J Neurol (1982) 228: 1–16

Journal of
Neurology
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Survey of Progress

Therapeutic drug monitoring: A comprehensive and critical review of analytical methods for anticonvulsive drugs

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Summary. In the last 20 years, there has been considerable improvement in the determination of anticonvulsive drugs in body fluids. Gas-liquid chromatography, high-performance liquid chromatography and immunological methods (radio-, enzyme-, fluoro-, and nephelo-immunoassays) have progressively supplanted spectrophotometry and thin-layer chromatography. As the number of publications shows, these methods have included a great variety of procedures. At present, gas-liquid chromatography and enzyme-immunoassays are routinely performed, awaiting a wider spread of liquid chromatography and other immunological techniques.

This paper is a comprehensive review of the analytical literature on the determination of phenobarbitone, primidone, phenytoin, carbamazepine, valproic acid, ethosuximide, clonazepam, and some of their metabolites in physiological fluids. Gas-liquid chromatographic methods are more particularly reviewed.

In order to facilitate the choice between these procedures, a critical selection of the techniques is given and recommendations are made. Emphasis is laid on technical problems encountered with the assays, as well as the need for a rigorous analytical assessment and internal and external quality controls. This review is completed by considerations on the determination of the free drug fraction and on sample collection and storage.

Key words: Analytical methods – Anticonvulsive drugs – Drug monitoring – Epilepsy

Zusammenfassung. In den letzten zwanzig Jahren wurde die Bestimmung von Antiepileptica in Körperflüssigkeiten erheblich weiterentwickelt. Die Gas-Flüssigkeitschromatographie, die sehr leistungsfähige Flüssigkeitschromatographie und immunologische Methoden (Röntgen-, Enzym-, Fluor- und Nephelo-Immunprüfungen) haben allmählich die Spektrophotometrie und die Dünnschichtchromatographie verdrängt. Wie die Zahl der Veröffentlichungen

0340-5354/82/0228/0001/$ 03.20

zeigt, wurden diese Methoden in eine Vielfalt von Verfahren aufgeteilt. Heutzutage werden Gas-Flüssigkeitschromatographie und Enzym-Immunoprüfungen routinemäßig durchgeführt, in Erwartung einer weiteren Verbreitung der Flüssigkeitschromatographie und sonstiger immunologietechnischer Methoden.

Die vorliegende Abhandlung stellt eine Übersicht des analytischen Schrifttums über die Bestimmung von Phenobarbital, Primidone, Phenytoin, Carbamazepin, Valproicsäure, Ethosuximid, Clonazepam und einigen ihrer Metaboliten in Körperflüssigkeiten dar. Gas-Flüssigkeitschromatographiemethoden werden besonders eingehend dargelegt.

Um die Wahl zwischen diesen Methoden zu erleichtern, wird eine kritische Auswahl der technischen Methoden und Empfehlungen beschrieben. Technische Probleme bei den Untersuchungen sowie die Notwendigkeit einer genauen analytischen Technik und interner wie externer Qualitätskontrollen werden hervorgehoben. Vervollständigt wird diese Übersicht durch Überlegungen über die Bestimmung der freien Arzneimittel und über Entnahme und Aufbewahrung der Proben.

Since the appearance in 1960 of a study on the pharmacoclinical interpretation of phenytoin (DPH) and phenobarbitone (PB) serum levels observed in treated patients [7], the therapeutic monitoring of anticonvulsive drugs has been developed considerably and now forms a part of epileptology. Excellent reviews of the current situation and knowledge of the pharmacological and clinical aspects of the therapeutic monitoring of anticonvulsants are available in the literature [19, 28, 47, 56, 61, 62].

Following the increase in the number of laboratories performing determinations of anticonvulsive drugs, interlaboratory quality control programmes have been conducted on a vast scale [14, 26, 55]. In 1974, Pippenger et al. [57], supported by the Epilepsy Foundation of America, organized a blind survey among 197 North American laboratories. Three plasma samples, presented in such a way as to simulate patients' specimens, were evaluated for DPH, PB, primidone (PM) and ethosuximide (ES). The results demonstrated that it was urgently necessary to improve the quality of the assays: the interlaboratory coefficients of variation ranged from 38% to 505% and the levels for one sample between 0 and 6 times the threshold of the toxic level. These results showed the necessity to use more reliable methods and improve the quality control within the laboratories. The development of external quality control programmes has led to a decrease in the variation of laboratory results [14, 55].

All the procedures described for anticonvulsant assays refer to a limited number of basic methods [3, 56, 62]. The principal techniques used, as well as an indication of their selectively in respect of anticonvulsants, are given in Table 1.

The aim of this paper is to review and discuss the different methods available, to point out their advantages and disadvantages, in order to demonstrate the options open to the clinical chemist wishing to practise determinations in the non-specialized laboratory. Especially chromatography (GLC) is discussed.

Table 1. Selectivity and other quality criteria of analytical methods for anticonvulsive drugs

	Spectrophotometry		EIA	FIA	NIA	RIA	TLC	HPLC	GLC	GC/MS
	Wallace (modified)	Others								
Selectivity[a]										
Phenytoin (DPH)										
Phenobarbitone (PB)										
Primidone (PM)										
Carbamazepine (CBZ)										
Ethosuximide (ES)										
Valproic acid (VPA)										
Clonazepam (CZP)										
Specificity	good	poor	good	good	good	good	good	very good	very good	highest
Within-run precision (CV%)[b]	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 5	< 5	< 5
Detection limit (mg/l)[b]	0.5	1	2.5	1	2	2	1	0.2	0.1	0.01
Sample volume needed (µl)	200	2000	50	50	100	20	100	100	200	100
Rapidity	slow	slow	rapid	rapid	rapid	medium	slow	medium	medium	medium
Easiness of work-up	medium	medium	good	good	good	good	medium	medium	medium	weak
Possibility of automation	nõ	no	yes	yes	yes	yes	no	yes	yes	no
Apparatus	cheap	cheap	medium	medium/ costly	costly	costly	cheap	medium/ costly	medium/ costly	very costly
Reagents	cheap	cheap	costly	costly	costly	costly	cheap	cheap	cheap	medium

[a] Selectivity is represented by a solid line joining drugs simultaneously determined. If a method does not allow for simultaneous analysis, each dash of the dashed line represents a drug individually assayed

[b] Typically for phenytoin

EIA: enzyme-immunoassay; FIA: fluoro-immunoassay; NIA: nephelo-immunoassay; RIA: radio-immunoassay; TLC: thin-layer chromatography; HPLC: high-performance liquid chromatography; GLC: gas-liquid chromatography; GC/MS: gas chromatography/mass spectrometry

Spectrophotometry and fluorometric methods

The spectrophotometric methods developed by Goldbaum [24] for barbiturate determinations are based on the absorption difference at one wavelength (240 or 260 nm) of the two ionic forms obtained when barbiturates are examined at two different pH levels. Among these methods, one may cite the procedure of Svensmark and Kristensen [71], which also permits determination of DPH by measuring the absorption difference between 235 and 260 nm.

Photometric assay of DPH has also been developed in another method, initially proposed by Wallace et al. and later modified by several authors [27]. In these methods, DPH extracted from the sample by dichloromethane or chloroform is oxidized to benzophenone with hot alkaline $KMnO_4$. After extraction by heptane, the benzophenone formed is measured spectrophotometrically at 257 nm or fluorometrically in an H_2SO_4 medium.

With the exception of the modifications of the Wallace method, the previously cited procedures are to be discouraged on the grounds of their lack of specificity and sensitivity. They also appear to be less reliable in external quality control surveys [14, 26]. At present, these methods are mainly of historical interest. Nevertheless, the modified Wallace methods are accurate, sensitive and sufficiently specific, but they can only be applied to DPH and are relatively time-consuming.

Thin-layer chromatography

Despite its selectivity, thin-layer chromatography (TLC) is very rarely used because of its relative lack of specificity and sensitivity. The method of Wad et al. [76] should, however, be mentioned; it involves the separation of DPH, PB, carbamazepine (CBZ), PM and mephenytoin, followed by a densitometric measurement at 215 nm.

Immunological methods

The immunological methods [44] are based on the competition for a limited quantity of an antibody made against the substance to be determined, between the molecules of the substance present in the sample and those added as reagent. The latter are made detectable by isotopic (radio-immunoassays), enzymatic (enzyme-immunoassays), fluorometric (fluoro-immunoassays) or nephelometric (nephelo-immunoassays) measurements.

The amount of competition is determined either after separation of the labelled antigen molecules from those combined with the antibody (heterogeneous techniques), or after the appearance or inhibition of enzyme activity, fluorescence or nephelometric absorption at the moment of formation of the antigen-antibody complex (homogeneous techniques).

The common advantage of the immunological methods is their rapidity (little or no sample preparation). On the other hand, they cannot be used for simultaneous analysis of more than one constituent. In addition, their admittedly

very high specificity does not exclude the possibility of cross-reactions, i.e. the one given by the glucurono-conjugate of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, a metabolite of DPH that may considerably increase in the sera of uraemic patients.

Radio-immunoassay: Radio-immunoassay (RIA) [9] offers numerous advantages for large series of determinations. However, this very sensitive method requires costly equipment and the handling of radioactive isotopes. Presently, only DPH and PB assays are commercially available as reagent kits (Amersham International Ltd, Amersham, England; Clinical Assays, Cambridge, Massachusetts, USA; Wien Labs, Succasunna, New Jersey, USA).

Enzyme-immunoassay: The homogeneous method put on the market in 1972 under the name EMIT (Enzyme Multipled Immunoassay Technique, Syva Co, Palo Alto, California, USA) [4] links an immunological reaction with an enzymatic detection. The EMIT method requires a spectrophotometer of good quality equipped with a thermoregulated flow cell and an automatic print-out system. Several adaptations for automatic apparatus are possible, for example on Abbott ABA-100, ACA-Dupont, LKB Kinetic Analysis System and centrifugal analysers. Some possible sources of error, e.g. dealing with the accuracy of enzymatic activity measurements, are to be noted: lipaemic, haemolytic or icteric samples, or anticoagulants other than heparin for the valproic acid (VPA) determination.

Fluoro-immunoassays: Several fluoro-immunoassay (FIA) methods have been proposed for the determination of PB, DPH and CBZ. One of them, commercialized by Ames Div., Miles Labs (Elkhart, Indiana, USA) is a homogeneous enzyme substrate labelled technique (SLFIA, TDA) [79].

Recently, Abbott Laboratories (North Chicago, Illinois), have commercialized an automated fluoro-immunoassay (TDX) based on the principle of fluorescence polarization [29].

Nephelo-immunoassay: Nephelo-immunoassay (NIA), a new method, is also commercially available as a semi- or fully automated procedure (ICS, Beckman Instruments, Geneva, Switzerland) [49].

These fluoro- and nephelo-immunological methods require less temperature and reaction time control than the EMIT method. Moreover, the reagents have a longer shelf-life.

High-performance liquid chromatography

Like GLC, high-performance liquid chromatography (HPLC) is selective, sensitive and specific. Five of the main anticonvulsants can be simultaneously analysed after a simple sample preparation. Contrary to GLC, no thermal decomposition of the drugs or their metabolites appears in HPLC. The disadvantages linked with adsorption of the drugs on the column are also eliminated. All these qualities explain why HPLC is an increasingly popular technique [60]. The first publications proposed a chromatographic separation on a silica gel phase [20, 77]. Nowadays, reverse phases (e.g. C18 bonded phases) are widely used. In these methods, the mobile phase consists of a mixture of water or a buffer with acetonitrile [1, 30, 63]

or methanol [18, 45], or both [72]. The eluent is analysed photometrically at 254 nm or at any wavelength between 195 and 210 nm.

Two methods for sample preparation are described. In the first, an equal volume of acetonitrile [30] or acetone [72] is added to the sample, and after centrifugation the upper phase is directly injected. This preparation, in which proteins are precipitated, has two disadvantages: the concentration of the extract is relatively weak, and its purity is insufficient (owing to the possibility of microprecipitation in the supernatant and the presence of interfering peaks). In the second method, the sample is extracted with chloroform or dichloromethane, the extract is evaporated to dryness, and the residue is taken up in a small volume of eluent [1, 45, 77]. Automated extraction [78] or automated complete analysis (the FAST-LC system, Technicon Instruments Corp., Tarrytown, New York, USA) [16] have also been proposed.

PB, DPH, CBZ, PM and ES can easily be determined with very satisfactory precision and accuracy. 10,11-Epoxy-carbamazepine can also be easily analysed [1, 45, 77], as can 5-ethyl-5(*p*-hydroxyphenyl)-barbituric acid and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin [18]. Finally, clonazepam (CZP) determination by HPLC has more recently been proposed [65].

Gas-liquid chromatography

The first publications proposing determinations of anticonvulsants in serum by gas-liquid chromatography (GLC) date back to the late 1960s [8, 38, 42, 54]. Since then, more than a hundred methods have been described, the larger part of them being variations or improvements of the original procedures [59].

These methods can be divided into methods without derivatization of the drugs, and methods by derivatization ("off-column" or "on-column").

Chromatography of phenobarbitone, phenytoin, carbamazepine, phenylethylmalonediamide and primidone without derivatization: These drugs are easily adsorbed on the solid phase, the liquid phase and the glass of the column. In consequence, unless certain precautions are taken, broad peaks or tailing, or both, are observed. This leads to a loss of precision and sensitivity. These methods also necessitate high temperatures for the injection block and the column. However, good results can be obtained by using a high-grade liquid phase, a suitable deactivated support and a silanized glass column. The liquid phase GP 2510 DA or a mixture of SP 2510 DA and SP 2110 (all from Supelco, Bellafonte, Pennsylvania) [73] allow efficient separation of ES, phenylethylmalonediamide, methsuximide, phensuximide, 2-methyl-2-phenylsuccinimide, mephenytoin, PB, CBZ, PM and DPH. The problems of adsorption and resolution can also be reduced by working with a capillary column. The methods of Thoma et al. [73] and Rambeck and Meijer [58] may be used successfully.

The results of external quality control surveys have shown that methods without derivatization are reliable for PB and PM, but are less reliable for DPH and CBZ [14, 26].

Chromatography of phenobarbitone, primidone and phenytoin by pyrolytic alkylation: The main difficulty encountered in analysing underivatized anticonvulsants

by GLC is the interaction between the drugs and the solid phase, liquid phase and column. To decrease this interaction, as well as to increase the volatility of the drugs, the polar group of the barbiturates and hydantoins can be processed by silylation or by alkylation. Silylation of DPH [8] and ethotoin has been proposed. However, this derivatization is not recommended for the analysis of barbiturates or CBZ because of the instability of the silyl derivatives formed. Among the reagents used to alkylate prior to injection ("off-column" alkylation) are diazomethane, dimethyl sulphate, or different alkyl halides in solution in methanol/N,N-dimethylacetamide/tetramethylammonium hydroxide [25]. However, these methods have not been as well developed as the "on-column" alkylation methods.

The formation of methyl esters by pyrolytic methylation ("on-column" methylation, flash-heater methylation) has been applied to a considerable number of substances [32], including the barbiturates and DPH. The first description of a simultaneous analysis of PB, DPH and PM by this technique is that of Kupferberg [38]. The process of this alkylation involves the formation of a quaternary salt of an acidic substance and a quaternary N-alkyl ammonium hydroxide, followed by the formation of a tertiary amine and the alkyl derivative of the acid at a high temperature within the injector block. In the case of barbiturates and hydantoins, the alkylation is done on the nitrogen of the imide groups. The alkyl donor is generally tetramethylammonium hydroxide (TMAH) or trimethylphenylammonium hydroxide (TMPAH) (Methelute, Pierce Eurochemie, Rotterdam, The Netherlands).

The extraction procedures used in pyrolytic alkylation methods include: (a) evaporation to dryness of the extracting solvent, followed by taking up the residue with the quaternary hydroxide [2] or another solvent, which is in turn extracted with the alkylating reagent [31, 52]; (b) washing, a second evaporation to dryness and taking up the residue in the quaternary hydroxide [38, 67]; or (c) re-extraction of the extracting solvent by a small volume of quaternary hydroxide [42]. On the other hand, procedures involving liquid-liquid column extraction have been described. In particular, the automated method of St. Onge et al. [70] should be mentioned.

Certain endogeneous or exogeneous constituents can cause interfering peaks: plasma non-esterified fatty acids, plasticizers from the tube stoppers, caffeine or theophylline eventually present in the plasma sample. Several ways have been suggested to cut out some of these interfering factors. Same authors have recommended purification of the organic extract by re-extraction in NaOH and/or washing with hexane [38, 67]. In practice, to avoid this troublesome step, recourse can be made to the nitrogen selective thermionic detector in place of a flame ionization detector [2].

A third solution has been proposed by MacGee [42] who, profiting from the alkaline nature of the quaternary ammonium hydroxide, has developed a rapid, elegant method of extraction, purification and concentration. Since then, several methods of this type, especially those adapted to the simultaneous analysis of PB, DPH and PM, have been proposed [33, 69].

Pyrolytic methylation does, however, present two disadvantages:

A) The same methyl derivative is obtained starting from a drug and from its N-desmethyl metabolite. This is the case with mephobarbital (N-methyl PB) and PB,

which both methylate to form N,N-dimethyl PB, or with mephenytoin and 5-ethyl-5-phenylhydantoin. In these two cases, the metabolite is active and the parent drug is at a low level in the blood. This minor inconvenience can be easily overcome by replacing the methylation currently used with, for example, ethylation [21, 43].

B) The second disadvantage is coupled with the decomposition of barbiturates in alkaline medium (in the alkylating reagent), in particular at the moment of injection. Thus, pyrolytic methylation of PB produces several other substances in addition to N,N-dimethyl PB, including methyl-phenylbutyramide and dimethyl-phenyl-butyramide [32, 50]. The relative amounts of methylated PB and its degradation products are very variable and depend, amongst other things, on the concentration and nature of the ammonium hydroxide and on the viscosity and pH of the solution injected [31, 50]. To avoid the resulting poor analytical precision different modifications have been tried. Only two have proved to be sufficiently efficient to allow a quantitative methylation of PB, as well as that of DPH, if it is to be assayed simultaneously: injection by means of an automated apparatus [2, 67] or the use of suitable internal standards, i.e. of substances the structure of which is sufficiently alike for degradation to occur in a comparable and proportional way to that of the substance to be analysed [17]. Although no important degradation of PM and DPH has been observed, it is also preferable to use internal standards of similar structure, the extraction and chemical behaviour of which are as close as possible to those of the substances to be determined.

Dudley [17] had already indicated that the most suitable internal standards for PB, DPH and PM determined by pyrolytic methylation are their paramethyl derivatives, i.e. 5-ethyl-5-*p*-tolylbarbituric acid 5-*p*-methylphenyl-5-phenyl-hydantoin and 4-methylprimidone (all from Aldrich Chemical Co., Milwaukee, Wisconsin). It is clear that an internal standard must be added to the sample as early as possible in the procedure.

Chromatography of carbamazepine: CBZ differs from the other anticonvulsants in its tricyclic structure. Its monitoring by GLC poses particular problems: its instability at high temperature and the difficulty in carrying out a specific extraction.

When injected without transformation, CBZ partially decomposes into iminostilbene and 9-methylacridine [32]. The degree of this decomposition depends on the presence of methanol in the solution injected and on the temperature of the injector. It is variable from one injection to another. For this reason, several transformations of CBZ prior to injection have been proposed, i.e. formation of a conjugate with a dimethylformamide/dimethylacetal mixture [46], formation of a derivative with pentafluorobenzamide [66], or formation of silyl derivatives.

On the other hand, the analysis of CBZ can be carried out through the intermediate, iminostilbene. Decomposition of CBZ into iminostilbene is complete if a solution of a quaternary ammonium hydroxide is used during the injection [64]. Analysis of CBZ can thus be integrated in a pyrolytic methylation method. However, iminostilbene itself is in this case partially methylated to form methyl-iminostilbene. As in the case of PB methylation, in order to obtain satisfactory

precision and accuracy, the best solution is to use an appropriate internal standard such as 10-methoxy-carbamazepine or 2-methyl-carbamazepine.

When determining CBZ by thermal decomposition, after extraction into the alkylating reagent it was observed that some of the normal constituents of the biological sample, principally non-esterified fatty acids, interfered with the chromatogram. This interference can be removed by clean-up procedures [64, 67] or, more simply, by use of a thermionic detector [2, 35].

If a simultaneous analysis of CBZ with PB, PM and DPH is to be performed, the methods described by Sichler and Pippenger [67] or by Kumps and Mardens [35] should be referred to. Analyses of 10-11-epoxy-carbamazepine should not be carried out by GLC, because this metabolite decomposes into several substances at high temperature.

Chromatography of valproic acid and ethosuximide: These two anticonvulsive drugs can easily be analysed on a stationary phase such as FFAP or SP 1000 (Supelco, Bellafonte, Pennsylvania). Two types of methods have been described.

The first type consists of an extraction by a solvent (chloroform or dichloromethane) the volume of which is much greater than that of the sample. The extract is evaporated to dryness and the residue is redissolved in the injecting solvent. As VPA and ES are relatively volatile, evaporation at a moderate temperature in the presence of a small volume of isoamyl acetate avoids losses due to evaporation [6]. Although the method to which we refer has been proposed for ES, it can be easily applied to the simultaneous analysis of ES and VPA [39].

The principle of the second group of methods was initially described by van der Kleijn et al. [75] for the analysis of ES, and by Dijkhuis and Vervloet [15] for that of VPA. The serum is extracted by a small volume of chloroform or carbon tetrachloride after addition of $HClO_4$, concentrated H_2SO_4 or a saturated solution of KH_2PO_4. The organic extract is injected directly [15, 36, 75]. Several internal standards can be used: α,α-dimethyl-β-methyl-succinimide or α-methyl-α-propyl-succinimide (Aldrich Chemical Co., Milwaukee, Wisconsin) for ES; nonanoic acid, cyclohexanecarboxylic acid, or 2-methyl-2-ethylcaproic acid (not commercially available) for VPA.

ES has also been analysed simultaneously with PB, DPH and PM after pyrolytic methylation [69].

Chromatography of clonazepam: Determination of CZP, the plasma concentration of which is of the order of 50 ng/ml, requires techniques of high sensitivity and specificity. For this reason, this halogenated benzodiazepine is most often determined with the aid of an electron capture detector. A thermionic detector could, however, be a suitable alternative [13]. Likewise the use of mass spectrometry can perfectly resolve the problems of specificity and sensitivity [22].

As a result of the very low plasma concentration of CZP, it is also necessary to prevent adsorption by silanization of the column and glassware, and to have recourse to a suitable deactivated phase, e.g. SP 2250 (Supelco, Bellafonte, Pennsylvania).

Naestoft and Larsen [48] first described a GLC method for CZP that does not include the formation of a derivative, and has since been modified with the aim of simplifying the extraction procedure [68]. In order to solve the adsorption problem

and also to increase the volatility of CZP and the sensitivity of the assay, several transformations of the drug have been described: acid hydrolysis into a benzophenone derivative [12, 40], or formation of N-methyl [11] or N-ethyl [51] derivatives. The alkylation methods have the advantage of shortening the extraction procedure in comparison with those involving acid hydrolysis.

Gas chromatography coupled with mass-spectrometry: Gas chromatography coupled with mass-spectrometry is the most sensitive and specific method of determining drug concentrations in physiological fluids [22, 23, 74]. This technique, however, needs sophisticated and costly equipment, and so excludes its development in the non-specialized laboratory.

General remarks

A) Solutions of known anticonvulsive drug concentrations prepared in the same biological matrix (e.g. serum) as the samples to be analysed should be used for standardization and internal quality controls. These solutions can be prepared in the laboratory by addition of 0.01–0.02 vol. of an alcoholic or hydro-alcoholic solution of anticonvulsant to 1 vol. of serum previously tested for absence of drugs. Control samples are also commercially available. The standard solutions should always be assayed by comparing with calibration reference samples such as the Standard Reference Material (SRM 900) of the National Bureau of Standards, Washington D.C. These calibration reference sera are supplemented with PB, DPH, PM and ES in three different concentrations. Moreover, the calibration and control samples should cover subtherapeutic, therapeutic and toxic concentrations.

B) In addition to internal quality control, external control is necessary to monitor the analytical quality [2, 56, 62]. Five external quality control programmes have been organized on an international scale for anticonvulsants: British "Heathcontrol" [26], "Antiepileptic Drug Levels Quality Control Program" of the Epilepsy Foundation of America [55], "College of American Pathologists Therapeutic Drug Monitoring Interlaboratory Survey Program", "TDM-Continuing Education and Quality Control Program" of the American Association for Clinical Chemistry, and the programme of the U.S. Department of HEW, Public Health Service.

C) The stability of anticonvulsants in serum or plasma does not pose any problem: conservation is satisfactory over several months at −30° C or for 1 month at 4° C. Blood can be collected in a tube containing anticoagulants (however, anticoagulants other than heparin must be avoided when determining VPA by EMIT). Blood haemolysis only has a small influence on the plasma levels except in the case of drugs with a high protein-bound fraction. DPH, for which the concentration in whole blood represents 60%–75% of that in plasma, is such a drug.

D) Some of the studies comparing results obtained by EIA and chromatographic methods (GLC or HPLC) have shown a significant difference for PB [5, 14, 26, 37, 41, 56] and VPA [34], the former giving lower and the latter higher results by EIA.

However, this difference is not sufficient to modify the pharmacoclinical interpretation of the result. Nevertheless, it is obvious that, outside this purely clinical field, these differences are far from negligible. They deserve fuller attention in external quality control programmes or in pharmacokinetic and pharmacological studies.

E) The delay between drug administration and blood sampling influences the blood levels. This is especially true for PM, VPA and CBZ. It is therefore necessary to know and standardize this delay if the best is to be made of drug level determinations. Sampling before the morning dose is most often recommended. On the other hand, correct interpretation of the blood level for the purpose of therapeutic monitoring can only be carried out if this level has reached the steady state.

F) It is most desirable that a meaningful dialogue take place between the clinician and the clinical chemist. Each laboratory engaged in therapeutic drug monitoring should be in a position to provide practical pharmacoclinical information. The laboratory should be able to assist the physician in serum level interpretation, possess relevant references and participate in a continuing education programme. Furthermore, as emphasized by Pippenger [53]: "One major problem associated with interpreting abnormal levels is the laboratory's lack of information about the patient. I strongly recommend the adoption of a universal requisition form for drug monitoring studies that would contain patient name, age, weight, and sex, and a list of all drugs the patient is receiving, the total daily dose, and time of the last dose".

On the other hand, the physician has to familiarize himself with the concepts of serum level, therapeutic drug monitoring, pharmacokinetics, etc. He also has to know the conditions of validity for correct interpretation of these concepts (time of sampling, etc.), and to keep in mind their relativity (interindividual variations, etc.).

Analysis of the free drug fraction

Drugs are distributed in plasma into two fractions, one bound to the plasma proteins, the other not bound (called "free fraction"). It has been stated that the proportion of the free fraction shows interindividual variations that are not negligible (influence of the level of total proteins, renal or hepatic diseases, drug interactions). As it is admitted that the free drug concentration is more correlated with the therapeutic or toxic effects than the total drug level, it would be desirable to measure this free fraction [10, 19, 28, 62]. All the methods previously cited measure the total drug concentration without differentiating between the two forms. However, the free form can be determined after putting into operation special techniques such as dialysis or ultrafiltration. Unfortunately, these techniques have not been routinely applied so far. More practical techniques of ultrafiltration have recently appeared on the market (Ultrafree, Millipore Corp., Freehold, New Jersey, USA; Centriflo Cones, Amicon Corp., Danvers, Massachusetts, USA).

An interesting alternative lies in determining the salivary concentration [10], which is known to reflect the plasma free form concentration, at least for DPH, CBZ, ES and PB. However, a correction in function of the pH of the saliva is necessary for PB. Measurement of the salivary level, although sometimes desirable, is still not widespread. This can be explained by the risk of contamination of the saliva at the time of oral administration, the difficulties of sampling in very young patients, and the lower reliability of determining salivary levels, which are lower than the plasma levels.

Conclusion

Seen practically, the analyst who wishes to monitor anticonvulsant drugs in physiological fluids has to choose between three basic methods: gas-liquid chromatography, high-performance liquid chromatography and immunological methods. A comparison of the different quality criteria of these methods is shown in Table 1 and allows the analyst to account for the differences between them.

From an analytical point of view, it is our opinion that GLC and HPLC are more advisable, as only chromatographic methods allow for the simultaneous determination of several drugs. They may also afford very good accuracy, precision and sensitivity. The apparently greater sensitivity of the immunological methods (see the sample volume analysed) is compensated by the quantitation limit, which is very high in comparison with GLC and HPLC.

However, other criteria have to be weighed when the practical aspects are considered:

A) The immunological methods have recently been developed because of their practicability. On the other hand, these methods do not seem suitable if the number of analyses is limited to a few determinations per day. The cost of analysis is then very high, and the speed of the determinations is compensated by the time necessary to assay multiple drug samples and/or to establish the standard curve (this could be different on automated apparatus where the calibration curve would remain stable from run to run). If the number of analyses is greater, rapidity, easiness of the work-up (skilled specialists are not required) and automation could be decisive factors in choosing the immunological methods.

B) The rapidity of enzyme-, nephelo-, and fluoro-immunoassays may also be advantageous, i.e. for "bed-side" measurements.

C) The laboratory equipment, the competence of the personnel, the experience acquired from other determinations using similar techniques, and economic considerations are important criteria that must also be taken into account and can be specific to each laboratory.

In terms of the analytical and practical aspects, and based on the results of external quality proficiency surveys, we think that at the present time the following procedures are to be recommended:

A) Phenobarbitone: GLC without derivatization, GLC with pyrolytic methylation, HPLC, EMIT

B) Phenytoin: GLC with pyrolytic methylation
C) Primidone: GLC without derivatization, GLC with pyrolytic methylation, EMIT
D) Carbamazepine: HPLC, GLC with derivatization, EMIT
E) Valproic acid: GLC without the evaporation step
F) Ethosuximide: GLC without the evaporation step

Newer, promising methods (FIA, NIA) have not been included in this list, as they have not yet been sufficiently evaluated.

It must also be remembered that internal and external quality controls are indispensable to ensure the reliability of the results. This analytical monitoring is an essential preliminary to an efficient pharmacoclinical monitoring of drug-treated patients.

Acknowledgements. The author wishes to express his gratitude to Professors A. Lowenthal and Y. Mardens for helpful discussion of this work and for their review of the manuscript. Special thanks are also due to Mrs. S. Fourness for translating the paper and to Miss C. Simons for typing.

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Received May 27, 1982