taken to obtain a deactivated column. However, it is possible to obtain symmetrical peaks with the new stationary phases especially developed for the separation of underivatized antiepileptics [15]. It is also possible to obtain sharp non-tailing peaks of underivatized hydantoins with cross-linked capillary columns [18,86].

Nevertheless, procedures involving derivatization of hydantoins seem to be the most widely used in routine laboratories. Among those, on-column alkylation predominates, and most procedures reported in the literature are derived from the originals of MacGee with tetramethylammonium hydroxide [87] and of Kupferberg with trimethylphenylammonium hydroxide [22]. Antiepileptic therapy necessitates the administration of several antiepileptics, and quite a few methods involve the separation of phenytoin, phenobarbital, primidone and other antiepileptics. This problem will be addressed in section 3.

In order to differentiate between mephenytoin and its N-demethylated metabolite, an alkyl derivative other than methyl must be used. Küpfer and Bircher [88] proposed propyl derivatives formed prior to injection. De Sagher et al. [89] reported ethylation with ethyl iodide; they found that the reaction was dependent upon the solvent and would proceed smoothly in polar aprotic solvents such as acetone. Yonekawa and Kupferberg [90] improved the procedure by using a higher-boiling ketone, methyl ethyl ketone. This example is interesting in the fact that phenytoin reacts much faster than nirvanol, indicating an influence of the substituent at the 5-position on the reactivity at N-1.

Dimethylformamide dimethylacetal forms N,N′-dimethyl derivatives of phenobarbital but is unable to permethylate phenytoin. Diazomethane gives the 3-methyl derivative of phenytoin, though smaller quantities of dimethyl derivatives may also be formed. Dialkyl derivatives become preponderant with diazoethane [91].

Drug monitoring of hydantoin antiepileptics does not require a great sensitivity and most procedures involve flame ionization detection. Thermionic detectors specific for nitrogen may add selectivity if needed.

All the aforementioned methods measure mephenytoin and 5-phenyl-5-ethylhydantoin as their racemates. Recently, Wedlund et al. [92] reported the enantiomeric resolution of both compounds on a chiral capillary column following propylation at the 3-position. These authors found that *S*-mephenytoin had an elimination half-life of 3 h whereas the *R*-enantiomer had higher peak plasma levels and a half-life of over 70 h.

2.3.2. Gas chromatography—mass spectrometry

Using SIM, Rane et al. [93] followed the transplacental transfer of phenytoin with 0.1-ml samples of plasma. Horning et al. [66] have measured phenytoin excretion in milk with the same technique.

GC—MS may also be used for the simultaneous determination of hydantoins and their metabolites. Hoppel et al. [94] assayed phenytoin and *p*-HPPH following extractive alkylation. Yonekawa and Kupferberg [90] measured mephenytoin and its N-demethylated metabolite. Ethotoin and some of its metabolites were likewise determined by this technique [95].

Pharmacokinetic studies at steady state by pulse labelling, a procedure in which a dose of the drug is replaced by its stable isotope labelled variant, is also possible with GC—MS. Kupferberg [96] investigated the influence of genetic differences upon phenytoin disposition with this method.

2.3.3. Liquid column chromatography

There are no particular difficulties in measuring phenytoin or the other hydantoin antiepileptics by liquid column chromatography. These compounds can be analysed under normal- [97,98] or reversed-phase [99—102] conditions with UV detection. Many procedures have been reported for the separation of phenytoin, phenobarbital, carbamazepine and primidone; these will dealt with in section 3.3.

Phenytoin and its hydroxylated metabolites, *p*-hydroxylated, catechol, O-methylcatechol and dihydrodiol, can be separated by reversed-phase on a C_{18} column [9]. Recently Fritz et al. [103] were able to separate the enantiomers

of *p*-HPPH by chiral ligand exchange LC. Baseline separation of *R*- and *S*-HPPH were obtained with the chiral Ni complexes of C_8-(L)-prolinamide.

2.4. Carbamazepine

Carbamazepine, a carboxamide derivative of iminostilbene, is very poorly soluble in water (72 μg/ml) [104]. Due to its high partition coefficient (Table 2), quantitative extraction can be obtained with most organic solvents. Carbamazepine lacks the acidic character of many other acidic anticonvulsants and therefore cannot be purified through back-extraction in the same manner. However, carbamazepine can be separated from other acidic anticonvulsants such as phenytoin and phenobarbital by extraction at alkaline pH.

TABLE 2

PHYSICAL PROPERTIES OF ANTIEPILEPTIC DRUGS

Drug	Molecular weight	Melting point (°C)	pK_a	Solubility*	Partition coefficient**
Phenobarbital	232.2	176	7.3	Freely (as salt)	4.2
Primidone	218.2	286	>12	0.6 μg/ml (37°C)	0.7
Phenytoin	252.2	295—298	8.3	14 μg/ml pH 1.7	18.8
Mephenytoin	218.2	136		Insoluble	
Ethosuximide	141.1	64		190 mg/ml pH 3.8	4
Methsuximide	203.2	52		2.8 mg/ml	5.3
Phensuximide	189.2	72		4.2 mg/ml	1400
Carbamazepine	236.2	190—193	>12	Insoluble	97
Clonazepam	315.7	237—239	10.5	Insoluble	
Valproic acid	144.2	-***	4.5	Freely (as salt)	
Progabide	334.8	141—144	>12	Insoluble	

*At 25°C unless stated.
**Chloroform—water at pH 3.4.
***Liquid at room temperature.

Carbamazepine is metabolized to a stable active epoxide at positions 10 and 11, and this is further converted to the inactive 10,11-dihydroxy metabolite. This epoxide is found in plasma of patients in significant concentrations, albeit lower than those of carbamazepine. Hydroxylation of the aromatic ring gives inactive metabolites [105].

2.4.1. Gas chromatography

Carbamazepine may be determined by GC either as the free drug [15,106—114] or following derivative formation (Table 3). It was realized early on that carbamazepine is unstable in the injection port of the chromatograph and degrades to iminostilbene and to 9-methylacridine [106]. In the same conditions carbamazepine epoxide rearranges to 9-acridine carboxy aldehyde (Fig. 4). Hence, for quantitative work, three approaches may be taken: chromatography on deactivated phases to reduce degradation, formation of stable derivatives, or find conditions for which the conversion to iminostilbene is complete.

In the first approach it is essential to reduce any possible interactions of the

TABLE 3

GAS CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF VALPROIC ACID AND CLONAZEPAM

Reference		Year	Internal standard	Phase	Temperature (°C)	Detection*	Derivative
Valproic acid							
Ferrandes and Eymard	[183]	1973	Methylpentylacetic acid	10% SE-30	100	FID	TMS
Meijer	[151]	1973	Trimethylcaproic acid				
Dijkhuis and Vervloet	[152]	1974	Naphthenic acid	5% RA-350	160	FID	None
Schoben and Van der Kleijn	[153]	1974	Norflunitrazepam	10% DEGS	122	FID	None
Vree et al.	[171]	1976	Cyclohexane carboxylic acid	5% FFAP	170	FID	None
Wood et al.	[154]	1977	Cyclohexane carboxylic acid	10% SP-216-PS	130	FID	None
Fellenberg and Pollard	[155]	1977	Octanoic acid	10% DEGA	150	FID	None
Löscher	[156]	1977	2-Ethyl-2-methylcaproic acid	10% Carbowax 6000	125	FID	None
Dusci and Hackett	[172]	1977	Methylmyristic acid	10% Carbowax 20M TPA		FID	None
Berry and Clarke	[157]	1978	Caproic acid	1.5% SP-1000	140	FID	None
Kupferberg	[158]	1978	Cyclohexane carboxylic acid	10% SP-1000	190	FID	None
Libeer et al.	[159]	1978	2-Ethyl-2 methylcaproic acid	10% AT-1000	200	FID	None
Balkon	[160]	1978	Paramethadione	3% OV-225	110	FID	None
Levy et al.	[161]	1978	4-Methylvaleric acid	5% FFAP	140	FID	None
Willox and Foote	[175]	1978	Cyclohexane carboxylic acid	3% OV-17	93	FID	Methyl
Gyllenhaal and Albinson	[176]	1978	Heptanoic acid	2% SP-1000	80	FID	Methyl
Varma and Hoshino	[170]	1978	Cyclohexane carboxylic acid	3% OV-17	60	FID	None
Jakobs et al.	[184]	1978	2-Ethyl-2-methylcaproic acid	5% DEGS-PS	135	FID	None
Pileire	[185]	1979	Octanoic acid	5% Carbowax PS	150	FID	None**
Pisani et al.	[162]	1979	Trimethylsuccinimide	10% DEGS-PS	175	FID	None
Runci and Segre	[163]	1979	Thymolnic acid	5% FFAP	230	FID	None
Cartron et al.	[164]	1979	Octanoic acid	10% DEGA 2% Orthophosphoric acid		FID	None
Hershey et al.	[174]	1979	2-Ethylpentanoic acid or cyclohexane carboxylic acid	10% SP-1000	160	FID	None
Hulshoff and Roseboom	[179]	1979	Octanoic acid	3% OV-17	115	FID	Butyl
Grgurinovich and Miners	[166]	1980	Octanoic acid or terephthalic acid	10% Carbowax 20M	180	FID	None
Sioufi et al.	[167]	1980	Octanoic acid	10% SP-216-PS	175	FID	None
Chan	[182]	1980	Octanoic acid	3% PC-3210	210	ECD	Phenacyl
Odusote and Sherwin	[168]	1981	Cyclohexane carboxylic acid	10% DEGS-PS	155	FID	None
Yu	[169]	1981	Octanoic acid	5% FFAP	190	FID	None
Morita et al.	[178]	1981	2-Propylhexanoic acid	3% OV-17	90	FID	Propyl
Kupferberg	[150]	1982	Cyclohexane carboxylic acid	10% SP-1000	190	FID	None
Nishioka et al.	[188]	1983	Octanoic acid	3% OV-1	80	FID/ECD	Hexafluoroisopropanol ester
Rege et al.	[181]	1984	Caproic acid	3% OV-17	205	FID	Phenacyl
Degel et al.	[186]	1984	2-Ethylbutanoic acid	10% DEGS-PS	170	FID	None**

Clonazepam							
De Silva et al.	[201]	1974	Flunitrazepam	3% OV-17	230	ECD	Aminobenzophenone
Naestoft and Larsen	[211]	1974	RO-5-4435	1% OV-17	270	ECD	Aminobenzophenone
Knop et al.	[217]	1975					
Parry and Ferry	[202]	1976	Norflunitrazepam	4% OV-101	280	ECD	Ethyl
De Silva et al.	[203]	1976	Nitrazepam	3% OV-17	240	ECD	Methyl
Gerna and Morselli	[198]	1976	2-Aminobenzyl-5-chloro-benzophenone	3% OV-17	280	ECD	None
Min and Garland	[205]	1977	[^{15}N] Clonazepam	3% OV-25	260	SIM (CH_4)	None
Cano et al.	[206]	1977	Methylclonazepam	3% OV-225	231	ECD	None
Min et al.	[212]	1978	[^{15}N] Clonazepam	1% OV-25	275	SIM	None
De Boer et al.	[213]	1978	Nitrazepam	3% OV-17	230	ECD	None
Edelbrock and De Wolff	[216]	1978	Methylnitrazepam	3% OV-17	245	ECD	None
Garland and Min	[214]	1979	[^{15}N, ^{18}O] Clonazepam	1% OV-25	245	ECD	None
Larking	[218]	1980	Desmethylflunitrazepam	3% OV-17	25	ECD	Aminobenzophenone
Riva et al.	[207]	1981	Methylclonazepam	2% SP-2510-DA	245	ECD	None
Dhar and Kutt	[208]	1981	2-Amino (dichloroaminoethyl)-5-chloro-2'-fluorobenzophenone	3% SE-30	214	NPD	Aminobenzophenone
Badock and Pollard	[209]	1982	Methylclonazepam	SP-2510-DA	260	ECD	None
Löscher and Al-Tahan	[210]	1983	Desmethyldiazepam	3% SP-2250	270	ECD	None
Joyce et al.	[215]	1984		WCOT capillary SE-30	5 min^{-1}	MS	

*FID = flame ionization detection; ECD = electron-capture detection; SIM = selected-ion monitoring; NPD = nitrogen—phosphorus selective detection; MS = mass spectrometry.
**No extraction.

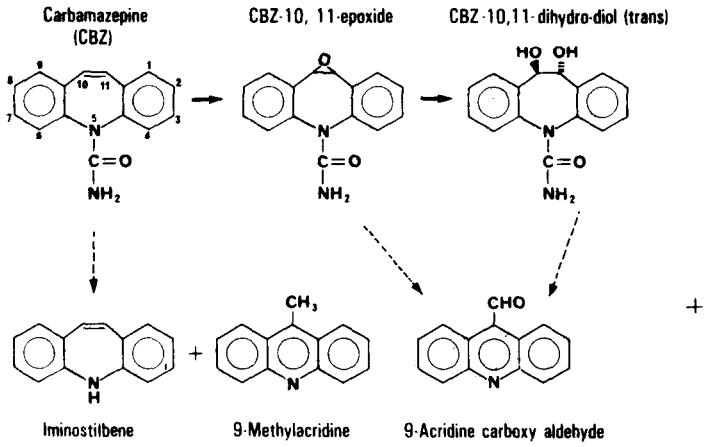


Fig. 4. Degradation of carbamazepine during gas chromatography.

underivatized compounds with the stationary phase and/or the support during the chromatographic process. This can be accomplished either by silanizing the column [106] or by using a stationary phase especially prepared for acidic compounds [15]. Numerous authors have reported methods for determining underivatized carbamazepine by these techniques.

Several stable derivatives of the amide function have been suggested to diminish the polarity of carbamazepine and thereby minimize absorption phenomenon leading to its degradation. Trimethylsilyl (TMS) derivatives have been proposed by Kupferberg [115] and by Least et al. [116]. Perchalski and Wilder [117] used the dimethylaminomethylene derivatives for this purpose. Reaction of a primary amide with trifluoroacetic anhydride may yield the expected trifluoroacetyl (TFA) derivative, but what is usually observed is a nitrile formed by dehydration of the amide. This amide was reported by Gerardin et al. [118], whose method is also noteworthy for including a clean-up procedure on a small silica gel column.

A few authors have followed the third approach, completely converting carbamazepine to iminostilbene [119—121]. This method is further complicated by the degradation of carbamazepine epoxide to 9-acridine carboxy aldehyde. Nevertheless, it is possible to analyse carbamazepine and its epoxide by this method [122].

It seems to us that the use of the correct internal standard is critical for the quantification of an unstable molecule like carbamazepine. In particular, the internal standard should follow the same rate of degradation as carbamazepine or its epoxide. Analogues of carbamazepine (e.g. 10-methoxycarbamazepine [118], 2-methylcarbamazepine [123]) should be used whenever possible.

Flame ionization detection is usually sufficient for monitoring carbamazepine in routine applications. Additional specificity may be obtained by thermionic detection and by SIM.

2.4.2. Gas chromatography—mass spectrometry

In GC—MS studies it is best to monitor the intact molecule, either derivatized or not. Palmer et al. [124] reported the quantitation of carbamazepine down to 50 ng/ml with 10,11-dihydrocarbamazepine as the internal standard. The

selectivity of MS detection allows the use of a very short column, and both carbamazepine and its epoxide can be simultaneously measured by SIM [125, 126]. GC—MS techniques are best suited for pharmacokinetic studies at steady state with pulse labelling. This methodology has been utilized to study the autoinduction of carbamazepine following administration of the ^{2}H- or ^{15}N-labelled compound [127,128].

2.4.3. Liquid column chromatography

Carbamazepine and its epoxide can be easily determined by liquid column chromatography. Recent procedures tend to favour reversed-phase chromatography [101,129—136], though normal-phase methods are equally valid [137—139]. Most routine separations have been carried out with columns packed with 5- or 10-μm particles. Shorter analysis times (less than 3 min) have been obtained in our laboratory for the simultaneous analysis of carbamazepine and its epoxide by using 7.5-cm columns packed with 3-μm particles.

Carbamazepine absorbs in the UV range at 214 and 288 nm. Some specificity may be obtained with the latter wavelength with a concomitant loss of sensitivity. A 254-nm fixed-wavelength detector also gives satisfactory results.

It is our opinion that the thermal instability of carbamazepine makes HPLC more attractive than GC application. This is all the more true in that carbamazepine and its epoxide can be analysed by HPLC with other antiepileptics (see section 3).

2.5. Succinimides

Ethosuximide, methsuximide and phensuximide are derivatives of succimide that have different alkyl groups on the 2-position (Fig. 1). Ethosuximide is an acid while the N-methylated compounds, methsuximide and phensuximide, are not. These three compounds are characterized by a high water solubility (190 mg/ml at 25°C for ethosuximide) and a low melting point [140]. The latter makes them amenable to GC analysis.

Methsuximide and phensuximide are extensively N-demethylated in the body [141]. The desmethyl metabolite of methsuximide but not of phensuximide accumulates in plasma and the concentration of desmethylmethsuximide can be several hundred times higher than that of the parent drug. As this metabolite is an active species, it is obvious that measurement of the N-desmethyl compound is more useful than that of the parent drug [142]. The difference in the N-demethylation rate between methsuximide and phensuximide lies in part in the fact that dihydropyrimidase preferentially cleaves succimide derivatives with only one substituent at the 2-position. Hydroxylated metabolites have been identified, but these are inactive.

2.5.1. Gas chromatography

Underivatized ethosuximide produces sharp peaks on polar phases such as OV-17 or OV-225 [142—145] at temperatures below 200°C. Methsuximide and phensuximide cannot be derivatized and thus are assayed as such.

Ethosuximide and the N-desmethyl metabolites of methsuximide and phensuximide contain an imine group, and they can be derivatized with procedures

developed for barbiturates, namely on-column alkylation [146,147] or alkylation prior to injection [53,148]. Butylation is favoured by a few authors for it decreases the volatility of the derivatives [146,148]. Most of these methods allow the simultaneous determination of ethosuximide with valproic acid and with other acidic antiepileptics.

These drugs are very volatile, and for methods requiring evaporation of an organic extract the addition of isoamyl acetate [149], isoamyl alcohol [142] or pyridine [53] may circumvent any possible loss during that step.

2.5.2. Gas chromatography—mass spectrometry

For GC—MS studies of ethosuximide Horning et al. [53] reported monitoring the MH^+ ions of the TMS derivatives. It should be noted that TMS derivatives of acidic substances like succimides are not the most stable. However, with an appropriate internal standard such as trimethylsuccinimide, the procedure gives reproducible results. Methsuximide and its N-demethylated metabolite can be determined underivatized under EI conditions using the fragment obtained by the loss of the imide group [142], or following butylation [149].

2.5.3. Liquid column chromatography

Ethosuximide, N-desmethylmethsuximide and phensuximide can all be measured by liquid column chromatography with UV detection. Ethosuximide is by far the most commonly utilized succinimide and HPLC methods for quantifying this compound usually include the concomitant measurement of other antiepileptic drugs. These techniques will be discussed in section 3.2.2.

2.6. Valproic acid

Valproic acid (2-propylpentanoic acid) is a clear liquid with a pK_a of 4.5. Its sodium salt, which is very hydroscopic, should be stored in a desiccator. This short-chain carboxylic acid is volatile and can be determined by GC without derivatization or following alkylation with methods described for barbiturates. Its acidic character allows the biological extract to be cleaned-up by back-extraction and most of the published methods are based on these principles [150].

2.6.1. Gas chromatography

As stated above, valproate can be assayed by GC underivatized following diffusion [151], single [152—171] or multiple [172—174] extractions of the acid (see Table 3 for a summary). The compound can also be quantitated as the methyl [175—177], propyl [178], butyl [179], phenacyl [180—182] or TMS [183] derivatives. A few authors have reported methods that allow the direct injection of biological samples [184—186]. In one of those [185], the salt is converted to the acid on-column by flushing with formic acid between injections.

Valproic acid can be extracted with diethyl ether, toluene, chloroform or carbon tetrachloride following acidification with a strong acid [152,153,158, 168,174,178,187]. Levy et al. [161] recommended an extraction pH of 4.5

to minimize degradation of conjugates in the plasma which may accumulate during chronic therapy. Back-extraction into 0.25 *M* sodium hydroxide has also been added for sample clean-up [174].

Valproic acid will be lost during solvent evaporation if preventive measures are not taken. These may include evaporation at ambient temperature, not completely evaporating to dryness, adding a small quantity of a high-boiling-point liquid (e.g. isoamyl acetate), or using a minimal volume of extraction solvent and injections without evaporation. The method of Morita et al. [178], which prepares the propyl derivative on-column, offers the advantage of double extraction without dilution in the organic phase. This procedure is obviously an adaptation of the MacGee [87] procedure described for phenytoin.

Chromatography of valproic acid does not create any particular problems. Most methods are based on the separation with columns developed for free fatty acids (FFAP columns or 10% SP-1000).

As usual, quite a few internal standards have been proposed. The most suitable are acids closely related to valproic acid. Hershey et al. [174] have shown evidence that, among those, 2-ethylpentanoic acid or 2-propylhexanoic acid give more erratic results than cyclohexane carboxylic acid. This may be corrected by periodically replacing the first few centimetres of the column packing.

Sensitivity is not a limiting factor for monitoring valproic acid at steady state, and flame ionization detection is sufficient for routine purposes. However, for additional sensitivity, Nishioka et al. [188] and Chan [182] have reported electron-capture detection (ECD) of the hexafluoroisopropanol and phenacyl esters.

2.6.2. Gas chromatography—mass spectrometry

Nau et al. [189] have proposed a GC—MS procedure for the determination of valproic acid and its metabolites [2-propyl-2-pentenoic acid (*trans*), 2-propyl-3-pentenoic acid (*trans*), 2-propyl-4-pentenoic acid, 3-hydroxy-2-propylpentanoic acid, 4-hydroxy-2-propylpentanoic acid, 5-hydroxy-2-propylpentanoic acid, 3-oxo-2-propylpentanoic acid and 2-propylglutaric acid] as their TMS derivatives. A sensitivity limit of 3—6 ng/ml was achieved for most metabolites with a sample size of 200 μl.

The pharmacokinetics of valproic acid and its metabolites were also investigated following administration of [1,2-^{13}C]valproic acid [190]. Hexadeuterated valproic acid was used for the identification of metabolites by Acheampong et al. [191]. Deuterated analogues of valproic acid have been separated from the unlabelled compound on a 60 m × 0.25 mm fused-silica column coated with OV-351 [187]. This could open the way for conducting bioavailability and pulse labelling studies without the need of a mass spectrometer.

2.6.3. Liquid column chromatography

Valproate has a weak UV absorbance which makes valproic acid detection difficult by HPLC. Nevertheless, it is still possible to quantitate this compound by HPLC following attachment of a suitable chromophore [192—198] on the carboxylic function. At this point in time, however, GC is to be preferred to HPLC until methods to detect valproic acid by liquid column chromatography improve.

2.7. Clonazepam

Clonazepam is not ionized at physiological pH as the imino nitrogen (N-4) protonates below pH 1.5 and the pK_a of the amido nitrogen is around 10.5 [199]. The pH for its extraction does not seem critical; however, at pH values greater than 9 acidic anticonvulsants would not be extracted.

The metabolic reduction of the nitro group of clonazepam yields an aromatic amine, which is further acetylated [200]. Neither compound seems to possess any pharmacological activity and thus need not be determined for routine drug monitoring.

As indicated in Table 1, steady-state concentrations are in the nanogram range, around one thousand times lower than those of most other antiepileptics. The problem in analysing clonazepam, therefore, lies in its determination without interference from the usual background endogenous substances.

2.7.1. Gas chromatography

Clonazepam may be determined underivatized [204—206, 209—216], but the amide moiety induces strong interactions through hydrogen bonding and great care should be taken to inactivate the column to obtain symmetrical peaks. Due to the high temperature of the analysis (around 250°C), a thermostable phase must be used; OV-17 or related phases have proved to have the needed thermostability for a polar phase. Table 3 should be consulted for a summary of GC methods concerning clonazepam.

Clonazepam has been reported to be stable using capillary columns [216]; however, recently, Joyce et al. [215] found that it may be partially transformed during the chromatographic separation. MS studies showed that the nitro group was reduced to the corresponding amine.

Polarity of clonazepam should decrease following alkylation of the nitrogen at the 1-position. Methyl and ethyl derivatives have been proposed for alkylation prior to injection [202,203]. Obviously, this derivatization increases sample work-up time, but the chromatographic behaviour of clonazepam improves.

Clonazepam may be hydrolysed to an aminobenzophenone in strong acidic conditions (0.5 *M* sulphuric acid) upon heating. This was the basis for methods reported by De Silva et al. [201] a decade ago, and more recently by Larking [218] and Dhar and Kutt [208]. The hydrolysis is reproducible under controlled conditions. 3-Hydroxyclonazepam gives the same benzophenone as clonazepam after hydrolysis, but interference is negligible.

Flame ionization does not provide the sensitivity required for the determination of clonazepam. ECD allows analysis in the nanogram range, and most GC procedures are based upon ECD with ^{63}Ni foil. Dhar and Kutt [208] have proposed the use of a nitrogen-specific detector, which allows the concomitant determination of other anticonvulsants. The standard ECD, while more sensitive, can measure only clonazepam unless specific derivatives are prepared for the other drugs.

Specificity and sensitivity may also be achieved with SIM. The methods were developed mainly by Min and co-workers [205,212,214], who found that greater sensitivity was obtained with detection of negative ions. These methods

required stable isotope labelled molecules with a sufficient difference in atomic mass. For pharmacokinetic studies and for reference methods, these GC—MS procedures would seem to be the methods of choice.

2.7.2. Liquid column chromatography

Clonazepam has been determined by HPLC under normal-phase [219] or reversed-phase [220—222] conditions. In order to obtain a gain in specificity, Rovei and San Juan [220] proposed detection at 306 nm instead of the usual 254 nm since clonazepam has an absorption maximum around 310 nm. We have found that an endogenous substance sometimes interferes with clonazepam when a Spherisorb C_6 column is used. This substance, unidentified as yet, was variable in intensity and could be separated on a C_{18} column with a higher percentage of capped silanol sites. Petters et al. [222] have recently reported a method for the determination of clonazepam and its 7-amino and 7-acetamide metabolites. However, the metabolites had to be assayed using a different set of chromatographic conditions.

Although, HPLC procedures are reproducible, they may, in particular cases, give problems due to spurious interference. It would be interesting to try a more specific detection. Electrochemical reduction of the nitro group warrants further studies for on-line detection of clonazepam by HPLC.

2.8. Progabide

Progabide is a new γ-aminobutyric acid mimetic compound with a broad spectrum of antiepileptic activity [223,224]. It has recently been approved for use in France and is undergoing clinical testing in a number of other countries. Progabide is metabolized to an active acid metabolite, which should also be measured during clinical monitoring.

2.8.1. Gas chromatography

Progabide was first measured in biological fluids by GC with ECD [225]. This method consists of a toluene extraction at pH 4.5 followed by derivatization with heptafluorobutyric anhydride. The glass column was packed with Gas-Chrom Q coupled with 3% OV-17 and maintained at 230°C. During derivatization, which occurs on the phenol moiety, the amide function is converted to a nitrile, and the resulting compound has good chromatographic properties. Though sensitive, this method is inadequate because it does not permit concurrent analysis of the acid metabolite.

More recently, our laboratory has developed a new GC method that enables simultaneous measurement of progabide and its acid metabolite. The compounds are extracted and reduced as described in the following section. Next, these reduced products are derivatized with heptafluorobutyric anhydride; potassium carbonate crystals are added to catalyse the reaction. It is important to note that the acid metabolite cyclizes to form a lactam following reduction (see Fig. 5), and, therefore, it is only necessary to derivatize the phenol hydroxy group to obtain a product with suitable chromatographic properties. Separation and quantification can then be effected by injection into a cross-linked methylsilicone capillary column (25 m × 0.2 mm). This method offers

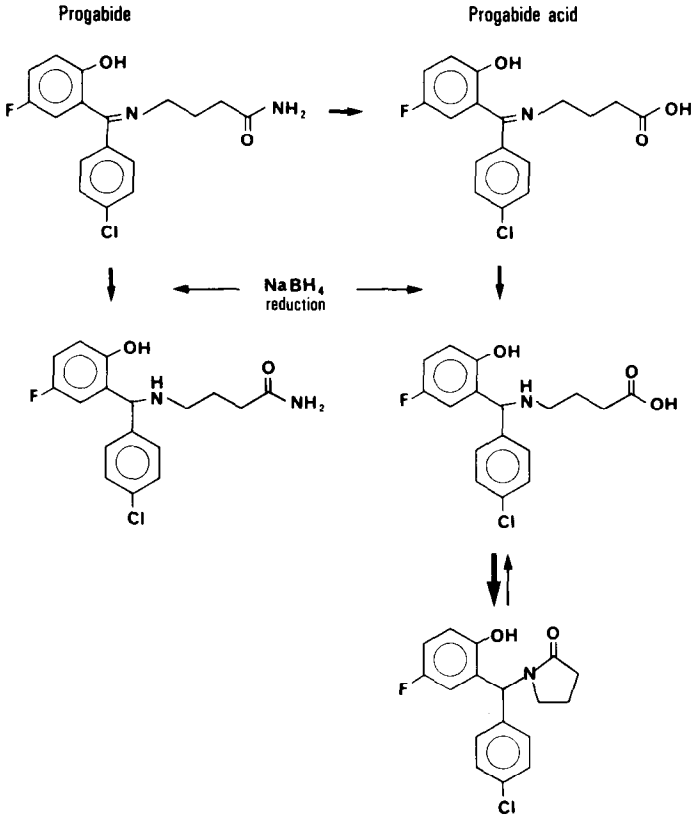


Fig. 5. Reduction of progabide and its acid metabolite by tetraborohydride.

excellent results, but derivatization along with the necessary clean-up steps to remove the catalyst make sample preparation time approximately 2 h longer than available HPLC methods.

2.8.2. Liquid column chromatography

Yonekawa et al. [226] and Padovani et al. [227] described similar techniques for measuring progabide and its acid metabolite by reversed-phase HPLC with electrochemical detection. The first step consists of extraction at pH 4.5—4.9 with hexane—2-propanol (96:4) [226] or toluene [227]. The imine bond in these compounds is then reduced by adding tetraborohydride to the organic phase. This reduction stabilizes the compounds, which are relatively rapidly degraded by both base and acid hydrolysis. The reduction is then followed by back-extraction into an acidic medium, and a final extraction at neutral pH (see Fig. 5).

The chromatographic properties of the compounds improve somewhat after reduction, but peak tailing of the resulting secondary amines is still evident. Adding a salt such as sodium chloride partially suppresses this phenomenon, but continued use of mobile phases with a high chloride content is not recommended for HPLC pumps. We have also found that a mobile phase consisting of 1% triethylamine in water (adjusted to pH 6 with orthophosphoric acid—acetonitrile—methanol (40:30:30) gives good results on Spherisorb ODS

(Phase Separations, U.K.) packed columns. However, this mobile phase is incompatible with electrochemical detection in the recommended range (+0.85 to +1.0 V).

In summary, HPLC coupled with electrochemical detection is currently the method of choice for studying the pharmacokinetics of progabide and its acid metabolite. UV detection with normal-phase chromatography may be suitable for monitoring plasma levels in the low microgram range, but this needs to be confirmed by clinical testing. The UV spectra of the reduced compounds are characterized by absorption maxima at 220 and 228 nm [226].

3. CHROMATOGRAPHIC SEPARATION AND SIMULTANEOUS QUANTITATION OF ANTIEPILEPTIC DRUGS

Simultaneous measurement of several antiepileptic agents is highly desirable. Epilepsy is usually treated with two or more antiepileptic agents, and multiple drug analysis therefore can be the most rapid and cost-effective means of monitoring drug levels in this patient population. This approach is possible because of the performance capabilities of modern chromatographic equipment along with the vast clinical and analytical experience concerning these drugs that has accumulated over the years. Additionally, the similar physicochemical properties and relatively high therapeutic concentrations of many antiepileptic compounds facilitates the task.

This section will emphasize HPLC and GC techniques for the concomitant analysis of all or various combinations of the following antiepileptics: phenobarbital, phenytoin, primidone, carbamazepine and ethosuximide along with certain of their metabolites. Sample preparation will be more fully discussed in a separate subsection.

3.1. Sample preparation

All of the principal sample pretreatment techniques (solvent extraction, deproteinization and solid-phase extraction) have been applied to the quantification of the anticonvulsant drugs. This section will discuss the relative merit of these various techniques with emphasis on their application to routine analysis of plasma or serum samples.

3.1.1. Solvent extraction

Solvent extraction remains the most widespread and universal technique of sample preparation for chromatographic analysis of the anticonvulsant drugs. This technique provides relatively clean samples, especially with the addition of back-extraction procedures, and also permits sample concentration for lower limits of detection. Liquid—liquid extractions are, however, time-consuming and necessitate adequate laboratory space for storing, evaporating and occasionally redistilling flammable solvents.

Several solvent systems have been proposed for concomitant extraction of all or various combinations of the following anticonvulsants: phenobarbital, phenytoin, primidone, carbamazepine and ethosuximide. The most popular solvents have been diethyl ether [32,228,229], ethyl acetate [230,231] and

chlorinated alkanes [22,232—235]. Ethosuximide, which is highly water-soluble, and primidone tend to give slightly lower recoveries than the other compounds. For a maximum recovery of ethosuximide, the evaporation temperature should be kept below 37°C [230]. These compounds, with the exception of carbamazepine, are weakly acidic and the pH of the aqueous phase is usually adjusted to a neutral or slightly acidic pH before extraction.

Solvent demixing with miscible polar solvents such as acetonitrile [236] and acetone have also been reported. These methods generally involve deproteinization with 1—2 vols. of the miscible solvent followed by the addition of an appropriate salt such as potassium chloride or ammonium sulphate to effect phase separation. Monaco et al. [236] further cleaned up the sample by agitating the acetonitrile—water phase with isooctane. We have also found that, following plasma deproteinization with acetonitrile, the phase separation may be realized without the addition of salt by centrifuging at −20°C and quickly transferring the organic phase. These techniques offer the advantage of better extraction of polar anticonvulsants and/or polar metabolites. Nonetheless, the extracts obtained by solvent demixing are contaminated with large quantities of endogenous substances, and with even partial evaporation the solvent may become milky in appearance.

Clean-up procedures may be added to eliminate interfering peaks. Hexane removes normal plasma constituents from a methanolic HCl phase. This back-extraction technique has been described for the simultaneous determination of phenobarbital, carbamazepine, primidone and phenytoin by GLC [117]. Phenobarbital, primidone and phenytoin can be extracted under acidic conditions and then back-extracted into 0.5 *M* sodium hydroxide [158].

As mentioned in the opening paragraph of this section, one of the disadvantages to classical liquid—liquid extractions is that agitation in extraction tubes, followed by centrifuging and pipetting of the organic solvent, is time-consuming. One alternative to decrease sample processing time during liquid—liquid extraction is to use extraction columns filled with porous diatomaceous earth (Extralut-1® from E. Merck, Darmstadt, F.R.G. is an example). These columns are employed by first mixing the plasma sample with the buffer and the internal standard. This mixture is applied to the column with the diatomaceous earth acting as a support matrix for the aqueous phase. The organic solvent is then passed through the column, a process taking approximately 0.5—2 min, and the solvent is collected in a clean tube for evaporation.

This technique has been applied to the chromatographic determination of phenytoin, primidone and phenobarbital in plasma [237] as well as the quantification of phenytoin and hydroxyphenytoin in urine [238]. The technique has also been found suitable for the analysis of progabide and its acid metabolite in our laboratory, and indeed, almost any liquid—liquid extraction involving water is easily amenable to this approach. We have found that, for a fifty-samples series, 40—60 min can be gained by performing extractions with these columns. Nonetheless, the extra cost necessitated by the use of these columns may not be justifiable to all potential users.

3.1.2. Solid-phase extraction

One of the promising additions to rapid sample preparation is solid-phase

extraction. A wide variety of bonded, silica and ion-exchange columns are currently available for this purpose from several manufacturers (Sep-Pak®, Waters Assoc., U.S.A.; Bond-Elut®, Analytichem, U.S.A.; Baker® disposable columns, J.T. Baker, U.S.A.). Bonded-phase extraction columns are of particular interest for anticonvulsant drug analysis because they are well suited for plasma or serum work-up for reversed-phase chromatography.

A high-speed HPLC method, which uses sample clean-up on C_{18} disposable columns, has recently been published for the simultaneous measurement of ethosuximide, primidone, phenobarbital, phenytoin and carbamazepine in serum [239]. Similar techniques have also been applied to anticonvulsants [240] and benzodiazepines [241,242]. In general, these techniques consist of four principal steps: column activation by rinsing with methanol and then water; adding the serum or whole blood, buffer and internal standard; rinsing with water and/or buffers; eluting with methanol and evaporating to concentrate the eluent if so desired. With the aid of vacuum devices sold by the column manufacturers, ten samples may be processed in this manner in 10—20 min. One manufacturer offers these columns in cassette form containing ten columns. These cassettes can then be placed into a specially designed automatic injector which assures, by diverting the mobile phase through the columns, that 100% of the compounds of interest present in the original sample are injected onto the HPLC column.

This approach offers numerous advantages. Although not as rapid as simple deproteinization, the technique is faster than classical solvent extraction. The chromatograms are much cleaner with column extraction than with deproteinization for the same sensitivity, and solid-phase extraction may rival or even exceed the results of liquid—liquid extraction. The columns may appear expensive, but this may be at least partially offset by reductions in technician time and solvent consumption. Furthermore, these columns may be regenerated and reused fifteen to twenty times [239]. Lack of column-to-column reproducibility, which hampered their acceptance when they were first placed on the market, seems to have improved. Also, we would like to mention that new method development with this technique, especially if the method is complex and entails several different types of columns for optimization, is not always evident, even for someone with a great deal of HPLC experience. However, with experience using this technique, efficiency improves, and one can appreciate all the advantages.

Column switching offers an on-line alternative to the column extraction techniques described in the previous paragraphs, and this technique has been applied for routine determinations of hydroxyphenytoin in urine [243] and antiepileptic drugs in plasma [244]. Notwithstanding the advantages offered by this technique, it has not been widely adopted for measuring antiepileptic drug levels. Few clinical laboratories are equipped with the necessary apparatus and the application of this technique is a novelty to most analysts. To date, this approach has generally been limited to trace enrichment and complex separations. This will undoubtedly change in the coming years.

3.1.3. Deproteinization

Deproteinization with acetonitrile [101,232,245—247] followed by injec-

tion of a portion of the supernatant is a very popular technique for preparing plasma samples for simultaneous HPLC analysis of phenobarbital, primidone, ethosuximide, phenytoin, and carbamazepine. The advantages of this technique are obvious: rapid sample work-up, very low cost, micro-scale applications and, essentially, 100% relative recoveries.

One important drawback is that, instead of sample concentration as is the case with solvent extraction, samples are diluted by deproteinization, and only a fraction of the sample is usually injected. This is not a serious limitation for routine clinical monitoring as therapeutic levels of the aforementioned anticonvulsants are sufficiently high. If desired, one may increase the fraction of the supernatant that can be injected by diluting it with an appropriate buffer; this decreases the percentage of organic solvent in the injection solution so it is less than that in the mobile phase [248]. The net gain in sensitivity is then obtained by injecting large volumes (0.1—1.0 ml) of these diluted supernatant fractions. These large injection volumes pose no problems when using columns with 5 μm particle size phases; however, it is often advantageous to use an automatic injector. This permits a regular time interval between the mobile phase disturbances produced by the injection of large volumes, and thereby gives more reproducible retention times.

Another shortcoming of protein precipitation is problems with interfering substances. Small quantities of virtually all drugs as well as a large number of endogenous plasma substances can be solubilized by this technique. Therefore, the solvent front is quite large and interfering peaks may also be present elsewhere in the chromatogram. This is even more of a problem when an increased fraction of the supernatant is injected by the technique described in the previous paragraph. Particulate contamination may also be bothersome and lead to column plugging. Chu et al. [249] have suggested increasing the acetonitrile/plasma ratio from 1:1 to 3:2, to avoid this problem, while Stafford and Kabra [250] analysing anticonvulsants have stated that it is not necessary if a 10 000 *g* capability centrifuge is used. Low-dead-volume (10 μl) 0.45-μm filters (Millex-HV$_4$®, Millipore, U.S.A., is an example) can also be used for sample clean-up following acetonitrile precipitation. The deproteinization of grossly lipaemic samples can give entirely unsatisfactory results, and it may be necessary to resort to an extraction technique [251].

Although acetonitrile is the most popular deproteinization solvent, acetone has also been applied with success, despite its high UV absorption, to anticonvulsant drug analysis by HPLC [252]. Haroon and Keith [253] have also suggested that an acetonitrile—2-propanol (1:1) mixture is a better deproteinizing solvent than acetonitrile alone. Trichloroacetic and perchloric acids may likewise be used, but cannot be recommended. One article stated that acetonitrile yields cleaner solvent fronts than perchloric acid [254]. We also have found that reversed-phase column performance deteriorates, rather rapidly, with repeated injections of supernatant solutions deproteinized with these acids. Furthermore, the relative recoveries can be quite low as drugs may bind to denatured protein in a completely aqueous system. This can lead to quantification errors if there are important quantitative and/or qualitative differences in plasma protein between the pooled control plasma utilized for the standard curve and the patient plasma.

3.2. Chromatographic separation and detection techniques

3.2.1. Gas chromatography

Rambeck and Meijer [112] compiled over one hundred methods for the determination of antiepileptic drugs by GC. Their review should be consulted as a reference to procedures published between 1969 and 1979. By looking at their impressive table one gets the impression that the search for the right method is an endeavour without end. This is confirmed by the fact that more procedures keep being published, albeit at a much slower pace.

First, it should be pointed out that GC preceded liquid column chromatography in the routine determination of antiepileptic drugs on a routine basis. Thus it is expected to find dozens of methods reported in the mid-seventies during the rapid development of GC methods in the biomedical field. In 1975, no less than sixteen articles described the determination of anticonvulsants.

From section 2, dealing with individual drugs, it is obvious that a mixture of antiepileptic drugs may be analysed either free or following derivatization of some sort. Furthermore, clinical considerations indicate that the major antiepileptics to be administered concomitantly would be phenytoin, phenobarbital, primidone and carbamazepine. Nevertheless, a few comprehensive methods have been reported for the determination of most antiepileptics [15, 255—263].

3.2.1.1. No derivatization. The analysis of a mixture does not change the intrinsic problem of the analysis of underivatized antiepileptics, i.e. the adsorption of these compounds onto the active sites of the column. Phenytoin and phenobarbital are acidic compounds. Primidone and carbamazepine are neutral, though these compounds are also capable of forming hydrogen bonds with the stationary phase. On the whole it is obvious that to minimize interactions, the column should be suited for acidic compounds.

Early on, the polar, thermostable phenylsilicone phase OV-17 was extensively used [228,263—267]. It is possible to achieve good chromatographic performance on an OV-17 column by carefully treating the support. Real progress was later made with the introduction of SP-2510-DA, a specially deactivated phase for anticonvulsants [15].

A potential problem arose, however, with the finding that cholesterol could interfere with primidone. This was solved by Godolphin and Thoma [261] who advocated adding a short precolumn of SP-2250-DA. Unfortunately, some variability could not be prevented in the preparation of these columns. A fixed combination 2% SP-2110 with 1% SP-2510-DA was then developed in order to circumvent the problem [15,268]. The cholesterol interference may be eliminated altogether by purifying the samples through short columns as described in section 3.1; these columns retain cholesterol while the antiepileptics are washed through with methanol.

Separation of phenytoin, carbamazepine, phenobarbital and primidone is achieved isothermally around 230°C; in these conditions, phenytoin, phenobarbital and primidone along with their *p*-methyl analogues are separated. Temperature programming becomes necessary if ethosuximide is to be analysed with the other drugs because of its much greater volatility. This is a point of debate; ethosuximide and valproic acid may be assayed in one set of chromato-

graphic conditions, then phenytoin, phenobarbital, carbamazepine and primidone at a much higher temperature [263].

Separation on capillary columns is not widespread in the routine laboratory analysis of anticonvulsants. Early on, working with breakable glass columns required a manual dexterity not always shared by the average technician. This is no longer true; nowadays, as fused-silica capillary columns have entered the market, there is no more technical difficulty in connecting capillary columns than in manipulating packed columns. Stationary phases chemically bonded to the column offer additional stability, and one may expect to see these columns become more prevalent in routine laboratory use in the near future.

3.2.1.2. Derivatization. Although various derivatives have been described, only the methyl derivatives formed by flash alkylation lend themselves to rapid preparation for clinical applications. The method described by Kupferberg in 1970 [22] allows the separation of phenytoin, primidone and phenobarbital. It has all the basic ingredients of the right method: extraction in acidic pH, purification by back-extraction and on-column methylation with trimethylphenylammonium hydroxide. If the pH is not high enough, primidone tends to be lost during the back-extraction step.

The procedure is greatly simplified using the reagent as the solvent for back-extraction. This step concentrates the solutes as ion pairs in the aqueous trimethylphenylammonium hydroxide. With this method the internal standard may also be added to the extraction solvent. Since the derivatized compounds may be degraded in strong alkaline conditions, the method may not be amenable to the analysis of a large batch of samples when they would stand for hours. The last point emphasizes the need for the right internal standard(s). *p*-Methylphenobarbital is the obvious internal standard to correct for the erratic degradation of N,N-dimethylphenobarbital; *p*-methylphenytoin and *p*-methylprimidone may also be added. Dudley et al. [228] have shown that multiple internal standards increase the reproducibility of the assay.

As was noted for the determination of underivatized antiepileptics, the separation of their methyl or ethyl derivatives requires temperature programming if volatile compounds like ethosuximide and methsuximide are assayed. A few comprehensive methods for the simultaneous determination of most antiepileptics have been reported. These include the separation of the antiepileptics on a packed column with OV-17 as the stationary phase, though capillary columns may also be used [269,270].

Even though a few procedures include the determination of carbamazepine along with the other compounds [258,260,262,269–275], the thermal instability of carbamazepine usually requires a special determination by preparing another derivative like the TMS or by analysing carbamazepine and its epoxide by HPLC. The determination of valproic acid, clonazepam, and progabide is not usually handled with the other antiepileptics and these drugs may be assayed as described in section 2.

3.2.2. Liquid column chromatography

In the decade that has passed since the first articles were published reporting the simultaneous analysis of two or more antiepileptic drugs by HPLC, the concept has flourished and many authors have made contributions. The outright

similarities amongst the various published methods testifies to the general agreement on the best approach. This likewise indicates that we have entered the fine-tuning stage of HPLC analysis of antiepileptic drugs. We will emphasize those articles that have been published since the review by Kraak and Crombeen [254]. Their article also includes a discussion of the theory describing the simultaneous analysis of anticonvulsant drugs. This paper should likewise be consulted for a review of normal-phase techniques for separating and quantitating antiepileptic drugs since little has been published subsequently.

A summary of selected HPLC methods is provided in Table 4, from which one may see that the typical method includes the following elements: small sample volume (25—500 μl); sample preparation consisting of a simple deproteinization step or a single extraction with a polar solvent; a barbituric acid derivative as an internal standard; a three-component mobile phase system; a reversed-phase column; manual injection; and ultraviolet detection at 195—210 nm with an option to monitor the effluent at 254 nm to maximize carbamazepine detection.

Although some methods describe the processing of 400—500 μl sample aliquots, most of them can be adapted to analyse samples of less than 100 μl. Solvent extraction produces somewhat cleaner samples, but this does not appear to result in a net gain in sensitivity. This is because only a small fraction of the solvent used to dissolve the evaporated residue is injected onto the column. Indeed, the suggested extraction solvents are sufficiently polar that interfering endogenous peaks with the same retention time as one or more of the measured compounds will become a problem if one tries to enhance the sensitivity by increasing the sample size and the fraction of extract injected. This does not normally pose a problem during clinical monitoring, but will become evident if one wants to measure concentrations in the submicrogram range. If this is the case, then the sample preparation and/or chromatographic system will have to be tailored to the individual compound.

The choice between extraction or protein precipitation, therefore, is not easily resolved. Deproteinization is faster and gives more reproducible recoveries, but is somewhat less selective. Solvent extraction gives cleaner chromatograms, although one should keep in mind that the recommended solvents are not very selective and will therefore extract many other drugs and drug metabolites. Also, solvent extraction is slower, more expensive and gives less reproducible recoveries. Solid-phase extraction, which is discussed in the section on sample preparation, may offer a noteworthy alternative.

Most of the authors quoted in Table 4 selected barbituric acid derivatives for internal standards. Soldin and Hill [101] used cyheptamide, a compound that resembles carbamazepine with the important exception that there is no tertiary nitrogen in the ring system. While this internal standard is acceptable for a protein precipitation method, it would not be a good choice for an extraction technique with most antiepileptic drugs. Phenacetin was cited by Kushida et al. [229], but this would be a poor internal standard for general patient populations that may use over-the-counter products containing this compound. Furthermore, although the authors reported good precision using this internal standard, the compound has little structural relationship with any of the antiepileptic drugs.

TABLE 4

LIQUID CHROMATOGRAPHIC METHODS FOR THE SIMULTANEOUS MEASUREMENT OF SELECTED ANTIEPILEPTIC DRUGS AND THEIR METABOLITES

Reference	Year	Drug analysed*	Sample preparation**	Internal standard	Mobile phase**	Column	UV detection (nm)
Soldin and Hill [101]	1976	PB, PRM, ESM, PHT, CBZ, (IS)	ACN ppt.	Cyheptamide	ACN—phos. buff. pH 8 (50:50)	μBondapak (C_{18})	200
Pesh-Iman et al. [232]	1979	PRM, PB, (IS), PHT, CBZ	CH_2Cl_2 ext., pH 5	Cyclopal	ACN—water—H_3PO_4 (27:72.8:0.2)	Spherisorb ODS (C_{18})	197
Christofides and Fry [231]	1980	ESM, PRM, PB, (IS), ethylphenacemide, CBZ, PHT	EtAC ext., pH 7	Heptabarbital	ACN—TBA—phos. buff. pH 7.5 (2:8)	SAS Hypersil (C_1)	200
Dolan et al. [285]	1980	PRM, PB, CBZ-E, (IS), PHT, CBZ	$CHCl_3$—IPA (75:25) ext., pH 5.5	Hexobarbital	ACN—MeOH—buff. pH 7.6 (32:2:64)	Fast-LC-8 (C_8)	200
Szabo and Browne [252]	1982	ESM, PRM, PB, N-desmethylmethsuximide, (IS), PHT, CBZ	Acetone ppt.	5-Ethyl-5-(*p*-methylphenyl) barbituric acid	ACN—MeOH—phos. buff. pH 6.8—7.0 (17:28:55)	μBondapak (C_{18})	195
Neels et al. [247]	1983	PEMA, PRM, ESM, PB, CBZ-E, (IS), PHT, CBZ	ACN ppt.	Allylisobutylbarbital	ACN—phos. buff. pH 6.7 (40:60)	LiChrosorb RP-18 (C_{18})	208
Kushida et al. [229]	1983	PRM, (IS), PB, chloramphenicol, CBZ, PHT	Ether ext., pH 6.5	Phenacetin	THF—MeOH—water (6.5:33.5:60)	LiChrosorb RP-18 (C_{18})	210
Kabra et al. [239]	1983	ESM, PRM, PB, (IS), PHT, CBZ	Bond elute (solid phase ext.)	Cyclopal	ACN—MeOH—phos. buff. pH 4.4 (135:350:515)	3 μm Applied Science Laboratories (C_{18}) or 5 μm Perkin-Elmer (C_{18})	210 (195 nm when ESM present)
Haroon and Keith [253]	1983	PB, PHT	EtAC ext., pH 3.9	5-(*p*-Methylphenyl)-5-hydantoin	ACN—MeOH—7 m*M* H_3PO_4 (10:30:60)	Zorbax CN (nitrile)	195
Gerson et al. [277]	1984	PRM, PB, (IS), PHT, CBZ	$CHCl_3$—MeOH (4:1) ext., pH 4.4	Hexobarbital	ACN—MeOH—phos. buff. pH 4.4 (280:420:1300)	μBondapak (C_{18})	195 or 254
Wad [230]	1984	PEMA, ESM, PRM, CGP10000, CP47779, desmethylphenytoin, PB, CBZ-E, mephenytoin, (IS), ethylphenacemide, CBZ, PHT	EtAC ext., pH 3.9	Hexobarbital	ACN—water gradient (ACN increased from 18% to 80%)	LiChrosorb RP-8 (C_{18})	207

*Drugs are listed in order of elution. Abbreviations: PB = phenobarbital; PRM = primidone; ESM = ethosuximide; PHT = phenytoin; CBZ = carbamazepine; CBZ-E = carbamazepine-epoxide; PEMA = phenylethylmalonamide; IS = internal standard.

**ACN = acetonitrile; MeOH = methanol; EtAC = ethyl acetate; IPA = isopropanol; TBA = tetrabutylammonium; THF = tetrahydrofuran; phos. buff. = phosphate buffer; ext. = extraction; ppt. = precipitation.

Columns packed with C_1, C_8 or C_{18} reversed phases were used in all of the listed methods with one notable exception; Haroon and Keith [253] employed a Zorbax-CN column. They claimed that this permitted a closer elution between phenytoin and its principal metabolite (HPPH) thereby facilitating their simultaneous quantification; however, they offered no data concerning the quantification of HPPH. This metabolite is inactive and is usually only measured in the urine, a technique which they did not discuss.

In general, the mobile phases cited in Table 4 elute the principal compounds in the following order: phenylethylmalonamide, ethosuximide, primidone, phenobarbital, phenytoin, carbamazepine. The pH can be varied to modify the selectivity of the chromatographic system and change the elution order of these compounds. The pH used by most authors was in the 6.5—7.5 range, and phenobarbital, which has a pK_a of 7.3, is therefore very sensitive to small pH changes in these systems. At pH 8 phenobarbital elutes before primidone and ethosuximide [101]. The temperature can also influence the resolution [101, 227], though the column may also be heated to reduce column pressure and stabilize retention times [239].

Ternary mobile phases have often been employed to enhance separation selectivity. A methanol—acetonitrile—buffer mixture was found to be useful in separating phenylethylmalonamide from ethosuximide [239] and phenytoin from carbamazepine [239,252]. Christofides and Fry [231] added an ion-pairing agent which reversed the elution order of phenytoin and carbamazepine. They also stated that this improved resolution of the other compounds, although they did not provide a chromatogram to support this claim. Furthermore, a new column must be conditioned with the ion-pairing agent to stabilize the retention times of carbamazepine and phenytoin. Kushida et al. [229] added tetrahydrofuran to their mobile phase, but mentioned no advantage for this mixture. Also, peak tailing could be observed with their system, and primidone was not adequately resolved from carbamazepine epoxide.

Both Wad [230] and Kabra et al. [239] offered unique approaches. Wad [230] used a gradient system to quantitate twelve antiepileptic drugs or metabolites. This work is well documented with data concerning drug recovery, precision and interferences. Nevertheless, the long analysis time (22.5 min) and the requirement for gradient equipment is not likely to appeal to many clinical laboratories, especially since very few need to analyse all the compounds quantitated by this method. Kabra et al. [239] took the opposite approach in developing a very fast isocratic HPLC method which enables the quantification of the five major antiepileptic drugs in less than 2.5 min with a 5 μm particle size column or less than 1.4 min with a 3 μm particle size column. This approach will become increasingly prominent as the number of laboratories equipped with the required micro-flow cell detectors increases.

In all methods, quantification is assured by UV detection. The absorption maxima for phenylethylmalonamide, ethosuximide, primidone and phenytoin are less than 200 nm [230,231,247]. Neels et al. [247], whose article included UV spectra of seven compounds, showed that the absorption maxima for phenobarbital, carbamazepine epoxide and carbamazepine were 205, 210 and 214 nm, respectively; carbamazepine also has a second maximum at 288 nm. Phenobarbital has a much higher UV absorption in strong alkaline media, but bonded silica phases do not support a pH over 7—8.

There was universal agreement that absorbance should be monitored in the 195—210 nm range. A few chose a wavelength of less than 200 nm because this resulted in the greatest sensitivity for most compounds of interest. Others selected a slightly higher wavelength (200—210 nm) as a compromise between the maxima of most compounds and one of the maxima for carbamazepine. One group of authors [247] also reported that monitoring the effluent at a higher wavelength (208 nm) rather than at 195 nm also prolongs the useful life of the UV lamp. Since the absorptivity of ethosuximide is much lower at wavelengths greater than 195 nm, Kabra [239] suggested setting the detector at 195 nm when ethosuximide is to be quantitated, and otherwise to monitor the absorbance at 210 nm.

A recent article [277] suggested using two detectors in series, one set at 195 nm for quantitating primidone, phenobarbital and phenytoin, the other set at 254 nm for quantifying carbamazepine. Indeed, the best solution may be to utilize one of the available detectors which permits one either to programme changes in the wavelength during the analysis, or better, to monitor several wavelengths simultaneously. We should emphasize, however, that excellent results can be attained by single-wavelength detection.

The subject of interfering substances is very important, but a detailed description of the reported interferences for the various methods largely exceeds the scope of this review. Nevertheless, a few general comments should be made. Certain articles [230,247] reported testing a large number of compounds and also gave the capacity factor, retention time or relative retention time of the tested substances, whereas another article [253] made no mention whatsoever of the topic. It is obvious that the number of possible comedications is quite limitless, and it is therefore very important to maintain a list of tested substances and possible interferences. The problem of metabolites is even more elusive and is best documented by testing serum from patients of subjects receiving a single chemical entity. This all points to the necessity for the analyst to have access to the names of all the drugs a patient is receiving when he receives samples for analysis. Since the selectivity may change as the column ages or from one batch of packing material to another, delicate separations with known interfering compounds should be re-verified from time to time.

Rapid sample processing time is frequently used as an argument to support one technique or another. Nonetheless, it is interesting to note that none of the cited articles mentioned the use of an automatic injector. It would therefore appear that, although rapid analysis time is important, the number of samples in a given run tends to be small in most laboratories. A fully automated system for on-line liquid—liquid extraction followed by HPLC analysis, "Fast-LC", has been proposed by Technicon (Tarrytown, NY, U.S.A.) for determining the serum levels of anticonvulsants and other drugs [278,279]. Despite the solid data and theoretical support that have been offered, this system has not attracted a large number of potential users either in research or in clinical laboratories. This is in part due to the complex appearance of the apparatus with mixing coils, tubes, pulleys, etc, which scares off analysts. Also, one must add that high-volume daily use is necessary to assure a return on the investment and adequate operator expertise.

3.3. Quantification and comparisons between methods

3.3.1. Internal standards

So far, we have discussed extraction and separation problems related to drug monitoring of antiepileptics. For quantitative analysis, chromatographic methods rely mainly, if not solely, on internal standards whereas immunoassay methodology can use only externally generated calibration curves. The right internal standard should behave in every respect like the compound to be measured during the extraction, derivatization and chromatographic processes. Indeed, the internal standard should be eluted near the compounds to be measured without impairing their separation.

In an international programme set up to control the quality of antiepileptic determinations, Dijkhuis et al. [280] found that around forty different internal standards were used for the simultaneous GC determination of phenobarbital, phenytoin and primidone. Although it is not obvious which one is the best, it is imperative to use an analogue of phenobarbital such as the *p*-methyl analogue for the internal standard as phenobarbital is unstable upon flash alkylation. The *p*-methyl analogues of phenobarbital, phenytoin and primidone are commercially available (Aldrich, U.S.A.). For the simultaneous determination of several antiepileptics by GC, it would be advisable to add several internal standards as discussed by Dudley et al. [281].

Several different internal standards are also employed in liquid column chromatographic methods, and this issue is discussed in more detail in section 3.2.2. With the exception of valproic acid, none of the LC methods require derivatization, and therefore selecting an internal standard is somewhat less critical. Nonetheless, physicochemical considerations during extraction and evaporation limit the choice of internal standards, and multiple internal standard methods should also be evaluated for liquid column chromatography.

3.3.2. Comparison between gas and liquid column chromatographic methods

Not all publications give sufficient data on accuracy and precision to allow a full assessment of the validity of the method. A few authors report the coefficient of variation obtained on a single day without evaluating the long-term reliability of the method. However, in general, the claimed within-day and day-to-day precisions are generally around 5% for both GC and LC methods. This value probably reflects the real-life reproducibility that may be obtained from skilled technicians.

Quality control programmes devised to compare precision amongst a large number of laboratories analysing antiepileptics have, however, reported values that are at least twice as high. In a recent evaluation by the Healthcontrol quality insurance programme, Wilson et al. [282] observed coefficients of variation ranging from 8.3% to 14.5% for various antiepileptics measured by LC and/or GC methods. They also reported that GC methods with derivatization tend to be less precise than those without derivatization. This was particularly noticeable for primidone, carbamazepine and valproic acid; an observation which may reflect a real difference or which may be the consequence of a lack of knowledge of derivatization procedures by a number of analytical laboratories.

Overall, the gas and liquid column chromatographic methods give similar precision, and the correlations between the two techniques are generally quite good [101,231,232,253,282,283]. Nevertheless, liquid column chromatography tends to give better results for carbamazepine and probably ethosuximide. Liquid column chromatography is a newer technique, still in rapid evolution, and, apart from valproic acid, most other antiepileptic drugs can be assayed by liquid column chromatography. The trend seems to be in switching from gas to liquid column chromatographic procedures.

3.3.3. Comparison between chromatographic and other methods

Antiepileptics are assayed principally by chromatography or by EMIT, and to lesser extent by RIA and other immunological techniques. One recent immunological technique based on fluorimetric polarization is promising, but has not yet been evaluated as extensively as has the EMIT methodology. Spectrophotometric techniques, which were the first widely used methods for determining antiepileptic drug levels, are less precise than the newer techniques and have largely fallen into disuse.

Most comparisons have been made between LC or GC methods and EMIT [236,284,285], though other authors [282,283,286] have also made comparisons with additional techniques. Precision with chromatographic methods and EMIT is similar; nevertheless, a small gain with the EMIT system may be noted for primidone [282]. It should be pointed out that EMIT and chromatographic techniques are only comparable for therapeutic concentrations, since outside this range the EMIT system performs poorly. Lack of specificity may occasionally pose problems with the EMIT system, and recently it has been reported that *p*-HPPH glucuronide may cross react with phenytoin resulting in falsely high estimates of phenytoin levels [287]. Also, EDTA interferes with valproic acid, and normethsuximide interferes with ethosuximide.

The analytical performance, practicality and cost of various methods have been compared by Meijer et al. [284]. This discussion points out that existing material as well as the volume of samples are also important factors, and that in certain circumstances chromatographic methods can be economically reasonable choices for routine antiepileptic level monitoring. It is also our opinion that chromatographic methods have an important place for antiepileptic drugs because reagents are not available for immunological assays of new drugs and metabolites; for instance, carbamazepine epoxide cannot be determined by EMIT. Furthermore, in the hands of an experienced analyst, more information can be gained from a chromatogram than from a system such as the EMIT, which tends to be operated in a "black box" fashion.

4. DISCUSSION AND CONCLUSIONS

For routine monitoring in a clinical setting, drugs are assayed in plasma or serum, and most of the methods described in the previous pages measure the total concentration of the drugs. Free drug concentrations theoretically give a better correlation with clinical response [53,288–290]; however, in everyday practice this is not always necessary, and until more convincing evidence is provided, it may be advisable to determine total drug concentrations rather

than the free concentration. Not all of the published methods are amenable to the analysis of unbound drug because the free fraction may only represent a small percentage of the total concentration (see Table 1) and sensitivity becomes the limiting factor. Saliva may give a good measure of free concentration. This is particularly true for phenytoin [291], but the correlation is not always so good with other antiepileptics such as valproic acid. Nevertheless, saliva monitoring becomes invaluable when repeated plasma sampling is not possible.

GC may still be the most widely used chromatographic technique for the determination of antiepileptics and such methods have achieved the status of reference methods, especially when coupled with MS detection. Certain antiepileptics can be assayed concomitantly by GC either free or derivatized following on-column alkylation with trimethylphenyl or tetramethylammonium hydroxide. The latter method requires the use of an analogue of phenobarbital in order to correct for degradation in the injection port.

Although virtually all antiepileptics can be quantitated by GC methods, the thermal instability of carbamazepine and its epoxide makes LC more attractive for these two compounds. The converse is true for valproic acid, which tends to be assayed mainly by GC due to its poor UV absorption. A few authors have, however, reported liquid column chromatographic analysis of valproic acid following derivatization.

Despite the historical pre-eminence of GC, the progress and interest in liquid column chromatography over the last ten years has been such that the next survey may show that liquid column chromatography has largely displaced GC from its favoured position. Liquid column chromatography is well adapted to simultaneous measurement of multiple antiepileptics, and since the mobile phase composition can be modified in an almost infinite way, the number of "new and improved methods for the determination of antiepileptics by HPLC" will undoubtedly increase in the near future.

Speed of analysis and power of separation can be improved in HPLC with columns packed with 3-μm particles. Advancements in detector sensitivity and selectivity, especially with rapid-scanning UV detectors, will also add impetus to method improvements in the clinical monitoring of anticonvulsant drugs. On-line and off-line column preparation techniques will likewise have an increasing impact on future methods.

Many of the comparative studies have indicated a similar degree of accuracy and precision between immunologic and chromatographic methods. The decision between the two types of methods depends mainly upon the number of samples to be analysed, the availability of dedicated chromatographs, the number of drugs to be analysed in the sample, and the presence of trained technicians. Running an automated EMIT system requires less skilled personnel, but chromatographic methods can be adapted easily to the determination of active metabolites and new drugs. Furthermore, chromatographic methods give more than a number on a printer, and a trained technician can respond to an unusual chromatogram.

Finally, there seems to be a wide variability in the precision and accuracy obtained in different laboratories. Although some methods may be inherently less precise than others (e.g. by the use of an improper internal standard), the

quality of the data depends principally on the human factor. Even sampling techniques can drastically affect the results as was recently pointed out by Bergqvist et al. [276] who examined the use of gel-barrier sampling tubes in the determination of some antiepileptics. Therefore it is essential to institute a strict intra-laboratory quality control programme and to adhere to one of the recognized inter-laboratory quality control schemes for anticonvulsant drugs.

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6. SUMMARY

The present paper reviews gas and liquid chromatographic methods for the determination of the most commonly monitored antiepileptic drugs: phenobarbital, phenytoin, carbamazepine, primidone, ethosuximide, valproic acid and clonazepam along with a new compound, progabide.

The individual classes of drugs are first treated separately to highlight specific aspects of their quantification, and this is followed by an overview of those methods permitting the concomitant analysis of two or more antiepileptic compounds.

Sample preparation techniques as well as comparisons between chromatographic and other techniques are treated more fully in separate sections.

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