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Review

Analysis of antiepileptic drugs

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CONTENTS

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1 INTRODUCTION

Epilepsy is one of the most common neurological disorders affecting 0.5–1% of the population The primary treatment is drug therapy and it is one of the first areas in which therapeutic drug monitoring was established. Therapeutic drug monitoring is based on a premise that plasma concentration of a drug is related in a reversible manner to the drug's concentration at its receptor site, which in turn determines its clinical effect It is widely recognized that there exists a considerably better correlation between clinical effects and plasma concentrations of drugs than there is between clinical effects and daily dosage of drugs. Advancements in technology associated with drug determinations are responsible for the progress and success in therapeutic drug monitoring which in turn led to the rapid advances in clinical pharmacology [1]. Antiepileptic therapy is one of the areas of clinical pharmacology where therapeutic window is relatively well defined. Therapeutic drug monitoring is an adjunct to rational drug therapy and it helps establish optimal drug therapy for individual patients [2]. It is especially important in fields like epilepsy where life-long multiple-drug therapy and drug–drug interactions are common.

The rapid pace of publications of analytical methods for quantitation of antiepileptic agents which was taking place in the late seventies and early eighties has subsided considerably. This is due to the fact that the existing antiepileptic drugs have been around for some time now and because of the early massive analytical effort dedicated to the development of suitable assays.

Many of the newer procedures are modifications of the older ones and many differ only in terms of sample preparation or the composition of the high-performance liquid chromatographic (HPLC) mobile phase. Many of the reports still lack adequate validation data (intra- and inter-day accuracy and precision assessment covering the range of experimental values) and do not check for interferences from commonly co-administered drugs and their metabolites Some of the assays proposed for clinical use were developed using biological fluids from animals. Experimental animals are kept under much more controlled conditions than people live in. Consequently chromatograms of extracts of biological fluids from animals tend to be more reproducible and cleaner than those from people and may be misleading.

Recent emphasis was placed on identification and quantitation of metabolites and on the measurement of free (non-protein-bound) drug concentrations In addition, there was a proliferation of non-chromatographic methods based on immunoassay techniques. These newer immunological methods are automated, rapid, and easily operable by relatively technically non-skilled individuals. They are suitable for routine clinical monitoring during chronic therapy and, under those circumstances, show similar degree of precision and accuracy to the chromatographic methods which are still being utilized as the standard reference methods However, immunoassays analyze for one drug at a time, have a limited

sensitivity for quantitation of drugs following a single dose, do not measure metabolites, and in some cases have problems with cross-reactive interferences.

It is estimated that overall about 20% of patients with epilepsy are not controlled by the current antiepileptic drugs. This number is much greater in adults with complex partial seizures, the most common type of epilepsy. Therefore, there is a continuing effort to develop new antiepileptic drugs, and several new candidates are at different stages of development. Some of the new candidate antiepileptic drugs are quite different from the older ones in their physicochemical characteristics and require a very different analytical approach. Standard antiepileptic agents (with the exception of benzodiazepines) are primarily weak acids, have no appreciable tendency for adsorption to various surfaces, and exhibit plasma concentrations in the μg/ml range. On the other hand, some of the new candidate antiepileptic agents tend to be weak bases, adsorb to many surfaces and exhibit plasma concentrations in the ng/ml range.

There is no single method or scheme for analysis of the antiepileptic drugs. The approach depends on the specific needs (*e.g.* clinical monitoring, pharmacokinetic studies, quantitation of metabolites, determination of new drugs) and the available resources (technical skill and instrumentation). The initial decision is between a chromatographic and an immunoassay method, and these two alternatives are discussed in more detail elsewhere in the text.

Chromatographic methods often require sample preparation prior to the actual chromatographic separation. However, there are chromatographic methods which allow direct sample injection (no sample pretreatment) and are primarily those using HPLC. Direct injection methods are faster and simpler and do not require additional solvents or solid-phase columns. However, in the long run they may require more frequent maintenance of instrumentation and replacement of analytical columns and are more susceptible to problems with interferences. Sample preparation is performed by deproteinization (*e.g.* acetonitrile), liquid–liquid (solvent) extraction (*e.g.* ethyl acetate, chloroform, methylene chloride) or liquid–solid (solid-phase) extraction (*e.g.* C_{18} disposable columns). While deproteinization is the simplest of three, it retains some of the drawbacks of the direct injection methods. On the other hand, direct injection and deproteinization methods, because of their non-selectivity, allow simultaneous analysis of compounds with different physicochemical properties unlike the extraction methods. Additional selectivity can be imparted to the extraction methods by using more than a single extraction step. Solid-phase extraction technique is gaining in popularity because of its rapidity. However, selection of an appropriate solid-phase column and solvents is not always obvious even to an experienced analyst.

Extracts usually require a concentration or evaporation step, most frequently for gas chromatographic (GC) analyses. In some cases, samples are derivatized (*e.g.* silylation, alkylation), especially for GC methods. However, there is a much lesser need for derivatization now after the introduction of capillary GC columns. In general, HPLC methods require simpler sample clean-up than GC ones, and as a result are often preferred. On the other hand, GC methods tend to offer a better sensitivity. Gas chromatographic–mass spectrometric (GC–MS) methods provide specificity and sensitivity, as well as structural information.

Analytical methods for determination of antiepileptic drugs have been reviewed in 1985 [3]. The present review is not meant as a replacement but a supplement to the 1985 review. It concentrates on developments in the analytical methodology for antiepileptic drugs since that review.

2 CHROMATOGRAPHIC METHODS FOR STANDARD ANTIEPILEPTIC DRUGS AND METABOLITES

Chromatographic methods are still utilized as the standard reference methods for the antiepileptic drugs.

2.1. Total drug concentrations

Therapeutic determinations of drugs and metabolites are most frequently carried out in plasma in serum. Plasma or serum concentrations of active compounds are thought to be in a reversible equilibrium with their respective concentrations at the receptor sites.

2 1.1. Analysis of individual drugs (and metabolites)

In some cases, analysis of only individual drugs is needed, such as during monotherapy or for specific therapeutic or research questions. In addition, with the advancements in analytical technology along with the recognition that some antiepileptic drugs have active and/or toxic metabolites, there is a growing interest in developing methods for quantitation of metabolites.

2.1.1 1. Valproate. Valproic acid is the most recently approved drug in the U.S.A. It has an unique structure, being a branched short-chain carboxylic acid. It has a large number of metabolites, some of which are believed to be active and some toxic. Some of the metabolites are found at concentrations several times lower than the parent. In addition, it posseses several properties which make it difficult from the analytical standpoint, namely high volatility, structure similar to many endogenous carboxylic acids, and metabolites with similar chromatographic and even MS characteristics to the parent drug. Evaporation step is usually avoided during sample preparation, and, instead, extracts are only concentrated or direct sample injections are made. Ethyl acetate is the preferred solvent for liquid–liquid extraction of valproate and its metabolites.

GC is the most frequently utilized chromatographic method for valproate. Unlike other antiepileptic drugs, valproate does not contain nitrogen or other structural moieties which would make it suitable for more sensitive and selective detectors such as nitrogen thermionic or electron-capture detectors. Instead, a flame ionization detector, which offers adequate sensitivity, is used for its analysis.

Nishioka *et al.* [4] described two different GC methods for the determination of valproate in human serum. Both methods used a packed column for sep-

aration, octanoic acid as the internal standard and 0.1 ml of serum. Neither method required derivatization or evaporation. One of these, the direct injection method, employed deproteinization with acetonitrile and hydrochloric acid followed by injection of the supernatant. In the extractive methylation method, valproate was extracted, using tetrabutylammonium chloride as a counter ion, into methylene chloride containing methyl iodide and the organic layer was injected. The limit of detection was 3 μg/ml A good correspondence was shown between the two methods and the direct injection method may be preferred due to its simplicity.

Kohda *et al.* [5] developed a novel "solvent flush" method requiring no extraction, evaporation, or derivatization and using 1 μl of rat plasma. Octanoic acid was used as the internal standard and the separation was achieved with a packed column In this approach, sample was "sandwiched" by phosphoric acid phases and injected directly. The limit of detection was 5 μg/ml and good accuracy and precision were demonstrated in the 12.5–150 μg/ml range. Because of the very small sample volume required, the method should be very suitable for small animals and pediatric patients. However, it needs to be validated using human samples prior to use for clinical monitoring. Another report [6] employed liquid–solid extraction using octadecyl-coated silica columns. The internal standard was α-methyl-α-ethylcaproic acid and the separation was performed on a packed column. The method utilized 100 μl of human plasma and did not require evaporation or derivatization The limit of detection was 1 μg/ml. The solid-phase extraction columns were reusable about five times. Solid-phase extraction are usually simpler, faster, avoid formation of emulsions and require considerably smaller quantities of solvents.

A capillary GC method was also reported for valproate and its 2-desaturated metabolite, 2-*n*-propyl-2-pentenoic acid [7]. This metabolite is the major monounsaturated metabolite of valproate and it lacks hepato- and embryo-toxicity. Cyclohexane carboxylic acid was the internal standard and trimethylsilyl derivatives were formed following the liquid–liquid extraction. The method was developed using rat plasma and brain tissue but the authors state that it was subsequently applied successfully to other biological tissues including human plasma. A sensitivity limit of 0.2 μg/ml for 150 μl of plasma was reported. Peak purity was established by the retention times and ratios of ion current intensities of the characteristic fragments using GC–MS.

Valproate exhibits poor ultraviolet (UV) absorption and lacks other structural features that would make it a suitable candidate (without a derivatization) for other common HPLC methods of detection (*e.g.* fluorescence or electrochemical).

Lovett *et al.* [8] measured valproate in 250 μl of human plasma using acetonitrile precipitation, a reversed-phase octadecyl column, and UV detection at 210 nm. Each isocratic elution of valproate and diazepam, the internal standard, was followed by a gradient to flush the column. The limit of detection was 1.5 μg/ml and good precision and accuracy were shown from 5 μg/ml and up Another

HPLC method analyzed valproic acid in human serum following precolumn derivatization with bromomethylmethoxycoumarin [9]. The method used 20 μl of blood or plasma, nonanoic acid as the internal standard, and acetonitrile for protein precipitation. Chromatographic separation was achieved using a C_{18} reversed-phase column. A fluorescent detector (excitation 325 nm, cut-off filter 398 nm) was employed but samples could also be monitored using a UV detector with about a ten-fold loss in sensitivity.

GC–MS is commonly employed for identification and quantitation of valproate and its metabolites [10–14]. A metabolic scheme for valproate is shown in Fig. 1 Capillary GC is most commonly used because of its resolving power, and usually more than one internal standard is used. Samples are derivatized, most commonly by silylation.

Determination of valproate and twelve metabolites was achieved after capillary GC separation of *tert.*-butyldimethylsilyl derivatives [10]. The analysis used 1 ml of human plasma or urine, and the limit of sensitivity was about 0.1 μg/ml.

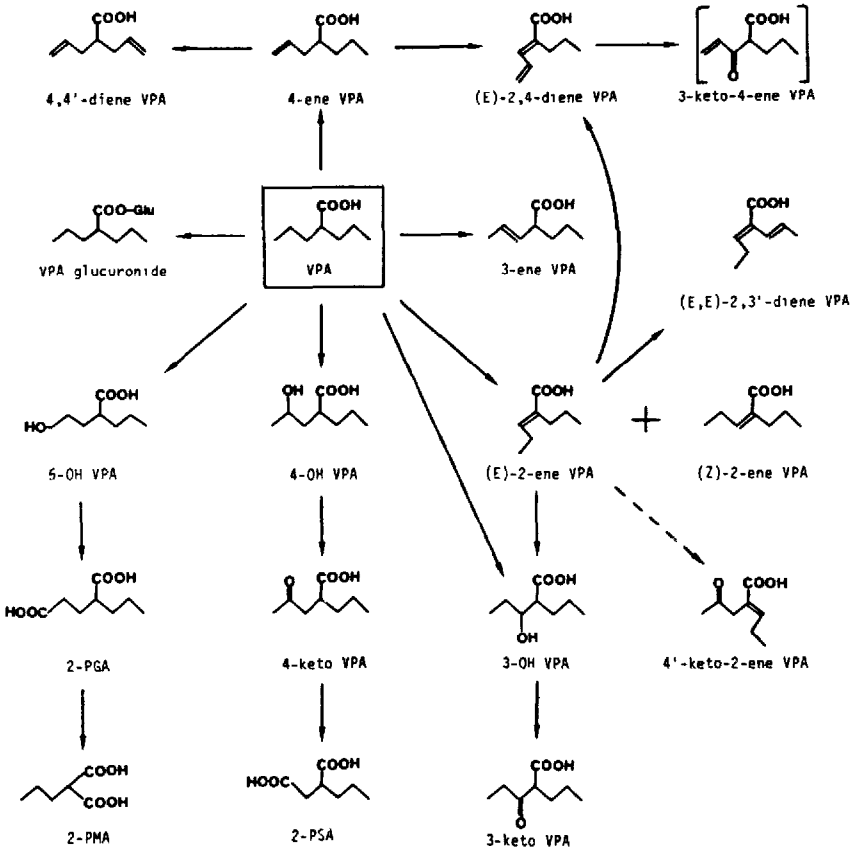

Fig. 1 Proposed metabolic pathways of valproate in man (Reproduced with permission from ref 14)

[$^2H_6$]Valproate was the internal standard for valproate and (*E*)-[$^2H_3$]2-ene-valproate was the internal standard for the metabolites. Samples were extracted with ethyl acetate and concentrated. Selected-ion monitoring (SIM) of the characteristic $[M-57]^+$ ions, in the electron-impact (EI) mode, was used for quantitation. It was suggested that mixed *tert.*-butyldimethylsilyl and trimethylsilyl (TMS) derivatization may be optimum because hydroxy metabolites were poorly derivatized with N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide.

Tatsuhara *et al.* [11] measured valproate and eleven metabolites in 10–100 μl of human serum or urine following ethyl acetate extraction, concentration, and formation of TMS derivatives. Undecylenic acid was used as the internal standard and packed column for separation. SIM in the EI mode was used for quantitation. Good precision and accuracy were shown. The limit of detection for the metabolites was 5–10 ng/ml using 0.10 ml of plasma

Rettenmeier *et al.* [12] described an automated method for determination of valproate and fourteen metabolites in human plasma (0.5 ml) and urine (0 05–0.50 ml) using a mass-selective detector. The assay involved ethyl acetate extraction, concentration, formation of TMS derivatives, capillary GC and SIM in the EI mode. Two internal standards were used: 2-*n*-propylhexanoic acid and 2-(3-hydroxy-*n*-propyl)hexanoic acid. A total cycle time of 70 min was required between injections, of which 38 min were chromatographic.

Abbott *et al.* [13] reported a negative-ion chemical ionization (NICI) method for analysis of valproate and fourteen metabolites in human serum (0.10–0.25 ml) or urine (0.25 ml) Four internal standards were used: [$^2H_6$]valproate, [$^2H_3$]2-ene-valproate, [$^2H_3$]3-ketovalproate and 2-methylglutaric acid. Samples were extracted with ethyl acetate, concentrated, derivatized to give pentafluorobenzyl esters and TMS derivatives of the hydroxyl and 3-keto groups. SIM of the $[M-181]^-$ ions was used for quantitation. A GC–NICI-MS method recently identified in human samples fifteen metabolites of valproate as their pentafluorobenzyl derivatives [14]. This method of ionization imparts additional sensitivity and specificity to the analysis.

2.1.1.2. Carbamazepine. Carbamazepine, an iminostilbene derivative, is a tricyclic compound with very poor water solubility. It is used therapeutically for treatment of epilepsy, trigeminal neuralgia, and some psychiatric disorders. Its stable, active metabolite, carbamazepine-10,11-epoxide, is found in significant amounts in plasma.

There is a tendency for degradation of carbamazepine and its metabolites under GC conditions and that is one of the reasons why HPLC analysis is preferred.

Carbamazepine and carbamazepine-10,11-epoxide were determined in plasma and urine following double liquid–liquid extraction [15] Separation was achieved using a C_{18} reversed-phase column, clonazepam as the internal standard, and UV detection at 215 nm. Hartley *et al.* described a liquid–solid extraction method for the determination of carbamazepine and carbamazepine-10,11-epoxide in plasma

and saliva [16] and carbamazepine-10,11-diol (*trans*-10,11-dihydroxy-10,11-dihydrocarbamazepine; *trans*-diol) along with the other two compounds in plasma [17]. The procedure utilized C_{18} solid-phase extraction columns, nitrazepam as the internal standard, a reversed-phase C_{18} HPLC column, and UV detection at 214 nm The compounds could be quantitated down to 50–100 ng/ml using 0.25 ml of human plasma and reasonable accuracy and precision were demonstrated. Chelberg *et al.* [18] developed a procedure for the determination of carbamazepine, carbamazepine-10,11-epoxide, carbamazepine-10,11-diol and 2-hydroxycarbamazepine in human plasma (0.25 ml) and urine (0.10 ml). Following addition of 2-methylcarbamazepine as the internal standard and liquid–liquid extraction, compounds were separated on a C_{18} reversed-phase column and monitored at 212 nm. The limit of quantitation for carbamazepine and its metabolites was 10 ng/ml and good precision was achieved.

Alkalay *et al* [19] reported a GC–MS method for the simultaneous analysis of carbamazepine, carbamazepine-10,11-epoxide, and carbamazepine-10,11-diol in human plasma. After addition of the internal standard, 10-methoxycarbamazepine, samples were extracted with chloroform and TMS derivatives were formed. Carbamazepine epoxide, in addition to being derivatized, also underwent a rearrangement to 9-acridinecarboxyaldehyde. Both the derivative and the rearrangement product were suitable for quantitation of the epoxide. Compounds were separated using a capillary column and quantitated by SIM in a CI mode with ammonia as the reagent gas. The compounds were quantifiable from 40 ng/ml to 10 μg/ml and good accuracy and precision were observed.

2.1.1.3. Hydantoins. Phenytoin is one of the most commonly used antiepileptic drugs It can be analyzed by GC, HPLC, or GC–MS. Use of deactivated column packings and capillary columns has obviated the need for derivatization in GC methods. Either a flame ionization or a nitrogen-selective GC detector can be used. The latter offers additional specificity and sensitivity.

Bailey *et al.* [20] reported a GC method for analysis of phenytoin in human plasma (0.20 ml) with a nitrogen thermionic detector or SIM in the EI mode. After addition of the internal standard, 5-(*p*-methylphenyl)-5-phenylhydantoin, liquid–liquid extraction, and extractive methylation, samples were chromatographed on a capillary column. Quantitation was possible down to 0.5 μg/ml, good precision and accuracy were observed, and a good correspondence between the two methods of detection was evident

Several metabolites of phenytoin have been identified, some of which may have a toxicological relevance. Two reports [21,22] described HPLC methods for the determination of phenytoin and its known metabolites (*p*-hydroxy, *m*-hydroxy, catechol, methylcatechol and dihydrodiol) in mouse urine and other biological samples. Extracts were chromatographed on a C_{18} column and monitored at 225 or 235 nm. Phenytoin is a prochiral drug, and Maguire and Wilson [23] reported an HPLC method for quantitation of patient urinary diastereomeric dihydrodiol metabolites, (5*R*)- and (5*S*)-5-[(3*R*,4*R*)-3,4-dihydroxy-1,5-

cyclohexadien-1-yl]-5-phenylhydantoin Urinary samples (1 0 ml) were hydrolyzed with β-glucuronidase, extracted using a solid-phase C_{18} extraction cartridge, and analyzed on a C_{18} column at 210 nm.

An HPLC method with electrochemical detection was employed for kinetic studies of microsomal *p*-hydroxylation of phenytoin [24]. Electrochemical detector is sensitive to phenol moiety and transparent to the parent, phenytoin. Therefore, it is possible to measure small amounts of the product in the presence of huge excess of the substrate. This approach should be useful in kinetic studies of other drugs by measuring production of the common phenolic metabolites.

Shimada and Wakabayashi [25] described a GC method for phenytoin and *m*- and *p*-hydroxyphenytion in urine (1.0 ml) of epileptic patients. After acid hydrolysis and addition of the internal standard (*p*-methylphenytoin), samples were extracted with organic solvents, derivatized by an on-column methylation, chromatographed on a packed column, and detected with a flame ionization detector. Maguire [26] reported a GC method with on-column methylation for quantitation of methylcatechol metabolite of phenytoin in human urine. The assay utilized a packed or a wide-bore capillary column and a flame ionization detector. 5-(3,4-Dimethoxyphenyl)-5-(4-methylphenyl)hydantoin was the internal standard. Minimal detectable concentration was 1 μg/ml.

Wedlund *et al* [27] quantitated *R*- and *S*-enantiomers of mephenytoin and its active N-demethylated metabolite, 5-phenyl-5-ethylhydantoin (nirvanol) in human plasma and blood Internal standards, 3-methyl-5-phenyl-5-isopropylhydantoin and 5-phenyl-5-propylhydantoin, were added to 1 ml of plasma or blood and the samples were extracted with dichloromethane, evaporated and propylated. Separation was achieved using a chiral capillary column and detection was by a nitrogen thermionic detector. Quantitation was possible between 50 ng/ml and 5 μg/ml. Mephenytoin and its metabolites exhibited stereoselective pharmacokinetic properties Propylation was necessary for chiral separation of the mephenytoin metabolite, but Akrawi and Wedlund [28] have subsequently cautioned against an impurity in the derivatizing agent, 1-iodopropane. A small amount of methylation was detected in the presence of undistilled 99% iodopropane which converted the metabolite back to the parent drug. Redistillation decreased this phenomenon. However, this will only become a significant problem when the metabolite levels are high and greatly exceed those of the parent drug.

2.1 1.4. Phenobarbital. Benchekroun *et al.* [29] reported a GC–MS method for determination of phenobarbital and its metabolite, *p*-hydroxyphenobarbital, as well as their perdeuteroethyl analogues in human plasma (0.05 ml) or urine (0.50 ml). [1,3-$^{15}N_2$,2-^{13}C]Phenobarbital, the internal standard, was added prior to a liquid–liquid extraction, and the extracts were pentylated and chromatographed using a capillary column. Quantitation was achieved by SIM in the EI mode using a mass-selective detector. The limit of detection was below 0.05 μg/ml and good accuracy and precision were reported in the range 0 5–3 0 μg/ml.

Phenobarbital undergoes a somewhat unusual metabolic biotransformation,

N-glucosidation, which has quantitative importance in man. HPLC methods for the determination of 1-(β-D-glucopyranosyl)phenobarbital were reported for urine (0.20 ml) [30] and plasma (0 05 ml) and bile (0.20 ml) [31]. After addition of the internal standard, 5-methyl-5-phenylhydantoin, urine samples were extracted with ethyl acetate, chromatographed on a C_{18} column, and monitored at 204 nm. The procedures for plasma and bile were similar to that for urine except for the differences noted below. In case of plasma, supernatant after acetonitrile precipitation was injected and UV detection at 254 nm was employed On the other hand, bile was extracted twice with ethyl acetate and UV detection at 260 nm was used. Phenobarbital and *p*-hydroxyphenobarbital were also assayed concurrently and the lower limit of detection for all three compounds in all three biological fluids used was 1 μg/ml.

A GC–MS method for the analysis of phenobarbital, *p*-hydroxyphenobarbital and phenobarbital-N-glucoside in human urine was also reported [32]. Urine obtained from a healthy subject after the ingestion of [$^{15}N_2$]phenobarbital contained labeled analogues of the three compounds and was used for the internal standards. Urine samples (0 2–1.0 ml) were incubated with β-glucuronidase (for the deconjugation of *p*-hydroxyphenobarbital) and extracted with ethyl acetate. Dried extracts were methylated with diazomethane for the analysis of phenobarbital and *p*-hydroxyphenobarbital. After the methylation, a portion of each sample was silylated for the analysis of phenobarbital-N-glucoside. The additional derivatization with the silylating reagent was necessary for the analysis of the glucoside metabolite because the N-methyl derivative underwent pyrolytic decomposition to N-methylphenobarbital and permethylation with methyl iodide led to extensive decomposition. Samples were chromatographed using a packed column and measured using SIM in the CI mode with methane as the reagent gas. The limit of detection was 0.1 ng/ml for all three compounds.

A recent study [33] reported that 1-(β-D-glucopyranosyl)phenobarbital undergoes decomposition to 1-(1-β-D-glucopyranosyl)-3-(2-ethyl-2-phenylmalonyl)-urea followed by decarboxylation to 1-(1-β-D-glucopyranosyl)-3-(2-phenylbutyryl)urea under physiological conditions of temperature and pH. Therefore, the authors suggested that the reports on the quantitation of phenobarbital-N-glucoside may need to be reevaluated depending on the conditions used for sample collection, storage and analysis.

2.1.1.5. Benzodiazepines Several benzodiazepines are used in the treatment of epilepsy. These include diazepam, nitrazepam, clonazepam, and clobazam (also discussed in Section 4.6). Benzodiazepines differ from the other antiepileptic drugs in that they are weak bases as opposed to weak acids and because they exhibit plasma therapeutic concentrations in the ng/ml and not μg/ml range Therefore, they require a different analytical approach and are not commonly analyzed simultaneously with other antiepileptic drugs.

Numerous methods have been published on the determination of various benzodiazepines as cited in a recent report [34] Bun *et al.* [34] described a capillary

GC method for the simultaneous determination of some 1,4- and 1,5-benzodiazepines, which are used clinically for treatment of epilepsy, and their metabolites (diazepam, N-desmethyldiazepam, oxazepam, nitrazepam, clonazepam, clobazam, and N-desmethylclobazam) in human plasma (0.50 ml). The assay utilized butyl acetate extraction and electron-capture detection The lowest measurable concentration was 0.5–1.0 ng/ml.

A GC–MS method for the analysis of diazepam, clobazam, flunitrazepam, triazolam, midazolam, oxazepam, lorazepam and some of their desmethylated and hydroxylated metabolites was also reported [35] Human plasma samples (0.5 ml) were extracted with butyl acetate, silylated (for derivatization of hydroxylated metabolites) with N,O-bis(trimethylsilyl)trifluoroacetamide, chromatographed using a capillary column, and analyzed using a mass-selective detector in the EI mode The detection limit was between 1 and 5 ng/ml depending on the compound.

Confirming several prior surprising and puzzling reports, diazepam and desmethyldiazepam were detected in brain of different species (including man) and plants [36]. Levels were low but measurable by GC–MS. It was proposed that these benzodiazepines may be of natural origin and incorporated into animal and human body via the food chain.

Clonazepam is the most commonly used benzodiazepine in the treatment of chronic epilepsy. Several HPLC methods have been reported since the last review. However, HPLC methods appear more suitable for determinations of clonazepam levels at the steady state than for pharmacokinetic studies.

One method [37] employed a solid-phase extraction of human serum (1 ml), methylclonazepam as the internal standard, chromatography on a C_{18} column, and detection at 254 nm The detection limit was 2 ng/ml and quantitation of 5 ng/ml in 0.5 ml of serum was possible. There were no interferences from the common antiepileptic drugs. Another method [38] employed liquid–liquid extraction, flunitrazepam as the internal standard, reversed-phase chromatography, and detection at 313 nm. A detection limit of 20 ng/ml was achievable using 0.20 ml of human serum. Common antiepileptics did not interfere with the assay. The authors claimed that it was possible, with modifications, to use the procedure also for the determination of nitrazepam. Lin [39] described a method using methylclonazepam as the internal standard, chloroform extraction, a cyanopropyl HPLC column, and detection at 306 nm. Additional sensitivity was achieved using an electronic filter. It was sufficient to use 0 1 ml of serum for analysis. The detection limit was 2 ng/ml using 0 5 ml of human serum and there were no interferences from commonly used drugs, including antiepileptics The author also stated that the assay can be directly applied for the determination of diazepam and desmethyldiazepam Doran [40] also employed a cyanopropyl HPLC column for quantitation of clonazepam in human serum. Two internal standards were employed, flunitrazepam and desmethyldiazepam, to avoid occasional interferences. A three-step liquid–liquid extraction was used and the detection

wavelength was 245 nm. Chromatographic stability was achieved using a guard column, a silica saturating column, recycling of a mobile phase, and *n*-butylamine as an amine mobile phase modifier to deactivate exposed column silanol groups. The assay used 2 ml of serum and the limit of detection was 5 ng/ml. The assay was free of interferences from commonly used drugs, including antiepileptics. Wad [41] used an HPLC method to confirm light-caused decomposition of clonazepam in serum

2 1.1 6. Other antiepileptic drugs. Methsuximide, a succinimide derivative, and its active metabolite, N-desmethylmethsuximide, were quantitated in patient serum (1 ml) after a solid-phase extraction using a C_{18} extraction column [42] 5-Methyl-5-phenylhydantoin was the internal standard and the extracts were analyzed by GC using a wide-bore capillary column and a flame ionization detector. Sensitivity of 0.5 μg/ml for the parent and 5.0 μg/ml for the metabolite was reported.

The oxazolodinedione antiepileptic, trimethadione, and its metabolite, dimethadione, were quantitated in human serum (0.10 ml) by GC with flame thermionic detection [43]. Maleinimide was used as the internal standard, and the samples were extracted with ethyl acetate and chromatographed on a packed column. Quantitation of both compounds was done in the range 0 05–10.0 μg/ml.

An HPLC method for quantitation of phenacemide (phenacetylurea) in patient plasma (0.05 ml) was reported [44] After precipitation with acetonitrile, supernatant was chromatographed on a C_{18} column and the effluent was monitored at 205 nm. The method did not use an internal standard.

2.1 2. Simultaneous analysis of several antiepileptic drugs and metabolites

Multiple-drug therapy is very common in the treatment of epilepsy and therefore methods which can quantitate several antiepileptic drugs simultaneously are desirable. Out of 15 000 samples received by one laboratory for analysis of the antiepileptic drugs, only about 10% were from patients on monotherapy [45]. About 20% of the patients were treated with two, 40% with three, and 30% with four or more antiepileptic drugs concommitantly.

Over the last few years, published methods on concurrent analysis of several antiepileptic drugs primarily utilized HPLC. HPLC generally requires simpler sample work-up, does not require derivatization, does not cause thermal instability, and the instrumentation is easier to operate than GC instrumentation. These are probably some of the reasons for recent preference for HPLC over GC. HPLC, however, is not as sensitive as GC for the antiepileptic drugs since their detection (without derivatization) is limited to the UV absorption and they do not possess good extinction coefficients. However, sensitivity in the HPLC is adequate for most of the antiepileptic drugs, especially for routine analysis.

The two drugs which are usually not included in the simultaneous analysis of the antiepileptics are valproate and clonazepam. Valproate lacks an adequate chromophore and therapeutic levels of clonazepam are several orders of magni-

tude lower than those of the other antiepileptics. One recent report [45] stated that it was more efficient to analyze valproate by a GC method than to derivatize and analyze it together with other antiepileptics for HPLC.

Ideally, in addition to appropriate data regarding the accuracy, precision, and limit of detection, analytical methods should be suitable for analysis of all the commonly used antiepileptics and their active and toxic metabolites. In addition, they should be shown to be free from interferences from less commonly used antiepileptics and their metabolites, as well as from substances like caffeine and commonly used over-the-counter medications as acetaminophen. Furthermore, they should be cross-validated by an independent method Unfortunately, many of the methods do not take some of these points into consideration. This is especially important for HPLC analysis of the antiepileptics because they are routinely monitored using relatively non-selective UV absorption wavelengths (around 200 nm) and frequently without or with a minimal prior sample clean-up.

Several HPLC methods for the simultaneous analysis of various antiepileptic drugs and their metabolites have been published over the last few years [45–60] and are summarized in Table 1. Only one method [57] attempted a concurrent analysis of the six most commonly used antiepileptics: carbamazepine, phenytoin, primidone, phenobarbital, ethosuximide, and valproate (as well as two main metabolites of carbamazepine). However, it appears that primidone and carbamazepine-10,11-diol peaks would overlap (Fig. 2). In fact, only two methods included valproate [50,57]. One of these methods [50] reported a limit of detection of 3 μg/ml for valproate. The other method [57] reported that determination of valproate below 20 μg/ml was unreliable.

Several criteria were usually employed in the selection of sample preparation, namely speed and simplicity, lack of interferences, adequate recoveries, and the reproducibility of ethosuximide values

Ethosuximide was troublesome due to its volatility during sample evaporation which was further accentuated by failure to use a suitable internal standard. Most of the methods employed one or at the most two internal standards for all the antiepileptic drugs and their metabolites One method did use an additional internal standard to take into the account the relative volatility of ethosuximide [58]. Methsuximide, structurally related to ethosuximide and an antiepileptic drug itself, was selected for the internal standard. Since methsuximide is rapidly metabolized, the authors felt that it was a suitable internal standard even when patients are receiving methsuximide. One method [52] was a modification of a previously reported method [61]. It eliminated the evaporation step and the resultant problems with ethosuximide.

Various sample preparation procedures were employed: none (direct injection with column switching), precipitation with an organic solvent, salting-out into a water miscible solvent, liquid–liquid extraction, and liquid–solid extraction One of the column-switching methods used a precolumn packed with octadecylsilyl

TABLE 1

HPLC METHODS FOR THE SIMULTANEOUS DETERMINATION OF ANTIEPILEPTIC DRUGS AND THEIR METABOLITES IN BIOLOGICAL FLUIDS

Abbreviations CBZ = carbamazepine, ESM = ethosuximide, MTH = mephenytoin, MSM = methsuximide, PAC = phenacemide, PB = phenobarbitol; PHT = phenytoin, PRM = primidone, VPA = valproate, ZNS = zonisamide, OCBZ = oxcarbazepine, DMMTH = desmethylmephenytoin (nirvanol), DMMSM = desmethylmethsuximide, PEMA = phenylethylmalonamide, DHCBZ = *trans*-10,11-dihydroxy-10,11-dihydrocarbamazepine (*trans*-diol), CBZE = carbamazepine epoxide, HCBZ = 10-hydroxy-10,11-dihydrocarbamazepine (a major metabolite of oxcarbazepine), HMCA = 9-hydroxymethyl-10-carbamoylacridane, PHPB = *p*-hydroxyphenobarbital, PHPHT = *p*-hydroxyphenytoin, HB = hexobarbital, ETB = 5-ethyl-5-(*p*-tolyl)barbituric acid, TB = tolylbarbituric acid, PMEPB = *p*-methylphenobarbital, PMEPHT = *p*-methylphenytoin, DCBZ = 10,11-dihydrocarbamazepine, MEPRM = *p*-methylprimidone, ACB = 5-allyl-5-cyclopentylbarbituric acid, BB = butobarbitone, MeCN = acetonitrile, MeOH = methanol, THF = tetrahydrofuran, isoPrOH = isopropanol, $CHCl_3$ = chloroform, CH_2Cl_2 = dichloromethane, EtOAc = ethyl acetate

Reference	Compounds[a]	Sample[b]	Sample preparation	Mobile phase	Analytical column	UV detection	Run time
Juergens, 1984 [46]	PEMA, ESM, PRM, DHCBZ, PB, DMMSM, CBZE, PHT, CBZ, no I S	0 05 ml serum	None, column switching	Gradient, MeCN–phosphate buffer pH 4 0	5-μm Shandon ODS Hypersil, 250 × 4 6 mm, precolumn, 70°C	205 nm	*ca* 11 min
Kuhnz and Nau, 1984 [47]	PEMA, ESM, PRM, DHCBZ, PB, CBZE PHT, CBZ, no I S	0 10 ml serum	None; column switching	Flow gradient, MeCN–H_2O	5-μm Spherisorb 5 ODS-2; 250 × 4 6 mm; precolumn; 60°C	200 nm	30 min
Ou and Rognerud, 1984 [48]	ESM, PRM, PB, PHPHT, DMMSM, TB (I.S), CBZ, PHT	0 05 ml serum	MeCN precipitation	Isocratic, acetone–MeOH MeCN–phosphate buffer pH 7 95	5-μm Radial NOVA-PAK C_{18}, 100 × 8 mm, ambient	200 nm	10 min
Kumps *et al* 1985 [49]	DHCBZ, PB, HCBZ, ACB (I S), CBZE, HMCA (I S.), PHT, CBZ	0 50 ml serum	EtOAC extraction	Isocratic, MeCN–MeOH–H_2O	5-μm Spherisorb ODS. 150 × 4 6 mm, ambient	254 nm	*ca* 14 min
Kushida and Ishizaki, 1985 [50]	PRM, PB, MEPRM (I S), VPA, PHT, CBZ	0 10 ml plasma	MeCN salting out in presence of saturated ammonium sulfate	Isocratic, MeOH–THF–phosphate buffer pH 5 9	5-μm LiChrosorb RP18, 250 × 4 mm, 50°C	210 nm	*ca* 12 min

Soto-Otero *et al*, 1985 [51]	ESM, PB, PHT, CBZ, PMEPHT (I S)	Rat, 300 mg brain	CH_2Cl_2 extraction in presence of ammonium sulfate at low pH	Isocratic, MeCN–MeOH–phosphate buffer pH 4 0	Spherisorb 5 ODS, 250 × 4 6 mm; precolumn, ambient	195 nm	8 min
Wad, 1985 [52]	PEMA, ESM, PRM, DHCBZ, HCBZ, DMMTH, PB, CBZE, MTH, HB (I S), CBZ, PHT	0 40 ml serum	MeCN salting out in saturated phosphate buffer pH 3 4	Gradient & isocratic MeCN–H_2O	10-μm Lichrosorb RP-8, 250 × 4 mm; 35°C	204 nm	*ca* 22 min
Fry *et al*, 1986 [53]	ESM, PRM, PB, DHCBZ, CBZE, BB (I S.), HMCA, PHT, CBZ	0 20 ml serum	Elution of Extrelut solid-phase columns with EtOAc–Ch_2Cl_2 (25 75)	Isocratic, MeOH , MeCN–THF–H_2O	5-μm Shandon Hypersil ODS, 100 × 5 mm, 40°C	206 nm	*ca* 6 min
Juergens, 1986 [54]	PEMA, ESM, PRM, DHCBZ, PB, DMMSM, CBZE, ETB (I S), PHT, CBZ	0 10 ml serum	Automated extraction using C_8 and C_{18} solid-phase cartridges	Gradient, MeCN–phospate buffer pH 4 0	5-μm Shandon Hypersil ODS, 250 × 4 6 mm, 70°C	205 nm	*ca* 11 min
Juergens, 1987 [45]	PEMA, ESM, PRM, ZNS, DHCBZ, DMMTH, HCBZ, PB, DMMSM, CBZE, OCBZ, MTH, ETB (I S), PHT, CBZ	0 50 ml serum	EtOAc extraction	Gradient, MeCN–perchloric acid	5-μm Shandon Hypersil ODS, 200 × 2 1 mm, in-line filter, 65°C	207 nm	*ca*. 12 min
Juergens and Rambeck, 1987 [55]	PRM, PB, DMMSM, CBZE ETB (I S), PHT, CBZ	20 200 mg brain	Acetone extraction of homogenate followed by extraction from Extrelut tubes by CH_2Cl_2 isoPrOH (97 3)	Gradient, MeCN phosphate buffer pH 4 4	5-μm Shandon Hypersil ODS, 200 × 2 1 mm, in-line filter, 65°C	207 nm	*ca* 12 min
Mendez-Alvarez *et al*, 1987 [56]	DHCBZ, PHPB, PB, CBZE, PMEPB, (I S), CBZ, DCBZ, (I S)	Rat, 0 20 ml serum, 0 20 ml urine, 300 mg brain	CH_2Cl_2–isoPrOH (90 10) extraction	Isocratic, MeCN MeOH–phosphate buffer pH 4 4	Spherisorb 5 ODS, 250 × 4 6 mm, precolumn, ambient	195 nm	*ca* 8 min

(Continued on p 436)

TABLE I (*continued*)

Reference	Compounds[a]	Sample[b]	Sample preparation	Mobile phase	Analytical column	UV detection	Run time
Matsumoto *et al.*, 1988 [57]	ESM, PRM, DHCBZ, PB, CBZE, PHT, CBZ, VPA, no I S	0 02 ml serum	None, column switching	Step gradient, MeCN–phosphate buffer	TSKgel ODS-120A, 250 × 4 mm, precolumn, 40°C	210 nm	*ca* 25 min
Meatherall and Ford, 1988 [58]	ESM, PRM, PB, TB (I S.), MSM (I S.), PHT, CBZ	0.10 ml serum	CH_2Cl_2–isoPrOH (95 5) extraction	Isocratic, recycled MeCN–MeOH–phosphate buffer pH 6 3	5-μm Supelcosil LC-1, 250 × 4 6 mm, precolumn, ambient	204 nm	*ca* 8 min
Soto-Otero *et al.*, 1988 [59]	PHPB, PHPHT, PB, PRM, EPB (I S), PHT	Rat, 0 20 ml serum, 0 05 ml urine, 300 mg brain	*tert* -Butylmethyl ether extraction at low pH in presence of excess ammonium sulfate	Isocratic, MeCN–phosphate buffer pH 4 0	Spherisorb 5 ODS; 250 × 4 6 mm, precolumn, ambient	195 nm	*ca* 10 min
Svinarov and Dotchev, 1989 [60]	ESM, PRM, DHCBZ, PAC, DMMTH, PB, CBZE, MTH, ETB (I S), CBZ, PHT	0 05 ml plasma	Elute from Chromosorb P microcolumn with $CHCl_3$ isoPrOH (6 1)	Isocratic, recycled MeCN–H_2O	5-μm Supelcosil-LC-8, 150 × 4 6 mm, ambient	208 nm	*ca* 18 min

[a] Compounds are listed in the increasing order of elution

[b] All samples were human except where specified otherwise

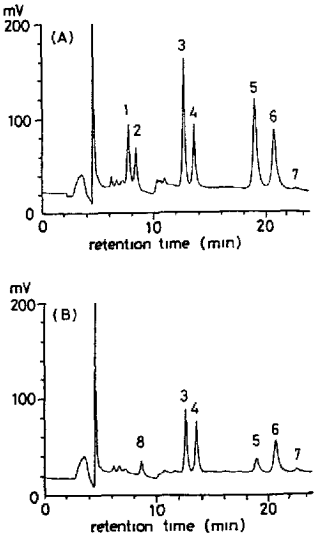

Fig 2 HPLC profiles of antiepileptic drugs and metabolites in spiked serum (A) and patient serum (B) For analytical conditions see Table I Peaks 1 = ethosuximide, 2 = primidone; 3 = phenobarbital; 4 = carbamazepine epoxide, 5 = phenytoin, 6 = carbamazepine, 7 = valproate, 8 = carbamazepine *trans*-diol (Reproduced with permission from ref 57)

resin to which bovine serum albumin was covalently bound [57]. This allowed serum proteins to pass through unretained, while retaining lipophilic drugs. Another method [54] employed automated extraction analysis with AASP (Advanced Automated Sample Processor) which elutes solid-phase cartridges (preloaded with sample) directly onto the HPLC column. Solid-phase cartridges were reusable for about ten times. The results obtained using three different sample preparation techniques, automated solid-phase extraction (C_{18} cartridges), direct injection with column switching, and ethyl acetate extraction, were in good agreement with each other [54]. All sample preparation methods utilized 0.5 ml or less of plasma (or serum) and one as low as 0 02 ml. In general, direct injection methods used the smallest and liquid–liquid extraction methods the largest plasma volumes However, limits of detection were often not specified and, thus, making comparisons was difficult

One important piece of practical information often neglected in the published reports is the life-time of columns. This is especially important for the methods which employ minimal sample clean-up Some methods have used in-line filters and/or precolumns or guard columns to extend the life of their analytical column. Juergens [45] reported that by using an in-line filter (0.5-μm frit changed every 500–600 injections), it was possible to make over 4000 injections of ethyl acetate extracts without a loss of resolution as compared to 350 injections without the filter. Other methods replaced a precolumn or a guard column roughly on month-

ly basis [51,59] or after *ca.* 1000 injections [62]. Another method replaced the end-fittings of columns after every 50 samples [57].

All methods employed reversed-phase analytical columns, and column temperatures ranged from ambient to 70°C. Most of the methods used 5-μm analytical columns. In two reports [45,55], a narrow-bore analytical column (2.1 mm instead of conventional 4.0 or 4.6 mm I.D.) with a low flow-rate (0.3 ml/min) was used. In comparing narrow-bore and conventional columns, Juergens [62] found that analytical results and column lifetimes (using an in-line frit filter for narrow-bore and a guard column for conventional columns) were comparable. Chromatograms obtained using these two columns were also very similar (Fig. 3). The savings resulting from much lower solvent consumption with narrow-bore columns more than made up for their higher cost.

Various chromatographic conditions were employed to separate different combinations of antiepileptic drugs and their metabolites (Table 1). One of the methods resolved fourteen different antiepileptic drugs and their metabolites (Fig 4) Some of the procedures reported problems with chromatographic interferences. Wad [52] reported an occasional interference in drug-free serum affecting primidone and therefore did not recommend the use of this method for its quantitation. Ou and Rognerud [48] reported overlap of primidone and carbamazepine *trans*-diol which necessitated modification of the mobile phase for the analysis of samples containing these two compounds

Two methods [58,60] were suitable for concurrent analysis of other therapeutic agents with the antiepileptics, *e g.* common barbiturates, theophylline, acetaminophen, caffeine, and chloramphenicol. A C_1 column was used by one of the methods [58] in order to resolve a wide polarity range of analytes in a reasonable time. In order to conserve solvents and minimize solvent preparation time, a continual recycling of the mobile phase for periods of two months (about 3000 injections) was also employed. The analytical column was replaced every two months (6000 injections)

In addition, a GC–MS procedure was also reported for the simultaneous analysis of carbamazepine, phenobarbital, phenytoin, primidone, and ethotoin in human serum (0.10 ml) [63]. Hexobarbital was used as the internal standard and samples were extracted into chloroform after addition of saturated sodium chloride solution. After on-column methylation and chromatographic separation using a packed column, compounds were quantitated using SIM in the EI mode.

2.2. Free drug concentrations

Up to this point, this review has only dealt with total (unbound plus protein-bound) drug levels. It is widely recognized that monitoring of the total levels of the antiepileptic drugs has significantly improved patient therapy. However, clinical relevance of free (unbound) antiepileptic drug monitoring is still uncertain [64–67]. Convincing evidence that therapeutic effects or toxicities are better corre-

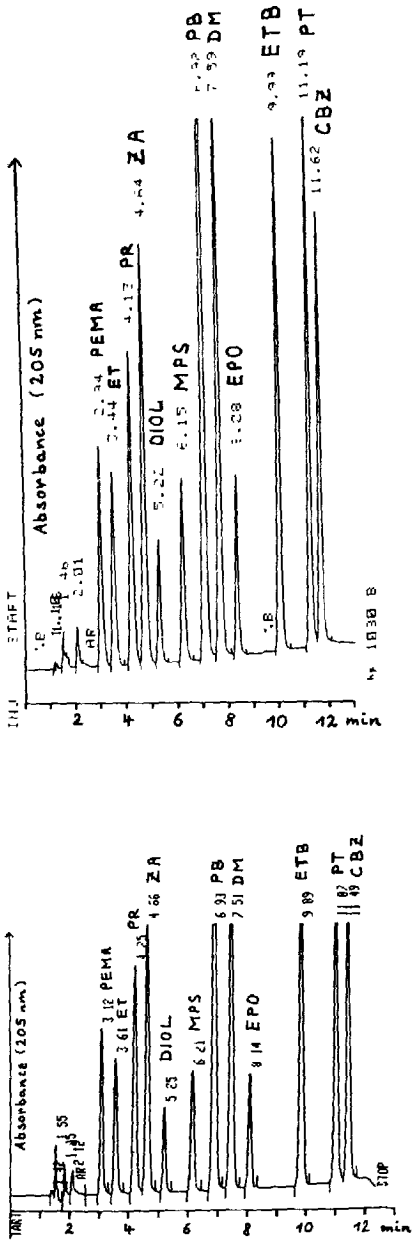

Fig 3 HPLC profiles of a calibration sample containing antiepileptic drugs and metabolites The top tracing was obtained using a conventional (125 mm × 4 6 mm I D) and the bottom a narrow-bore (200 mm × 2 1 mm I D) analytical column Abbreviations PEMA = phenylethylmalonamide, ET = ethosuximide; PR = primidone, ZA = zonisamide, DIOL = carbamazepine *trans*-diol, MPS = methylpropylsuccinimide (internal standard), PB = phenobarbital; DM = desmethylmethsuximide, EPO = carbamazepine epoxide, ETB = 5-ethyl-5-(*p*-tolyl)barbituric acid (internal standard), PT = phenytoin, CBZ = carbamazepine Both columns were packed with 5-μm Shandon Hypersil® ODS packing Gradient elution with acetonitrile and phosphate buffer was used (Reproduced with permission from ref 62)

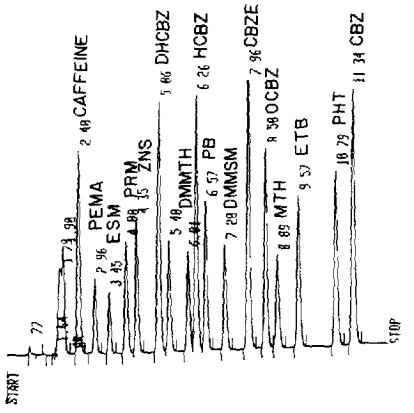

Fig. 4. HPLC profile of a test solution of fourteen antiepileptic drugs and metabolites. For abbreviations and analytical conditions see Table 1. (Reproduced with permission from ref. 45.)

lated to the free than the total antiepileptic drug concentrations is still lacking. However, in the situations where alterations in drug protein binding have occurred (renal disease, displacement by other drugs or endogenous substances), free levels provide useful information. Recently, Friel *et al.* [68] have shown a significantly better correlation for phenytoin between brain (human grey matter) and free serum concentrations than between brain levels and total serum concentrations.

The interest in free drug levels is based on pharmacological principles that free drug crosses biological membranes and interacts with receptors to ellicit an effect and that the free drug at the receptor site (site of action) is in a rapid, reversible equilibrium with the free drug in plasma.

Most commonly used antiepileptic drugs are extensively metabolized and exhibit a low extraction ratio (restrictive clearance), and their clearance is dependent on the free fraction and the intrinsic clearance. Therefore, total concentration is inversely dependent on the free fraction and the intrinsic clearance. Free concentration, on the other hand, is inversely dependent on intrinsic clearance but is independent of the free fraction. During chronic therapy, a displacement of a drug will not effect its free concentration but will decrease its total concentration (by increasing its free fraction). In that case, dosage adjustment based on the total levels would be inappropriate [64].

Commonly used antiepileptic drugs exhibit the following percentages binding (primarily to albumin): phenytoin 87–93%, valproate 70–92% (saturable, concentration-dependent), carbamazepine 60–83%, phenobarbital 45–50%, primidone 0–30%, and ethosuximide 0–10% [64]. Monitoring of the free levels is theoretically warranted for drugs with the following characteristics: (a) highly bound, (b) variable extent of binding, (c) small volume of distribution (< 2 l/kg), (d) narrow therapeutic index, and (e) established relationship between pharmacolog-

ical response and free drug concentrations [64,67]. Therefore, of the antiepileptic drugs, phenytoin, valproate and carbamazepine are reasonable candidates for free drug level monitoring.

Determination of free levels of the antiepileptic drugs introduces several methodological problems [66]. For example, a separation of free and bound forms of drugs is required and since free levels are much lower than the total levels, greater sensitivity is needed and there is a greater problem with interferences. Several different approaches have been used for obtaining biological fluid for measurements of free concentrations (equilibrium dialysis, ultrafiltration, ultracentrifugation, saliva, and tears) and their potential problems were reviewed [66,67].

Ultrafiltration, using small disposable filter units, has become the most frequently employed method for obtaining samples for determinations of free levels. Its advantages include speed and simplicity, requirement for small sample size, and no sophisticated instrumentation Comparisons between ultrafiltration, ultracentrifugation, and equilibrium dialysis have generally demonstrated a good agreement between these methods, *e.g.* for phenytoin [69].

An HPLC method was reported for the simultaneous determination of free levels of phenytoin, carbamazepine, phenobarbital, and primidone using ultrafiltration [70]. Ultrafiltration served not only to obtain samples containing the free drug concentrations but also to deproteinize the samples and allow direct injection of the ultrafiltrate.

3 IMMUNOASSAY METHODS FOR STANDARD ANTIEPILEPTIC DRUGS

There is a growing effort to automate and simplify methods for determination of the antiepileptic drugs. The objectives are to minimize sample preparation and sample size, use simple instrumentation or no instrumentation at all, obviate the need for highly skilled technical staff, expedite availability of results, minimize cost, and make the procedure as fool-proof as possible. A variety of immunoassay techniques were developed to meet these demands: enzyme-multiplied immunoassay technique (EMIT, Syva, Palo Alto, U.S.A.), radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), substrate-labeled fluorescence immunoassay (SLFIA, Ames TDA, Ames Division, Miles Labs., Elkhart, IN, U.S.A.), flourescence polarization immunoassay (FPIA, Abbott Labs, Diagnostics Division, Irving, TX, U.S.A.), nephelometric inhibition immunoassay (Beckman ICS, Beckman Instruments, Brea, CA, U.S.A), apoenzyme reactivation immunoassay (ARIS, Ames Seralyzer, Ames Division, Miles Labs.), and ACCU-LEVEL enzyme immunochromatography (Syntex Medical Diagnostics Division of Syva).

Chromatographic and immunoassay methods are compared in Table 2. Most reports found reasonable precision, accuracy, and correlation between these immunoassay techniques and the traditional reference methods, GC and HPLC. For example, FPIA (for phenobarbital, phenytoin, primidone, carbamazepine,

TABLE 2

GENERAL COMPARISON OF CHROMATOGRAPHIC AND IMMUNOASSAY METHODS FOR ANALYSIS OF ANTIEPILEPTIC DRUGS

	Chromatography	Immunoassay
Commonly used serum/plasma sample size	50–1000 μl	< 50 μl
Technical skill and experience requirements	Considerable	Minimal
Relative analysis time	Usually slower	Rapid
Data output	Chromatogram and numerical	Numerical
Ability to measure multiple compounds simultaneously	Yes	No
Ability to measure metabolites	Yes	No
Ability to measure new drugs	Yes	No
Problems with interferences	Resolvable	Occassional
Sample clean-up	Usually	No
Sample derivatization	Sometimes	No
Sensitivity for single-dose pharmacokinetic studies	Adequate	Limited

and valproate) yielded results with reasonable precision and accuracy and showed a good correlation with the HPLC method (GC method for valproate) [71].

Metabolites, however, are not quantitated by immunoassay techniques and in some cases they even interfere. Carbamazepine epoxide was reported to cross-react with carbamazepine in the EMIT assay thus yielding overestimated carbamazepine levels [72,73]. Phenytoin levels were also overestimated using the EMIT method in patients with renal failure due to cross-reactivity with *p*-hydroxyphenytoin in their plasma [74]. Cross-reactivity between ethosuximide and N-desmethylmethsuximide was reported in EMIT and FPIA assays, and neither of these compounds could be measured using either of these methods in patients treated with ethosuximide and methsuximide (rare combination) [75] In fact, the authors took the advantage of this cross-reactivity to quantitate N-desmethylmethsuximide by slightly modified EMIT and FPIA methods in serum of patients which were not treated with ethosuximide. Results showed good accuracy, precision, and agreement with valued obtained using HPLC.

The ARIS method is portable and employs solid-phase chemistry (reagent impregnated into a strip) and an Ames Seralyzer reflectance photometer In evaluating this assay for phenobarbital and phenytoin, acceptable accuracy and precision were demonstrated and reasonable correlation with other methods was shown (GC, EMIT, FPIA, SLFIA) [76–80].

The ACCULEVEL enzyme immunochromatographic method measures drug concentrations by the migration and binding of the drug along the chromatographic paper coated with monoclonal antibodies. The method requires only 12 μl of blood, no instrumentation, and no skilled technical staff, no venipuncture (only a finger prick). It can be performed at a remote site, is completely self-

contained, and does not require reconstitution or calibration (provided by the manufacturer). It takes about 25 min per assay but assays can be done in parallel unlike with other immunoassay techniques (sequential). Acceptable precision, accuracy, and correlation with other methods (EMIT, FPIA, HPLC) was demonstrated for phenytoin and phenobarbital [81] and carbamazepine [82]. Carbamazepine epoxide did not interfere with carbamazepine determination.

Recently, a large study using external quality assurance samples was performed to evaluate eight techniques (RIA, EMIT, SLFIA, FPIA, nephelometry, GC with derivatization, GC without derivatization, and HPLC) for measurement of eight antiepileptic drugs (phenytoin, phenobarbital, primidone, carbamazepine, carbamazepine epoxide, ethosuximide, valproic acid and clonazepam) [73]. A significant amount of cross-reactivity of carbamazepine epoxide with carbamazepine was evident in immunoassays Precision and accuracy were generally acceptable with the possible exception of nephelometry and GC with derivatization.

4 CHROMATOGRAPHIC METHODS FOR NEW CANDIDATE ANTIEPILEPTIC DRUGS

As discussed in the Introduction, many epileptic patients are not adequately controlled by the existing drugs Therefore, there is a continuing effort to develop new drugs for the treatment of epilepsy. This section will concentrate on drugs at various stages of development ranging from those in early clinical trials to those which have been already approved in some countries [83,84]. New antiepileptic drugs are usually added to the existing therapeutic regimen and therefore it is beneficial to develop methods that can quantitate the new candidate drug simultaneously with commonly used antiepileptic drugs and their active metabolites. However, this is often technically difficult because several new candidate drugs (Fig. 5) have physicochemical characteristics quite different from those of traditional antiepileptic agents, and the former often exhibit much lower clinically relevant plasma concentrations.

4.1 Progabide

Progabide, 4-{[(4-chlorophenyl-5-fluoro-2-hydroxyphenyl)methylene]amino}butanamide, is a new antiepileptic drug which has been approved for use in some countries. It is a derivative of γ-aminobutyric acid (GABA) which readily crosses the blood–brain barrier and its activity is thought to be due to its GABA-mimetic properties

An HPLC method with UV detection was reported for the determination of progabide and its active acid metabolite (deamidated progabide) in human blood (1 ml) and plasma (1 ml) [85]. After addition of the internal standard (structural analogue of progabide), samples were extracted with toluene, chromatographed on a 10-μm silica column, and analyzed with UV detection at 340 nm. The limit of sensitivity was 50 ng/ml for both compounds. This method is simpler than previ-

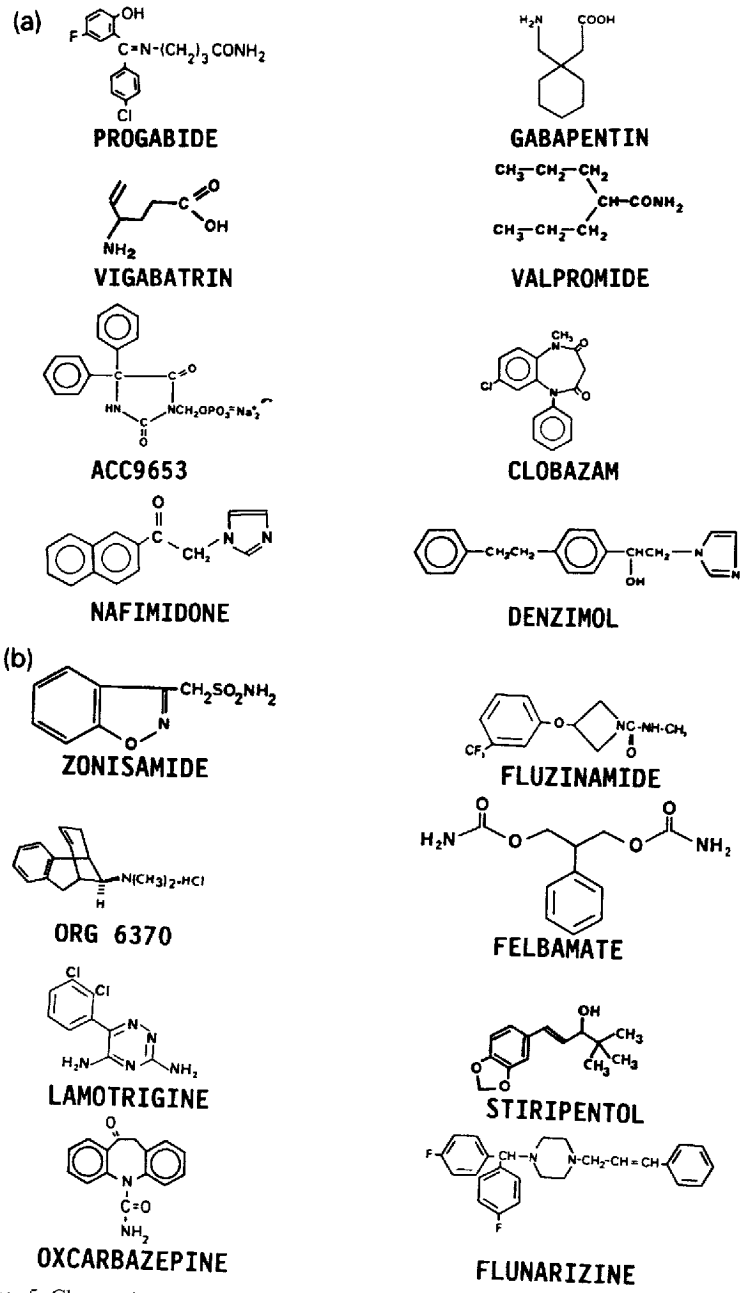

Fig 5 Chemical structures of new candidate antiepileptic drugs

ously published HPLC methods [86,87] in that it does not require an initial reductive hydrogenation of the imine bond, it utilizes a simpler one-step extraction, and it uses UV detection as opposed to a more complex electrochemical detec-

tion. However, it was not as sensitive and chromatograms did not appear as clean as those obtained using the electrochemical detector. In a subsequent publication [88], the authors suggested that the analytical column be washed with a methanol–25% ammonia–methylene chloride (4:0.2:95.8) solution after 150–200 injections of biological extracts. This served to regenerate the column and maintain its efficiency. There were no interference from other commonly used antiepileptic drugs.

4.2. Gabapentin

Gabapentin, 1-(aminomethyl)cyclohexaneacetic acid, is a GABA analogue which crosses the blood–brain barrier. An HPLC method [89] was reported for its analysis in human plasma (0.5 ml). Its analogue, 1-(aminomethyl)cycloheptaneacetic acid, was used as the internal standard, samples were deproteinized with perchloric acid, derivatized with 2,4,6-trinitrobenzenesulphonic acid, chromatographed on a 10-μm C_{18} column using acetonitrile–water–acetic acid (58:41.5:0.5), and monitored with a UV detector. Limit of detection was 10 ng/ml.

4.3. Vigabatrin

Vigabatrin (γ-vinyl-GABA; GVG; DL-4-aminohex-5-enoic acid) is an enzyme activated, irreversible inhibitor of GABA transaminase. It is a structural analogue of the inhibitory neutotransmitter GABA and the most specific known inhibitor of the enzyme responsible for GABA degradation.

A GC–MS method [90] was reported for the determination of the *R*(−)- and *S*(+)-enantiomers of GVG in human plasma (100 μl). *S*(+)-γ-acetylenic-GABA was used as the internal standard and, after protein precipitation with methanol, samples were derivatized first with trifluoroacetic anhydride and then with diazomethane. Separation of the compounds was achieved using a capillary column coated with a chiral phase (L-valine-*tert.*-butylamide coupled to an alkylsiloxane polymer). SIM in the EI mode was used for the detection. Plasma, urine, and cerebrospinal fluid were shown to be free of endogenous interferences.

An HPLC method [91] was also reported for the determination of vigabatrin in human plasma (100 μl) and urine (10 μl). γ-phenyl-GABA was used as the internal standard, and after the addition of copper chloride, samples were derivatized with dansyl chloride. Copper(II) ions were used to complex endogenous α-amino acids and thereby prevent their derivatization which enhanced specificity and simplified chromatography. A 6-μm reversed-phase HPLC column was used for separation at room temperature. Fluorometric detection was used for monitoring (excitation 345 nm, emission 418-nm cut-off filter). The limit of detection was 0.5 μg/ml for plasma and 10 μg/ml of urine.

4.4. Valpromide

Valpromide (2-propylvaleramide) is an amide of the commonly used antiepileptic drug valproic acid A GC method for the analysis of valpromide and valproic acid in human plasma (1 ml) was reported [92]. Caprylic acid was used as the internal standard, compounds were extracted into chloroform, chromatographed on a glass column packed with 5% free fatty acid phase, and detected with a flame ionization detector. The minimal detectable concentration was 1–2 μg/ml.

4.5 Disodium phosphate ester of 3-hydroxymethyl-5,5-diphenylhydantoin

The disodium phosphate ester of 3-hydroxymethyl-5,5-diphenylhydantoin (ACC-9653) is a prodrug of phenytoin. It was developed primarily to overcome the solubility problems with phenytoin While phenytoin exhibits poor solubility in aqueous solutions and precipitates easily in physiological solutions and at the site of intramuscular injection, its prodrug is readily soluble in aqueous solutions and is rapidly and quantitatively hydrolyzed (probably by phosphatases) in the body to phenytoin.

An HPLC method was reported for the analysis of phenytoin and its prodrug in human plasma (1 ml) [93]. Diphenylphosphate and 5-(4-methylphenyl)-5-phenylhydantoin were used as the internal standards for phenytoin prodrug and phenytoin, respectively. After protein precipitation with acetonitrile, methylene chloride was added to the supernatant. The aqueous phase was used for the analysis of phenytoin prodrug. The organic phase (acetonitrile–methylene chloride) was extracted with sodium hydroxide, and the alkaline phase neutralized and used for the analysis of phenytoin. Phenytoin and phenytoin prodrug were analyzed by HPLC using a C_{18} column but different mobile phases. UV detection at 214 nm was used.

4.6. Clobazam

Clobazam, 7-chloro-1-methyl-5-phenyl-1*H*-1,5-benzodiazepine-2,4-(3*H*,5*H*)-dione, is a 1,5-benzodiazepine with antiepileptic properties. A GC method was reported for the analysis of clobazam and its active metabolite, N-desmethylclobazam, in human plasma (0.1 ml) [94]. Methylclonazepam was used as the internal standard and ethyl acetate extracts were chromatographed on a packed column and monitored using electron-capture detection. None of the commonly used antiepileptic drugs and benzodiazepines interfered with the assay The limit of detection was 7 and 15 ng/ml for clobazam and N-desmethylclobazam, respectively.

An HPLC method was developed for the determination of clobazam and three metabolites, N-desmethylclobazam, 4′-hydroxyclobazam and 4′-hydroxy-N-des-

methylclobazam, in human plasma and urine [95]. Clobazam, N-desmethyl-clobazam and the internal standard, diazepam, were extracted with diethyl ether at pH 13, followed by extraction of the hydroxy metabolites and the internal standard, nitrazepam, with diethyl ether at pH 9. The chromatographic separation was carried out on a 10-μm C_{18} column and detection was at 230 nm. The limit of detection was about 10–20 ng/ml for each compound. Interferences from some commonly employed antiepileptic drugs were detected for the hydroxy metabolites of clobazam. Another HPLC method [96] described an analytical procedure for clobazam, clonezapam, and nitrazepam in human serum (1 ml). Flunitrazepam was used as the internal standard, samples were extracted with a hexane–ethyl acetate mixture, chromatographed on a 5-μm C_8 column, and monitored at 220 nm. The lower limit of detection for the three benzodiazepines was about 0.5–1 ng/ml. Commonly used antiepileptic drugs did not interfere with the assay. A simultaneous analysis of clobazam, N-desmethylclobazam, and clonazepam in human plasma (1 ml) was reported [97]. Methylclonazepam was the internal standard, samples were extracted with diethyl ether, chromatographed on a C_{18} column, and monitored at 313 nm. The limit of detection was 25 ng/ml for clobazam and N-desmethylclobazam and 5 ng/ml for clonazepam. The assay appeared free of interferences from commonly used antiepileptic drugs as well as some other drugs.

A capillary GC method for the determination of clobazam and some other benzodiazepines used in the treatment of epilepsy was also reported [34] (see Section 2.1.1.5).

4.7. Nafimidone

Nafimidone, 1-(2-naphthoylmethyl)imidazole hydrochloride, and its active major metabolite nafimidone alcohol, 1-[2-hydroxy-2-(2-naphthyl)ethyl]imidazole, were determined in human plasma (0.5 ml) and urine (100 μl) [98]. Methylnafimidone and methoxynafimidone were used as internal standards. Compounds were extracted using a three-step liquid–liquid extraction procedure and chromatographed using a 10-μm reversed-phase column and an ion-pairing reagent, sodium dodecylsulfate. Nafimidone alcohol was detected at 225 nm using a UV detector while the parent drug, nafimidone, was monitored with a fluorescence detector (245 nm excitation, 456 nm emission) for greater sensitivity because its levels were considerably lower than those of its metabolite. The limits of detection for nafimidone and nafimidone alcohol were 5 0 and 12.5 ng/ml, respectively. Commonly used antiepileptic drugs did not interefere with the method

4.8. Denzimol

Denzimol, N-{β-[4-(β-phenylethyl)phenyl]-β-hydroxyethyl}imidazole hydrochloride, is another imidazole-containing candidate antiepileptic agent. GC and

HPLC methods were developed for the determination of denzimol and its main metabolite, N-{β-[4-(β-phenyl-β(α)-hydroxyethyl)phenyl]-β-hydroxyethyl}imidazole, in human plasma (1 ml) and urine (0.5 ml) [99]. Denzimol homologue, N-{γ-[4-(β-phenylethyl)phenyl]-γ-hydroxypropyl}imidazole, was used as the internal standard. For a GC analysis of plasma samples, a solid-phase extraction with C_{18} cartridges was employed, followed by chromatography on a wide-bore capillary column and a detection with a nitrogen-selective detector. Urine samples were treated with β-glucuronidase–arylsulfatase and supernatants were analyzed by HPLC using a C_{18} reversed-phase column and detection at 214 nm. The limit of detection for denzimol and its metabolite was 2.5 and 15 ng/ml, respectively, using GC and 0 5 and 1 μg/ml using HPLC. There were no interferences from commonly used antiepileptic drugs.

4.9. Zonisamide

Zonisamide, 1,2-benzisoxazole-3-methane-sulfonamide, is a new candidate antiepileptic drug being evaluated for the treament of refractory partial seizures. Zonisamide was analyzed in human serum (0.50 ml) concurrently with several other antiepileptic agents and their metabolites by HPLC [45,62] (see Section 2 1.2). Ethyl acetate extracts were chromatographed on conventional (125 mm × 4 6 mm) and narrow-bore (200 mm × 2 1 mm) 5 μm C_{18} columns and monitored at 205 nm. Comparable results were obtained with the two column and the saving in the costs of solvents more than offset the higher costs of the narrow-bore columns [62].

4.10. Fluzinamide

Fluzinamide, N-methyl-3-[3-(trifluoromethyl)phenoxy]-1-azetidine carboxamide, and three of its active metabolites (N-hydroxymethyl, N-formyl and N-desmethyl) were analyzed in human plasma (0 5 ml) by HPLC [100]. A single extraction of samples with a hexane–methylene chloride–butanol mixture was followed by a chromatographic separation on a 10-μm reversed-phase column and UV detection at 220 nm. Commonly used antiepileptic drugs were shown not to interfere with the assay. Quantitation was possible down to 0.05 μg/ml of the compounds. For optimum separation of all compounds, it was necessary to adjust the composition of the mobile phase, especially the percentage of tetrahydrofuran, for different column lots and age of the analytical column. During the evaporation, its was necessary to maintain the temperature below 35°C in order to avoid conversion of the N-hydroxymethyl metabolite to the N-desmethyl metabolite.

4.11. dl-[5α,9α,11S]-5,6,9,10-Tetrahydro-N,N-dimethyl-5,9-methanobenzocycloocten-11-amine hydrochloride*

dl-[5α,9α,11*S**]-5,6,9,10-Tetrahydro-N,N-dimethyl-5,9-methanobenzocyclooctene-11-amine hydrochloride (Org 6370) is a rigid aminobenzobicyclonone. A capillary GC method [101] was developed for analysis of Org 6370 and its active N-desmethyl metabolite, Org 6363, in human plasma (0 5 ml). A stereoisomer of Org 6370 was used as the internal standard, samples were extracted using C_2 solid-phase columns and analyzed using a nitrogen-selective detector. Org 6370 and its metabolite were measured down to 4 and 8 ng/ml, respectively. The report also discussed several precautions necessary when working with these amines, *e.g.* deactivation of glassware and capillary injection liners, maintenance of alkaline conditions during solid-phase extractions, solvent composition for elution of solid-phase columns, and use of pure ethyl acetate kept under nitrogen for reconstitution of dried extracts

4.12. Felbamate

Felbamate, 2-phenyl-1,3,-propanediol dicarbamate, is a new candidate antiepileptic drug with a structure similar to the antianxiety agent, meprobamate. An HPLC method with a C_{18} column was used for its analysis in human plasma [102]. The lowest measurable concentration was 0.5 μg/ml.

4.13. Lamotrigine

Lamotrigine, 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4,-triazine, is a potential antiepileptic agent undergoing clinical trials An HPLC method was reported for its analysis in human plasma (0.2 ml) and urine (0.2 ml) [103]. A structural analogue, 3,5-diamino-6-(2-methoxyphenyl)-1,2,4-triazine, was used as the internal standard, samples were extracted with ethyl acetate, chromatographed on a silica column, and monitored at 306 nm The lower limit of detection was 50 ng/ml.

4 14. Stiripentol

Stiripentol, (±)-1-(3,4-methylenedioxyphenyl)-4,4-dimethyl-1-penten-3-ol, is a new candidate antiepileptic drug. An HPLC method was reported for analysis of stiripentol in human plasma (0.2 ml) [104]. The internal standard was 3-bromo-N-propylcinnamamide, samples were extracted into diethyl ether, chromatographed on a C_8 column, and monitored at 254 nm Three metabolites resulting from the opening of the methylenedioxy ring (*p*-hydroxy, *m*-hydroxy, and dihydroxy) were analyzed in urine by HPLC using a reversed-phase column. A GC–MS study [105] identified thirteen urinary metabolites of stiripentol after hydrolysis of human urinary samples with β-glucuronidase, solid-phase extraction, and formation of TMS ether derivatives.

4.15. Oxcarbazepine

Oxcarbazepine, 10,11-dihydro-10-oxo-5*H*-dibenz[*b,f*]azepine-5-carboxamide, is a keto homologue of carbamazepine or 10,11-dihydro-10-oxocarbamazepine. It has fewer unwanted side-effects than carbamazepine, possibly because it is not metabolized into an epoxide.

HPLC methods have been reported for simultaneous analysis of oxcarbazepine, carbamazepine, and their metabolites (10,11-dihydro-10-hydroxycarbamazepine, the main and active metabolite of oxcarbazepine; 10,11-epoxycarbamazepine, a metabolite of carbamazepine; 10,11-dihydro-10,11-*trans*-dihydroxycarbamazepine, a common metabolite of oxcarbazepine and carbamazepine) [106,107]. Major metabolic pathways for oxcarbazepine and carbamazepine are depicted in Fig. 6. HPLC is well suited for the analysis of these compounds since its avoids the problem of thermal instability and a need for derivatization associated with GC methods. Both methods used liquid–liquid extraction (ethyl acetate or diethyl ether–methylene chloride) of 0.5 ml of human plasma and chromatography on a 5-μm reverse-phase column (C_{18} or C_8) with UV detection (254 or 210 nm). The internal standard was 9-hydroxymethyl-10-carbamoylacridane [106] or 5,6-dihydro-11-oxo-11*H*-dibenz[*b,e*]azepine-5-carboxamide [107]. Both methods were shown to be free of interferences from other commonly used antiepileptic drugs The limit of detection for all the compounds was below 0.2 μg/ml in both assays.

As discussed in Section 2.1.2., oxcarbazepine and/or its major metabolite

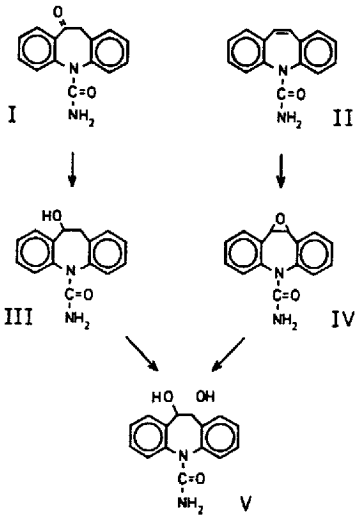

Fig 6 Major metabolic pathways for oxcarbazepine (I) and carbamazepine (II) in man. Both drugs form carbamazepine *trans*-diol (V), but by a different route Oxcarbazepine is metabolized via 10-hydroxy-10,11-dihydrocarbamazepine (III) and carbamazepine via carbamazepine epoxide (IV) (Reproduced with permission from ref 109)

10,11-dihydro-10-hydroxycarbamazepine were also measured concurrently with other commonly used antiepileptic drugs [45,49,52]. Oxcarbazepine is rapidly metabolized to 10,11-dihydro-10-hydroxycarbamazepine in man and plasma levels of the latter greatly exceed those of the former. Therefore, some of the methods were only concerned with quantitation of the metabolite [49,52].

Von Unruh and Paar developed GC [108] and GC–MS [109] procedures for the analysis of oxcarbazepine and its main metabolites (10,11-dihydro-10-hydroxycarbamazepine and 10,11-dihydro-10,11-*trans*-dihydroxycarbamazepine) in 0.5 ml of human plasma. In order to avoid thermal instability associated with GC-based methods, samples were derivatized with N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) resulting in bis(trimethylsilyl)derivatives of enol of oxcarbazepine and its 10-hydroxy metabolite and a tris(trimethylsilyl) derivative of its *trans*-diol metabolite. The derivatives in MSTFA were stable at room temperature for several hours and at −20°C for at least a month. The GC method used carbamazepine as the internal standard, methylene chloride extraction for all compounds, a capillary column, and a flame ionization detector. It was more sensitive than HPLC methods and the limit of detection was 10 ng/ml for oxcarbazepine and its 10-hydroxy metabolite and 25 ng/ml for its *trans*-diol metabolite. The GC–MS method employed two extractions. Methylene chloride was used for the extraction of oxcarbazepine and its 10-hydroxy metabolite and charcoal adsorption for the *trans*-diol metabolite in order to improve its recovery. Both extractions used the 10,11-*cis*-diol metabolite of oxcarbazepine as the internal standard. Chromatographic separation was achieved with a capillary column and the detection was by SIM in the EI mode. The GC–MS method was even more sensitive than the GC method. The limit of detection was 0.1, 0 1 and 1.0 ng/ml for oxcarbazepine, its 10-hydroxy metabolite and its *trans*-diol metabolite, respectively. Both GC and GC–MS methods are shown to be free of interferences from other commonly used antiepileptic drugs.

4.16. Flunarizine

Flunarizine, (*E*)-1-[bis(4-fluorophenyl)methyl]-4-(3-phenyl-2-propenyl)piperazine, is an effective calcium channel blocker with a variety of clinical activities. It exhibits antihistaminic, antiarrhythmic, and anticonvulsant activity and is used in the treatment of peripheral and cerebral circulatory disorders, vestibular disorders, and migraine prophylaxis. It is presently undergoing clinical trails for treatment of epilepsy.

Flunarizine is a weak base with a tendency to adsorb to many surfaces, and in the presence of acids, especially sulfuric acid, an irreversible loss of flunarizine is observed. Early GC methods [110,111] utilized packed columns and were shown to be satisfactory in studies using normal volunteers. However, they did not have adequate sensitivity for pharmacokinetic studies in epileptic patients treated with other antiepileptic drugs (phenytoin and/or carbamazepine) which are known

enzyme inducers. A GC method was developed using a capillary column, three-step liquid–liquid extraction, and a nitrogen-selective detection [112]. The flunarizine analogue, (*E*)-1-[(4-chlorophenyl-4-fluorophenyl)methyl]-4-(3-phenyl-2-propenyl)piperazine, was used as the internal standard. It allowed quantitation of flunarizine as low as 0.25 ng/ml in plasma (1 ml) and was shown to be suitable for pharmacokinetic studies in patients on other antiepileptic medications. Another GC method [113] required only 250 μl of human plasma and utilized a liquid–solid extraction with Extrelut columns (E. Merck, Darmstadt, F.R.G.). Cinnarizine was used as the internal standard, the chromatographic separation was on a packed column, and detection with a nitrogen-selective detector. The limit of quantitation was reported as 1 ng/ml even though the methods was not validated below 5 ng/ml.

HPLC methods do not have the sensitivity of the GC methods but can be used for analysis of samples from patients treated chronically with flunarizine Several HPLC methods employed a single extraction step [114–116]. However, this led to discolored and sometimes cloudy samples, rapid degradation of chromatographic conditions, filter occlusion, rising system pressure, and a loss of column efficiency [117]. An HPLC method [117] was developed using the same extraction procedure as was used with the previously reported GC method [112] except that it used cinnarizine as the internal standard Chromatographic separation was achieved on a short (10-cm) 3-μm C_8 column and detection was at 254 nm. Isocratic conditions were used but a somewhat complex mobile phase was necessary to resolve flunarizine from apparent unidentified metabolites. Under these conditions, a good agreement between GC and HPLC methods was shown. However, this also illustrated the importance of cross-validation of analytical methods. It was the initial failure to obtain satisfactory correspondence between GC and HPLC methods (even though there were no visible signs of interferences) that led to modifications of the HPLC conditions.

5 DISCUSSION AND CONCLUSIONS

Monitoring of antiepileptic drug concentrations (and their metabolites) in biological tissues has had a major impact in developing and optimizing the treatment of epilepsy. It will continue to play an important part in antiepileptic therapy. Most of the standard, commonly used antiepileptic drugs have been around long enough that analytical methods have been developed. Consistent with this, the number of published methods for the analysis of standard antiepileptic drugs is on a steady decline. Many of the recent reports describe modifications of the previously published methods. Identification and quantitation of metabolites have received lot of attention Determination of free drug levels was also a subject of many studies, but there is still no concensus on the importance of the free levels. There are also numerous new candidate antiepileptic drugs at different stages of development. Many of these differ from the standard antiepileptic drugs

in that they are weak bases and exhibit clinically relevant concentrations in ng/ml range This requires a very different analytical approach.

There is no one chromatographic technique as the method of choice for the antiepileptic drugs. Following the technological developments, capillary GC columns are replacing packed GC columns and HPLC methods are being favored over the GC methods. Capillary columns offer greater resolving power and sensitivity and have a lesser need for prior sample derivatization. HPLC techniques require a simpler sample clean-up, do not cause thermal decomposition, do not require derivatization, and are easier to use. However, HPLC lacks the sensitivity of GC. HPLC of the antiepileptic drugs is primarily limited to relatively non-specific UV detection at non-specific wavelengths. GC–MS offers both specificity and sensitivity, but the instrumentation is relatively expensive and requires more sophisticated users. However, with the advent of mass-selective detectors, GC–MS instrumentation is becoming cheaper, simpler to operate, and much more compact It is just a matter of time before LC–MS reaches a level of development which would make it a dominant technique.

Large emphasis has been placed on sample preparation in an effort to simplify and expedite the analysis. There is a trend away from complicated liquid–liquid extractions to single-step liquid–liquid extractions, liquid–solid extractions, column switching, simple deproteinizations, and direct sample injections. The simpler sample preparations are time-efficient and also result in large savings of solvents. However, the ones requiring little or no sample preparation are primarily suited for HPLC and may lead to more frequent maintenance of the chromatographic system and a shorter life of the analytical column. One of the advantages of the methods using the minimal sample preparation is that they are suited for the simultaneous analysis of compounds with different physicochemical properties. However, this is a two-edged sword in that there is a greater chance of interferences.

New methods are being developed for direct analysis of drugs, including antiepileptics, in biological matrices. One of these new approaches, which has been reported for analysis of phenobarbital and carbamazepine, involves shielded hydrophobic phase (SHP) [118]. Hydrophilic groups on this new chromatographic phase prevent the larger, water-soluble biopolymers from interacting with the hydrophobic component of the bonded phase and they pass unretained through the column. Small analytes penetrate to the hydrophobic regions of the phase and are chromatographed by a reversed-phase mechanism.

The use of robotics in sample preparation is growing and in the near future it will probably become as prevalent as the use of autosamplers.

Recently, there was a proliferation of immunological, as opposed to conventional chromatographic, methods, for the analysis of antiepileptic drugs. This is especially true in clinical monitoring. These immunological methods generally require less sophisticated operators and instrumentation than the chromatographic methods. Within the therapeutic range, immunological methods are re-

ported to have accuracy and precision similar to chromatographic methods. Chromatographic methods are more suited for single-dose studies. Immunological methods analyze for one compound at a time, do not analyze for metabolites, are not available for new drugs, generate only a single numerical value for the output (*i.e.* no chromatogram), and in some cases they may have cross-reactivity problems.

6. SUMMARY

This review discussed various analytical methods for the determination of antiepileptic drugs and their metabolites in biological tissues. The emphasis was on the reports published since the last review [J. T. Burke and J. P. Thenot, *J. Chromatogr.*, 340 (1985) 199]. Both chromatographic and immunological procedures were cited and compared. Methods for individual and simultaneous quantitation of standard antiepileptic drugs and their metabolites were considered. In addition, a discussion of free drug determination and procedures for new candidate antiepileptic drugs were included.

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