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Review

Determination of antiepileptic drugs in biological material

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Abstract

Current analytical methodologies applied to the determination of antiepileptic drugs in biological material are reviewed. The role of chromatographic techniques is emphasized. Special attention is focused on new chemical entities as well as current trends such as high-speed liquid chromatographic techniques, hyphenated techniques and electrochromatography techniques. A review with 542 references. © 2002 Published by Elsevier Science B.V.

Keywords: Reviews; Antiepileptic drugs

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1. Introduction

About 3000 years ago in Mesopotamia, a secondary generalized convulsion was fully described in Akkadian, the oldest written language [1]. Cases of epilepsy were described in Egypt [2] and Babylonia [3] more than 2000 years ago. Hippocrates (460–377 B.C.) [4,5] was the first to recognize epilepsy as an organic process of the brain. Epilepsy is the most common serious neurological disorder affecting people of all ages. Anyone can develop epilepsy, it occurs in all ages, races and social classes. Seizures tend to start in infancy or by late adolescence, but the incidences rise again after 65. Statistics showed that epilepsy affects 1% of the world population and that more than a hundred people every day are newly diagnosed as having epilepsy. The cost of epilepsy in the United States has been recently estimated from population-based clinical and survey data [6]. Epilepsy can be treated, but the success depends on many factors: type of epilepsy, accuracy of diagnosis, accuracy of treatment, compliance, associated handicaps and social problems. With appropriate drug treatment, seizures can be completely controlled in about 80% of people. The first drug for the treatment of epilepsy, potassium bromide, was introduced by Locock in 1857 [7]. It remained the sole effective compound for more than 50 years. Phenobarbital, introduced by Hauptmann in 1912 [8], had become the drug of choice in 1918. The discovery of phenytoin in 1938 [9], then primidone in 1952 and ethosuximide in 1960 opened the search for new chemical entities such as carbamazepine (1963), clonazepam (1974), valproic acid (1974), vigabatrin (1989), lamotrigine (1991), gabapentin (1993), topiramate (1995) and tiagabine (1997). Nowadays, as a result of improved understanding of seizure neurochemistry and mechanisms of action of antiepileptic drugs (AEDs), physicians have established therapeutic strategies involving first line and second-line drugs for treatment of epilepsy including special situations such as emergency treatment, other drug treatment, alcohol and pregnancy. The predisposition to seizure intractability and expression of brain neuromolecules consequent to seizures is under genetic control [10]. This discovery may allow a

more rational approach to AED choice. In the future, treatment may be guided by a series of pharmacogenetic tests in order to choose the most appropriate AED and to monitor the antiepileptogenic and the evolution status of the disease [10].

The rationale for the determination of AEDs and their metabolites in body fluids and tissues arises from different fields of investigations. Either drug or metabolite levels are required for regular monitoring of therapeutic drug levels, for adverse drug reaction studies, for drug to drug interaction studies, for issues of toxicity concern, for absorption/distribution/metabolism/excretion (ADME) studies, for pharmacokinetics as well as for pharmacokinetics/pharmacodynamic studies. AEDs are often used in polytherapy including up to three different AEDs, each of them having several metabolites. Hence, the specificity of the analytical techniques to be applied is of paramount importance in therapeutic drug monitoring (TDM).

Hundreds of papers have been published describing the determination of specific AEDs in biological material including the simultaneous determination of miscellaneous AEDs. As an example, literature search (CAS Online) by using keywords ‘‘carbamazepine’’ and ‘‘determination’’ revealed more than 1000 articles dealing with the determination of that specific drug. To our knowledge, since the last comprehensive review on the determination of AEDs in 1985 (291 references [11]) only a few specific reviews have been published having less than 17 references [12–19]. Fazio et al., reported an overview of high-performance liquid chromatographic (HPLC) analysis of AEDs but they restrained the review to both the well known established drugs phenobarbital (**PB**), phenytoin (**PHE**), primidone (**PRM**), ethosuximide (**ESM**), carbamazepine (**CBZ**), valproic acid (**VPA**) and to HPLC methods [20]. Finally, a general review with over 60 references has been recently published on the measurement of the common anticonvulsants and their metabolites in biological fluids [21].

The objective of this paper was to give a comprehensive survey of current techniques applied to the determination of AEDs in biological media in emphasizing the role of chromatographic techniques including the determination of new chemical entities and current trends such as high-speed liquid chromatography, hyphenated and electrochromatography techniques.

The main AEDs currently either commercialized or under clinical evaluation are given in Table 1 with their major active and inactive metabolites. References to comprehensive drug descriptions, PK data, protein binding data and both rationale and normal ranges to therapeutic monitoring are also given.

2. Determination of individual antiepileptic drugs

2.1. GABA uptake inhibitors and antiepileptic drugs structurally or functionally related to GABA

*2.1.1. Vigabatrin (**VGB**)*

VGB, γ-vinyl-γ-aminobutyric acid, is a structural analogue of GABA. It inhibits the enzyme GABA transaminase irreversibility [52]. **VGB** is a chiral molecule commercialized as the racemate, where only the *S*-(+)-enantiomer is pharmacologically active [53]. *R*-(−)-**VGB** does not undergo chiral inversion and does not interfere with the action of *S*-(+)-**VGB** [52]. Literature data suggest a linear correlation with a mean (*R*)/(*S*) ratio of 1.3 [54,55].

Enantioselective methods using HPLC [56] and GC [57] have been published but, until recently, only GC assay methods have been applied to biological fluids involving either MS detection [58] or TID [55]. The GC–TID method is an alternative to the GC–MS method. Both methods require a deproteinization of the plasma followed by both the esterification of the carboxylic acid moiety and the acetylation of the amino functional group. The GC–TID method was used to evaluate steady-state **VGB** enantiomer levels in epileptic patients. The latter assays imply the use of expensive chiral capillary columns and time-consuming sample preparation. A HPLC procedure for the enantioseparation of mixtures of DL-amino acids after converting them to the diastereomeric isoindolyl derivatives using OPA and chiral *N*-acylated cysteines was developed for food matrix [59]. This procedure was recently applied to the separation and quantification of both optical isomers of serum **VGB** [54]. However, (*R*)-(−)-**VGB** is only partly resolved from the next eluting amino acid

Table 1
Major AEDs and their metabolites

Drug and metabolites, Antiepileptic drug Main active metabolite(s) Main inactive metabolite(s)	Chemical properties, Comprehensive drug description	PK data, ADME data, drug interactions, enzyme induction	Protein binding	TDM, Rationale, normal ranges
Acetazolamide	[22–24]	[24]	[24]	
Clobazam	[23,24]	[24,27]	[24,27]	[27]
Desmethyl-clobazam (clophazine)		[24]		
Clonazepam	[23–25]	[24,27]	[24,27]	[24,27]
7-Amino-clonazepam		[24]		
Carbamazepine	[23,24,26]	[24,27,28]	[24,27,156,157]	[24,27,29,30]
Carbamazepine-10,11-epoxide		[110]		[30]
10,11-dihydro-10,11-*trans*-dihydroxy-carbamazepine		[37,110]		
Diazepam	[23,24,31]	[24]	[24]	[24]
Desmethyldiazepam (nordazepam)	[23,24]	[24]	[24]	
Oxazepam	[24,32]	[24]	[24]	
Temazepam	[23,24]	[24]	[24]	
Eterobarb	[23]	[33,279,281]		
N-Monomethoxymethylphenobarbital		[33]		
Phenobarbital	[23,24,41]	[24,27,281]	[24,27]	[24,27,29]
Ethosuximide	[23,24]	[24,27]	[24,27]	[27]
Felbamate	[23]	[27,38,33,34,348]	[27,38,34]	[27,29]
Flunarizine	[23]	[38]	[38]	
Fosphenytoin	[262]	[33]	[33,264]	
Phenytoin	[23,24,42]	[24,27,246,247]	[24,27,28,251]	[24,27,29]
Gabapentin	[23]	[27,28,38,33,34]	[27,38,33,34]	[27,29]
Lamotrigine	[23]	[27,28,38,33,34,346]	[27,38,33,34]	[27,29,33]
Lorazepam	[23,24,35]	[23,24,35]	[24,35]	
Losigamone		[38,327]	[38]	
Levetiracetam (ucb L059)	[27]	[33,97,98]	[97]	
Nitrazepam	[23,24,36]	[23,24]	[23,24]	[24,27]
Oxcarbazepine	[23]	[27,28,37,38,33,34,39]	[27,34]	[27,29]
10-hydroxy carbamazepine (**MHD**)		[37]		
10,11-dihydro-10,11-*trans*-dihydroxy-carbamazepine		[37,110]		
Pheneturide	[23]	[40]		
Phenobarbital	[23,24,41]	[24,27,281]	[24,27]	[24,27,29]
Phenytoin	[23,24,42]	[24,27,246,247]	[24,27,28,251]	[24,27,29]
m-Hydroxyphenyl-5 phenylhydantoin	[24]	[24]		
p-Hydroxyphenyl-5 phenylhydantoin	[24]	[24]		
Piracetam	[23]	[33,84,497]		
Pregabalin	[459]	[459]		
Primidone	[23,24,43]	[24,27]	[24,27]	[24,27,29]
Phenbarbital	[23,24,41]	[24,27,281]	[24,27]	[24,27,29]
Phenylethylmalodiamide	[24]	[24]		
Progabide	[23]	[102]	[103]	
Progabide acid metabolite		[102]	[103]	
Ralitoline	[464]	[38,464]	[38]	
Remacemide	[377]	[38,33,377]		
Desglycinyl-remacemide (FPL 12495)		[377]		
Retigabine	[474]	[475]		
Rufinamide (CGP 33101)	[385]	[385]		
CGP 47292	[385]	[385]		
Stiripentol	[390]	[38,33,390]	[38,33,396]	
Talampanel	[462]	[462]		
Tiagabine	[23,44]	[27,38,33,34,45,46]	[27,33,34]	[27,39]
Topiramate	[23]	[27,28,38,33,34,47–49]	[27,33,34]	[27,29]
Vigabatrin	[23]	[27,28,38,33]	[27,33]	[27,29,50,65]
Valproic acid	[23,24,51]	[24,27,51,401,402]	[24,27,28,444]	[24,27]
Zonisamide	[23]	[27,38,33,195,196]	[27,33]	[27]

Abbreviations: see text.

derivative. The pH of the mobile phase is found to be very critical for the separation of these peaks [54].

Racemic **VGB** is determined in plasma and serum usually by RP-HPLC–FD following pre-column derivatization with reagents such as Dns-Cl [60] and OPA [61–63]. These reagents were primarily applied to the analysis of amino acids. An analogue of **VGB**, γ-phenyl-γ-aminobutyric acid, was used as internal standard. Determination of **VGB** in plasma and in urine by means of a Liquimat II amino analyzer is also reported [64]. The OPA derivatization of samples previously deproteinized with either MeOH or ACN is the method of choice because it allowed the full automation of the derivatization and chromatographic steps as well as the simultaneous determination of **VGB** and **GBP** [62,63,65]. Gradient elution is applied to separate peaks due to **VGB**, **GBP** and γ-phenyl-γ-aminobutyric acid from the large number of peaks originating from endogenous compounds, amino acids and metabolites [62,63]. A decrease in intensity of the fluorescence signal due to the reuptake of oxygen during HPLC separation is reported [62]. The experimental conditions given in Ref. [63] were reported to have some advantages: the peaks of interest are well resolved, no decrease in peak intensity is observed, the serum volume required is only 50 μl and the method can be applied to both serum and urine. However, the necessary run times using gradient elution programs are typically 22 to 35 min. An isocratic elution procedure using a cycloheptane analogue of **GBP** as internal standard (see Section 2.1.2) allows one to reduce the run times down to 10 min for the simultaneous determination of **VGB** and **GBP** in serum [65]. The stability of **VGB** in biological fluids is reported [55,65,150].

*2.1.2. Gabapentin (**GBP**)*

1-(Aminomethyl)cyclohexane acetic acid, **GBP**, is designed to act as a $GABA_A$ receptor agonist that can freely cross the blood–brain barrier [66]. The drug is not metabolized and it shows no appreciable protein-binding [38]. Nowadays, HPLC–FD methods involving an automated pre-column derivatization with OPA are recommended as method of choice for the TDM of **GBP**. These methods allow the automated simultaneous determination of both **GBP** and **VGB** in a single run [62,63,65] (for details see Section 2.1.1). The HPLC–FD assay methods, which are dedicated specifically to the assay of **GBP** alone, involve also the derivatization of **GBP** with OPA [67–69]. However, in one procedure **GBP** is not baseline resolved from a large unknown peak present both in a patient sample and in a drug-free serum spiked with **GBP** but not present in the blank drug-free serum [68]. The investigations performed for the study of potential interference with **GBP** in another procedure [69] are focused on many drugs and endogenous compounds that do not react with OPA. In this case, only a few AEDs are investigated and neither their metabolites nor **VGB** are tested for possible interference. As an alternative to FD, **GBP** is determined in biological fluids by HPLC–UV using 2,4,6-trinitrobenzenesulfonic acid as UV labeling agent [70–72]. The derivatization step is performed on deproteinized serum samples as for the OPA derivatization, but it requires a reaction time of one hour and includes several tedious additional steps. Several factors were found to affect the derivatization yield [72]. The derivatives are separated isocratically in RP mode. GC–FID [73,74] and GC–MS [75] assay methods are also reported. **GBP** is first extracted from serum by SPE on C_{18} DECs and then derivatized with MTBSTFA + 1% *tert*.-butyldimethylsilane prior to GC separation [73]. A tedious sample preparation including a deproteinization step followed by clean-up on ion-exchange resin columns, the evaporation of the eluate, the methylation of the carboxylic moiety with methanolic hydrogen chloride followed by the acetylation of the amine moiety using TFAA, was also applied prior to chromatography [74]. The **GBP** analogue, 1-(aminomethyl)cycloheptane acetic acid [65,70–74], γ-phenyl-γ-aminobutyric acid [63] and Gö-3609 [92] are used as internal standard for **GBP** assays. A CE method is reported as less time-consuming, less labor-intensive and cheaper than the HPLC and GC methods [76]. It involves the derivatization of deproteinized serum with fluorescamine and UV detection at $\lambda = 200$ nm. Some unchanged AEDs such as **CBZ**, **PHT**, **PRM** and **VPA** do not interfere with **GBP**. However, **GBP** is not baseline resolved from an endogenous peak and no internal standard is used. The sensitivities obtained by HPLC and GC methods allow the reliable determination of levels below 0.5 μg/ml while the CE method has a detection limit of

1 μg/ml. The stability of **GBP** in biological fluids has been investigated [65,68,71,150].

*2.1.3. Tiagabine (**TGB**)*

The GABA uptake inhibitor, **TGB**, is a chiral compound originating from the development of nipecotic acid derivatives [77]. Its enantiomeric purity is better than 99% *R*-(−) [44]. HPLC methods have been developed for the separation of **TGB** enantiomers [78,79] but no application to biopharmaceutical analyses has been reported. Sensitive analytical techniques are required for the determination of **TGB** in human plasma because through levels are as low as 17 to 21 ng/ml after multiple oral doses (9 mg/day) [80]. Only a few methods are published about the determination of **TGB** in biological media. RP-HPLC with coulometric detection of plasma samples extracted by SPE on C_8 DECs is reported to give satisfactory results with a quantification limit of 8 ng/ml [81]. However, large negative and positive baseline drifts are observed under the experimental detection conditions. Replacing the EC detection by UV detection at 260 nm also leads to a quantification limit of ca. 8 ng/ml [82]. A GC–MS assay method has been recently proposed as an alternative to HPLC methods for the TDM of **TGB** [83]. It involves the derivatization of **TGB** extracted by LLE to its methyl ester followed by GC–MS (EI 70eV) analysis in SIM mode. The esterification step, which is performed by means of a safe and stable diazomethane derivative, does not require the evaporation of the organic solvent used for extraction. An LOD of 0.5 ng/ml is reported. Stability data of **TGB** in biological fluids are also reported [44,81].

*2.1.4. Piracetam (**PCT**)*

PCT, a cyclic analogue of GABA, was the first commercially available nootropic drug. It has been shown to have beneficial effects in patients with numerous disorders such as memory impairment, alcoholism, senile dementia, Alzheimer's disease and epilepsy. However its mechanism of action is unknown [84]. No metabolites of **PCT** have yet been found [84]. Various analytical approaches including GC, HPLC and CE are described for the determination of **PCT** in biological fluids. The first chromatographic methods reported involve GC [85,86] or HPLC [87–90] techniques. All suffer from strong interferences from biological matrixes [91] and a lack of sensitivity [92]. The sample preparation techniques applied to the extraction of **PCT** from plasma, serum and CRF are mainly deproteinization by means of either perchloric acid [92] or acetone [84,86,91] or ACN [93] as well as LLE [94] and SPE [87]. For urine, LLE is reported to be the extraction method of choice despite an extraction recovery of order of 41% [94]. **PCT** has no strong chromophore. Consequently, UV detection at wavelengths below $\lambda = 210$ nm is applied in most of the HPLC and CE assays. EC detection has been successfully applied for the simultaneous sensitive determination of **PCT** and its hydrolysis products [95]. NPD is applied to GC analysis. In recent papers, either ephedrine [93] or **PCT** analogues [84,91] are used as an internal standard. No internal standard is used in the procedure for determining **PCT** levels in plasma and urine involving the LLE [94]. Typical LODs obtained by means of HPLC methods range from 0.1 [92,95] to 1 μg/ml [84,93,94] while those obtained by GC seem to be similar. In addition to the HPLC–EC assay developed, a photometric method suitable for physicians' laboratories is reported [95]. The specificity of the assay against other AEDs is reported only in one paper [84].

*2.1.5. Levetiracetam (**LVT**)*

(*S*)-α-Ethyl-2-oxo-pyrrolidine acetamide, ucb L059, is the *S*-enantiomer of the ethyl analogue of **PCT** [27]. It probably acts indirectly on the GABA–benzodiazepine–chloride ionophore complex and NMDA receptors [96]. Blood and CSF PK of **LVT** have been studied [97,98]. For monitoring drug levels in human serum, two methods were developed and compared [99]. An SPE procedure allowing a drug recovery exceeding 97%, is followed by either RP-HPLC separation and UV detection or GC–NPD separation using on-column injection on a megabore column. Agreement between both methods was excellent and both methods were found to be suitable for PK studies as well as TDM. Later, an automated HPLC–UV method was reported involving gradient elution [100]. However, the procedure requires a manual LLE involving centrifugation, phase separation and evaporation. No interference from commonly prescribed AEDs is observed. Both serum and heparinized plasma give the same results. Free non-

protein bound serum **LVT** concentrations have been monitored by means of this method following serum ultrafiltration [97]. The method was also applied to the determination of **LVT** in CSF [97]. An enantioselective analysis of **LVT** and its enantiomer (*R*)-α-ethyl-2-oxo-pyrrolidine acetamide using GC and ion trap spectrometric detection has been recently reported [101].

*2.1.6. Progabide (**PGB**)*

PGB is a Schiff's base derived from γ-aminobutyramide and 2-hydroxy-5-fluoro-4′-chlorobenzophenone. It is metabolized to other GABA agonists: **PGB** acid metabolite (**PGBa**), gabamide and GABA. **PGBa** is more potent than the parent compound [102]. **PGB** undergoes rapid hydrolysis to benzophenone in aqueous and in methanolic solutions at acidic pH. The instability of the imine bond presents the main difficulty in the quantitation of the drug. Procedures have been developed to prevent the hydrolysis in blood samples [103], plasma samples [103,104], urine samples [103] as well as during extraction and work-up procedures [103]. However, no serious drug losses occur before or after the initial extraction procedure unless vigorous conditions are employed according to Ref. [106]. The blood/plasma partition ratio of both **PGB** and **PGBa** is reported [103]. Stability data of **PGB** in biological fluids and aqueous solutions are also reported [103,107].

A GC–ECD method has been developed for the PK studies of **PGB** in rhesus monkey [105]. The procedure involves the extraction of **PGB** and an analogue used as internal standard followed by derivatization with HFBA. It was further found to be difficult to apply as analytical method hence unsuitable for the determination of **PGBa** [106]. HPLC methods involving either sensitive amperometric detection [103,104] or UV detection [106–108] were further developed. A good correlation is found between the GC–ECD method and the HPLC method previously developed, which was routinely used for over a year [103]. However, a tedious sample preparation is required. It involves first a LLE, the reduction of the imine bond by means of sodium borohydride followed by a back LLE. In contrary, sample preparation for the other HPLC methods involves a single LLE. The use of toluene for extraction frequently leads to emulsion formation, which affected the extraction recoveries. Sonication is reported to be the most effective way for breaking this emulsion [108]. With the exception of Ref. [107] where NP-HPLC separation is applied, only RP-HPLC separations on C_{18} columns are performed. Thiopental is used as an alternative internal standard [108] instead of miscellaneous **PGB** analogues including SL 79,182 and SL 78,050.

2.2. Iminostilbenes

The iminostilbenes class of AEDs is constituted by the derivatives of carbamazepine (**CBZ**) (Fig. 1). **CBZ** is an established AED widely used alone and as co-medication in the treatment of both partial and

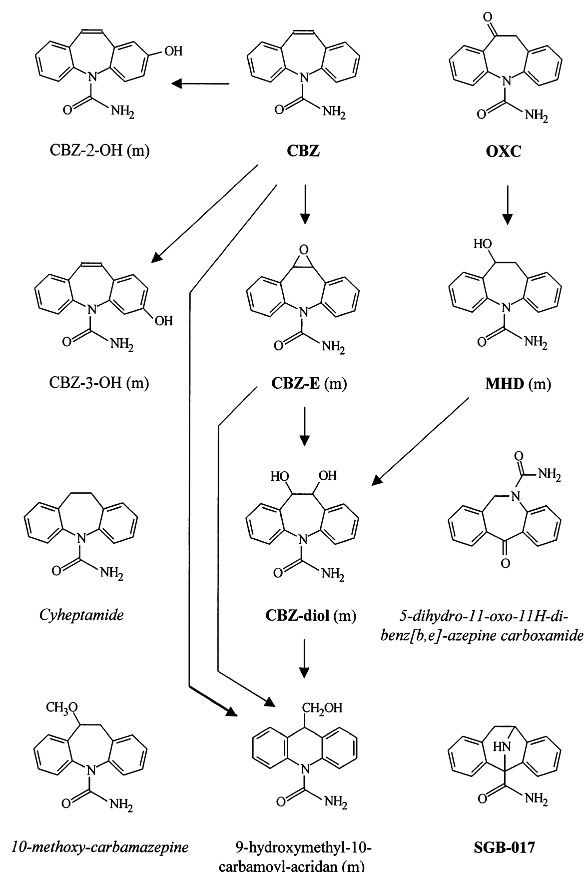

Fig. 1. Chemical structures of AEDs from the iminostilbenes class, of their metabolites (m) and of related compounds used as I.S. (italicized). Metabolic pathways according to Refs. [37,115,116,119].

generalized tonic-clonic seizures [109]. Oxcarbazepine (**OXC**) is the keto-homologue of **CBZ**. The metabolic pathways of both **CBZ** and **OXC** are shown in Fig. 1. Despite their similar structures, **CBZ** and **OXC** have different PK and metabolism [37,110,111]. New iminostilbenes such as SGB-017, (+)-5-aminocarbonyl-10, 11-dihydro-5H-dibenzo[*a*, *d*]-cyclohepten-5,10-imine (Fig. 1), are currently under clinical evaluation [112].

2.2.1. Carbamazepine (CBZ)

The active metabolite of **CBZ**, carbamazepine-10,11-epoxide (**CBZ-E**) is thought to be partially responsible for the side effects of a **CBZ** therapy [113]. Its plasma concentration is about one third of that of **CBZ** in healthy volunteers under steady-state conditions. Under the same conditions, **CBZ-diol**, the inactive *trans*-diol **CBZ** metabolite, can reach concentrations even higher than those of **CBZ-E** [110,114]. A new pharmacologically active metabolite of **CBZ**, 9-hydroxymethyl-10-carbamoyl-acridan has been detected in serum and as glucuronide conjugate in urine (Fig. 1) [115]. The HPLC–ESP-MS identification of these compounds and their corresponding synthetic products as well as an HPLC method for its determination in serum is reported [116]. The detection of 2-hydroxyiminostilbene in the urine of patients taking **CBZ** and its oxidation to a reactive iminoquinone intermediate has been recently investigated [117]. HPLC assays have been also recently reported for a new epoxide-diol metabolite in the plasma of an epileptic child [118] and for **CBZ** phase I metabolites and their glucuronides in urine [119].

Only a few GC studies were performed since the last comprehensive review [11] on the determination of **CBZ** and its two major metabolites, **CBZ-E** and **CBZ-diol** because those can be easily determined by HPLC with UV detection. A GC–FID procedure for the determination of **CBZ** and **CBZ-E** in human plasma is compared with both a HPLC assay method and an enzyme-multiplied immunoassay [120]. **CBZ** levels are found to be in good agreement, but GC seems to have a relatively poor reproducibility.

Numerous conventional RP-HPLC–UV assay methods are reported for the simultaneous determination of **CBZ** and its major metabolites in plasma, serum, human breast milk or urine following either LLE [110,120–129] or SPE [130–132,170] or chemical deproteinization [126,133–137] or column-switching [138,139] or on-line dialysis [140]. The separation of **CBZ** and metabolites on microbore columns was investigated. The column with internal diameters ranging from 2.0 to 2.9 mm are packed with either 5 μm C_{18} particles [141,142] or RP-phenyl 10 μm particles [143]. Many of the reported methods were developed for the determination of **CBZ** and metabolites following either **CBZ** monotherapy or as add on drug. As a consequence, most of those are not suitable for TDM purposes where the assay specificity should be carefully validated. However, some methods allowed the simultaneous detection of **CBZ**, **OXC** and their metabolites [110,125,126]. In the last years, investigations were performed to apply the recent developments made in the field of HPLC stationary phases and columns to the determination of **CBZ** and its metabolites. A new C_{18} column packing with a particle size of 2 μm is reported to give a higher sensitivity and shorter analysis time than the conventional C_{18} column packing [144]. However, the reported run times are comparable to those described in most of the procedures mentioned for the determination of **CBZ** and metabolites following **CBZ** monotherapy. A high-speed HPLC procedure allowing the separation of **CBZ** and **CBZ-E** from nine other AEDs and their metabolites within 2.5 min is reported [145]. The procedure involved a fast chemical deproteinization of the plasma samples and the use of a conventional HPLC system with a short Kovasil RP column packed with monosized 1.5 μm non-porous silicon dioxide microspheres. Silica columns with packing having a semipermeable surface allow the simultaneous determination of **CBZ** and **CBZ-E** following direct injection of 5 μl of human plasma [146,147]. HPLC conditions are also reported allowing the direct injection of rat urine on a regular RP column [148].

Fully automated systems are described for the determination of **CBZ** and **CBZ-E** in plasma. These systems involve either a LLE by means of a robotic system such as the Zymark PyTechnology laboratory automation system [149] or an SPE. In the latter case, the SPE was performed either online with exchangeable C_{18} cartridges by means of a Merck OSP-2 device from Merck [150] or using off-line

SPE on DECs on a Gilson ASPEC device equipped with a Rheodyne type injector connected to the HPLC system [151–154]. The typical analysis throughput of this latter system ranges from 4-6 samples an hour.

Free and total **CBZ** levels have been determined simultaneously by direct injection of rat plasma on an internal-surface RP column and HPFA [155]. Internal-surface RP columns and HPFA are also applied to the study of unbound and total **CBZ** concentrations in protein binding equilibrium [156] and **CBZ** levels in albumin solutions [157]. A conventional RP-HPLC method is also reported for the determination of total and free **CBZ** and the principal metabolites in human serum samples and their ultrafiltrates [158].

A HPLC assay involving isocratic elution and UV detection rather than mass spectrometry is reported for the baseline resolution of serum unlabeled **CBZ** from its heavily deuterated analog, **CBZ**-d_{10}, which is used as stable isotope-labeled tracer for PK studies [179]. A thermospray HPLC–MS method for the separation and quantification of tracer concentrations of isotopically labeled (^{15}N, ^{13}C)-**CBZ-E** in the presence of steady-state levels of **CBZ** and **CBZ-E** is also reported [159]. MLC procedures involving direct injection of physiological fluids have been reported for the determination of **CBZ** levels [160–162] but no further investigations were carried out [163].

CBZ and its metabolites are determined in hair [164–168], brain tissue [169] and saliva [128,170–174]. Possible hair color effects discernible in the recovery of **CBZ** from hair were recently investigated [175]. Fallacious results from measuring salivary **CBZ** concentrations are reported [173].

HPLC with diastereomeric derivatization has been applied to isolate the enantiomers of the metabolite with two asymmetric carbons, **CBZ-diol**, which is excreted in the urine of patients receiving **CBZ** therapy in an enantiomeric excess of ca. 80% [176]. The absolute configurations of the enantiomers are determined by a circular dichroism exciton coupling method to prove the prevalent enantiomer having the (−)-*S*,*S*-form [176]. A trimethylcellulose-type chiral stationary phase was also applied to determine levels of (−)-*S*,*S*-**CBZ-diol** and (+)-*R*,*R*-**CBZ-diol** in serum [177]. The same authors developed a HPLC method for the determination of AEDs and their metabolites including chiral compounds. However, this method is not suitable for the chiral separation of **MHD** enantiomers in serum [178].

When an internal standard was required by the procedure, **CBZ** analogues such as cyheptamide (Fig. 1) [142], 2-methyl-carbamazepine [123], 5-dihydro-11-oxo-11H-dibenz[*b*,*e*]-azepine-5-carboxamide (Fig. 1) [110,152–154], 10,11-dihydrocarbamazepine [179], carbamazepine-10-hydroxide [124] and 10-methoxy-carbamazepine (Fig. 1) [173] are used. However, chemicals from other chemical classes such as 1,3-dimethyl-7-benzylxanthine [120], 5-ethyl-5-*p*-tolyl-barbituric acid [116,144,164], 5-(*p*-methylphenyl)-5-phenylhydantoin [147,137], phenacetin [135], cyclopal [126], halodiph [141], clonazepam [127] and nitrazepam [128,133] are also reported to be suitable. Procedures requiring the use of two internal standards, e.g., lorazepam plus nordazepam [143] are also recommended.

2.2.2. Oxcarbazepine (OXC)

The clinical pharmacology and PK of **OXC** have been reviewed [37]. In contrast to the oxidative metabolism of **CBZ**, **OXC** is rapidly reduced by cytosolic enzymes to its pharmacologically active monohydroxylated derivative **MHD** (see Fig. 1) in man. **MHD** predominates in blood after oral dosing whereas **OXC** reaches only low levels. A major route for elimination is via the glucuronide conjugate with a minor amount oxidized to the pharmacologically inactive **CBZ-*trans*-diol**. Formation of **MHD** is stereoselective, with the two optical isomers formed in the ratio of 80% (*S*)-+ to 20% (*R*)-− [180]. Plasma concentration–time profiles reflect this asymmetric reduction, although time to peak concentration and elimination half-lives are similar [180].

The stereoselective determination of the **MHD** enantiomers has been described [180,181]. Later, separation on a Chiracel OD column coupled on-line with a Chiracel ODH column has been reported for the simultaneous determination of **OXC** and the enantiomers of its metabolites **MHD** and **CBZ-*trans*-diol** [182] or only its metabolites [183].

In addition to HPLC assay methods developed for the specific determination of **OXC** alone [185] or

OXC and its metabolite [186–191], several HPLC methods have been developed for the simultaneous determination of **OXC**, **CBZ** and their metabolites in serum or plasma [110,125,126]. A narrow-bore liquid chromatographic assay using **CBZ** as internal standard is reported to be sufficiently sensitive to allow quantification of **OXC** and **MHD** in rat brain, liver and blood microdialysates [192]. Further developments led to the development of a liquid chromatographic method using a microcolumn (800 μm I.D.) coupled to a U-shaped optical cell for high-sensitivity UV absorbance detection. This system was applied to the determination of **OXC** and **MHD** in rat blood and hippocampus microdialysates [193].

OXC analogues such as 9-hydroxy-methyl-10-carbamoylacridane [125,126], 5-dihydro-11-oxo-11H-dibenz[*b*,*e*]-azepine-5-carboxamide [110,189], **CBZ** [192], **CBZ-E** [186,187,193] are used as internal standard depending on the applications.

GC–MS methods have been proposed for the determination of either **CBZ-*trans*-diol** [194] or **OXC** and **MHD** in human plasma and urine [194]. Thermal decomposition to the substituted iminostilbene derivatives occurred only to the extent of a few percent under the conditions described. The reported methods allowed the study of the kinetics of ^{15}N-labeled **CBZ-diol** in patients and volunteers receiving ^{15}N-**CBZ**.

Plasma **OXC** and its metabolites were found to be stable at −20°C for at least 1 month [187], 3 months [188] and 12 months [150].

2.3. Sulfonamides

2.3.1. Zonisamide (ZNS)

1,2-Benzisoxazole-3-methanesulfonamide, **ZNS**, is an antiepileptic drug recently developed in Japan. The clinical pharmacology and the PK of **ZNS** have been reviewed [27,33,195,196]. **ZNS** concentrations in erythrocytes were found to be 4–9 times higher than those found in plasma [33]. After oral administration, **ZNS** is excreted in urine partly as unchanged drug, *N*-acetyl-**ZNS** and as a glucuronide conjugate of a metabolite with an open isoxazole ring [197,198]. Rat urinary metabolites of **ZNS** have been identified by means of thin-layer chromatography–matrix-assisted-secondary-ion mass spectrometry [199]. The chromatographic methods commonly used for monitoring **ZNS** in serum involve GC [200,201], HPLC with UV detection at wavelengths ranging from 205 to 280 nm [202–206] and MECC [207]. An EIA was developed. However, some disadvantages are reported [204,207]. The recent HPLC methods are considered to be simpler, more rapid and more sensitive than the GC methods previously described. Chemical deproteinisation with ACN [207] or MeOH [208] and LLE with either dichloroethane [202] or ethyl acetate [203] is applied for the sample preparation. Off-line and on-line SPE are also described [204,205,209]. The use of disposable C_{18} cartridges allows the monitoring of **ZNS** in serum volumes as low as 20 μl with a detection limit of 0.1 μg/ml [204]. Ten serum samples can be prepared within 15 min and the described procedure can be easily automated as no evaporation of the eluates was required. A rapid routine method using a non-porous silica column has been recently reported to be accurate and to improve solvent consumption [206]. As a rule, isocratic elution is applied and is reported to have a suitable specificity against possible co-medication. However, Juergens considered that isocratic separations were not suitable for studying patients undergoing multi-drug treatment. Consequently, he developed a procedure involving a gradient elution for the separation and the quantification of **ZNS** together with nine other AEDs and their metabolites in 12 min [203]. Shibata et al. reported the co-elution of **ESM** and **ZNS** applying their procedure [208]. Conventional HPLC is also reported to be suitable for the determination of **ZNS** levels in human breast milk [205]. According to Ref. [207], MECC overcomes the troublesome maintenance of the HPLC systems, the use of expensive organic solvent and the problems of environmental concern. However, the procedure described requires a classical LLE with ethyl acetate followed by solvent evaporation to dryness. The detection limit found is 3.0 μg/ml. The correlation of **ZNS** levels assayed by means of MECC and HPLC is reported to be excellent ($r = 0.981$, $n = 25$), although the levels obtained with MECC were slightly lower than those found with HPLC (slope = 0.869). The stability of **ZNS** in calibration standards and in reconstituted quality control sera kept at 4°C is poor [202].

*2.3.2. Acetazolamide (**AZD**)*

Carbonic anhydrase inhibitors, such as the sulfonamide drug **AZD**, formerly used as diuretics, play a role in the treatment of epilepsy, glaucoma and altitude sickness. Many analytical methods developed for **AZD** are consequently focused on forensic medicine as well as on the screening of banned drugs in sports since diuretics promote the excretion of bodily fluids and may mask the use of anabolic steroids and other drugs. The analytical methods developed are summarized in Table 2.

2.4. Succinimides

Ethosuximide is the most effective and representative AED of this chemical class among several others, e.g., methsuximide and phensuximide evalu-

Table 2
Analytical methods for the determination of **AZD** in biological material

Technique	Medium	Application	Remarks	Ref.
CZE–UV	Urine, serum	Screening		[210]
GC–ECD	Whole blood, plasma, saliva	Assay		[211]
GC–ECD	Urine	Screening	Extractive alkylation	[212]
GC–FID	Serum	Assay	Clinical application to some cases of multiple AED administration	[226]
GC–MS	Blood, saliva	Assay		[213]
GC–MS	Urine	Screening	LLE, three different derivatization (methylation) procedures compared	[214]
GC–MS	Hair	Screening	SPE	[218]
GC–MS	Urine, serum	Screening		[210]
GC–MS	Urine	Screening	Use of a macroreticular acrylic copolymer for the efficient removal of the co-extracted phase-transfer reagent after derivatization by direct alkylation	[215]
GC–MS	Urine	Screening		[233]
GC–MS	Urine	Screening	Derivatization by direct alkylation	[216]
GC–MS	Urine	Screening	Study SPE versus LLE	[220]
HPFA	Albumin solutions	Assay	Determination of the free drug in protein binding equilibrium	[217]
HPLC–DAD	Hair	Screening	SPE	[218]
HPLC–DAD	Urine	Screening	Column-switching	[219]
HPLC–DAD	Urine	Screening	Study SPE versus LLE	[220]
HPLC–UV	Urine	Assay	Column-switching	[221]
HPLC–UV	Urine	Assay		[222]
HPLC–UV	Plasma, saliva	Assay	SPE. The method was suitable to determine **AZD** in plasma from pedriatric patients	[223]
HPLC–UV	Plasma	Assay	Protein precipitation with ACN	[224]
HPLC–UV	Whole blood, plasma, urine	Assay	LLE	[225]
HPLC–UV	Serum	Assay	Clinical application to some cases of multiple AED administration	[226]
HPLC–UV	Serum, plasma vitreous humors	Assay		[227]
HPLC–UV	Serum	Assay	LLE	[228]
HPLC–UV	Serum	Assay	Application to nine less common AEDs	[229]
HPLC–UV	Urine	Screening	LLE	[230]
HPLC–UV	Urine, plasma	Screening	Column-switching	[231]
HPLC–UV	Urine	Screening	Micellar liquid chromatography	[232]
HPLC–UV	Urine	Screening		[233]
HPLC–UV	Urine	Screening	Micellar liquid chromatography	[234]
Differential pulse polarography	Serum	Assay	LLE	[235]
Enzymatic assay	Serum	Assay		[184]

Abbreviations: see text.

ated for their anticonvulsant properties since the 1960s [236].

2.4.1. Ethosuximide (ESM)

Many papers have been reviewed in the last comprehensive review on AEDs published in 1984 [11]. Only one GC method [237] and a few HPLC methods [238–240] as well as a few immunoassays [241–243] have been published since that time. Possible losses of **ESM** during the LLE steps of sample preparation are described. These losses are due to the high solubility of **ESM** in water as well as its volatility [244]. The sensitive determination of **ESM** is performed by HPLC after tagging with suitable reagents because **ESM** has no strong chromophore nor fluorophore. BrMmC and the alkylsulfonate derivative, 2-(2-naphthoxy)ethyl 2-[1-(4-benzyl)piperazyl]ethane sulfonate are reported to be suitable reagents [239,240] for UV detection. However, the sample preparation remains laborious. In addition to the procedures mentioned, **ESM** is included in many chromatographic procedures dedicated to the simultaneous determination of AEDs in biological fluids (see Section 3). As a rule, the TDM of unchanged **ESM** is performed nowadays by means of automated analyzers that offer a high degree of automation and high throughputs [245]. These systems, which require low sample volumes, are mainly based on FPIA and EMIT techniques.

2.5. Hydantoins

Phenytoin, which was discovered in 1938 [9], is the AED of interest in the class of hydantoins. The determination of markedly less used AEDs from this class of compounds such as mephenytoin, methoin and ethoin have been previously reviewed [11]. With the exception of the PHT prodrug, fosphenytoin, no extensive development has been done for this class of compounds since that time.

2.5.1. Phenytoin (PHT)

PHT is one of the most widely used drugs for the treatment of epilepsy, hence extensively studied. It is metabolized by cytochrome P-450 (CYP) 2C9/10 producing various metabolites including ***p*-HPPH**, the major metabolite and ***m*-HPPH** [246,247]. Total serum concentrations of **PHT** correlate with the therapeutic efficacy as well as toxicity [248,249]. Consequently, the therapeutic range is well established for **PHT** [27]. Routine monitoring of **PHT** serum concentrations is performed in order to maintain therapeutic efficacy, i.e., seizure control while minimizing such dose-related toxicities as ataxia, nystagmus, blurred vision and drowsiness [250]. It is, however, the free unbound **PHT** moiety that is responsible for both the activity and toxicity of the agent [250]. The determination of free **PHT** concentrations can be of clinical importance when a reduction in binding takes place under special or pathological conditions because **PHT** is highly bound to proteins [251].

As the anticonvulsant properties of **PHT** were discovered in the 1940s, the determination of **PHT** and its metabolites in biological fluid by means of chromatographic techniques including GC, GC–MS and HPLC has been covered in the last reviews [11,20]. Nowadays, the routine monitoring of unchanged **PHT** concentrations is performed mainly by means of analyzers that offer a high degree of automation and high throughputs [245]. These systems, which require low sample volumes, are mainly based on FPIA and EMIT techniques. However, chromatographic methods such as HPLC remain the method of choice in the following cases: Simultaneous determination of **PHT** and **PHT** metabolites or other AEDs in various biological media including plasma from carbon tetrachloride intoxicated rats [252], equine plasma [253], human urine and plasma or serum [254–256], human brain [255,257], human hair [164]. A GC–MS method for the analysis of **PHT** and [$^{13}C_3$]**PHT** from plasma obtained from pulse dose PK studies has been recently reported [258]. Comparative studies are reported [135,259]. The application of internal surface columns and direct injection as an alternative to ultrafiltration coupled to FPIA for the determination of free **PHT** has been investigated. Both methods yield similar levels for the free **PHT** [250]. The comparison of total and free **PHT** serum concentrations measured by HPLC and a standard TDx assay and their implications for the prediction of free **PHT** serum concentration from the total **PHT** concentration are reported [259]. **PHT** and its metabolites *S*- and *R*-***p*-HPPH** are selectively determined among several AEDs via β-cyclodextrin inclusion complexes by a

HPLC method involving column-switching technique [178]. A HPLC assay involving isocratic elution and UV detection rather than mass spectrometry has been reported for the baseline resolution of serum unlabeled **PHT** from its heavily deuterated analog, **PHT**-d_{10}, which was used as stable isotope-labeled tracer for PK studies [179]. An assay method has been reported for the simultaneous determination of **PHT** and **CBZ** by HPLC in human hair for testing compliance of patients during therapy [164]. A kind of protease, biopurase, is useful for the digestion of hair and recovery of **PHT** and **PB** [260]. MLC procedures involving direct injection of physiological fluids are reported for the determination of **PHT** levels [160,161] but no further investigations were carried out [163]. MECC was recently reported to be suitable for the determination of **PHT** sodium and **PB** sodium in blood [261].

2.5.2. Fosphenytoin (FOS)

FOS, the disodium phosphate ester of 3-hydroxymethyl-5,5-diphenylhydantoin, is a newly developed prodrug for the parenteral administration of **PHT** [262]. Because of the structural similarity of **PHT** and **FOS**, significant cross-reactivity occurs in the classical immunoassays commonly used for the **PHT** level measurements [263,264]. This cross-reactivity, which is not linear, produces falsely elevated **PHT** levels when measured in presence of **FOS**. For this reason, a RP-HPLC method with UV detection has been developed for the simultaneous determination of **FOS** and **PHT** in both human plasma and plasma ultrafiltrates [263]. The method is suitable for both patient monitoring and PK studies. The study of protein binding of **FOS** in uremic sera has been reported [264].

2.6. Barbiturates and deoxybarbiturates

The AED class of barbiturates and deoxybarbiturates includes phenobarbital (**PB**), primidone (**PRM**), eterobarb (**ETB**) and other molecules such as barbexalal, methabarbital, methylphenylbarbital. Since the last comprehensive review on the determination of this class of AEDs in biological material [11], no extensive investigations have been carried out. A few papers report the HPLC determination of the decarboxylation product of **PB**, pheneturide (**PTD**) in human serum. Those cover the racemic **PTD** [122] and the **PTD** enantiomers [265].

2.6.1. Phenobarbital (PB)

PB, the eldest modern AED discovered in 1912 [8] and launched as AED in 1918, is still of widespread use. The major metabolites are *N*-glucopyranosylphenobarbital, *p*-hydroxyphenobarbital and its glucuronide conjugate as well as two dihydrodiol compounds and hydroxymethylphenylbarbituric acid [24]. The determination of **PB** in biological material by means of GC and HPLC has been previously reviewed [11]. No extensive analytical development has been reported since that time but, due to its widespread use, **PB** is included in most of the methods reported for the simultaneous determination of AEDs in biological fluids (see Section 3). The simultaneous determination of methylphenobarbital enantiomers and **PB** in human plasma by on-line coupling of an achiral pre-column to a chiral HPLC colunm has been described [266].

The application of HPLC–UV methods for the determination of **PB** and its metabolite *p*-hydroxyphenobarbital [267,268] suggested that glucuronide conjugation in rat and humans are different. The TDM of unchanged **PB** is performed mainly now by means of high throughput automated analyzers [245]. These systems, which require low sample volumes, are mainly based on FPIA and EMIT techniques. MLC procedures involving direct injection of physiological fluids have been reported for the determination of **PB** levels [160,161] but no further investigations were carried out [163].

The determination of **PB** and other AEDs in hair for the purpose of forensic interests was recently reviewed [269,270]. **PB** was determined in human hair by GC–MS after SPE [271,272]. **PB** in hair was found to give good information over a long period, especially when blood collection has not been made [271]. A kind of protease, biopurase, was found to be useful for the digestion of hair and recovery of **PB** and **PHT** [273]. The effect of pigmentation on pigmented and nonpigmented rat hair on **PB** concentrations was studied [274]. The determination of **PB** concentration in rat offsprings by HPLC has been recently reported [275]. A RP-HPLC procedure has been recently reported for the determination of **PB** and **CBZ** using **ETB** as internal standard [276].

MECC was recently reported to be suitable for the determination of **PB** sodium and **PHT** sodium in blood [261].

The stability of **PB** in urine as well as its postmortem stability in blood and tissues is reported [277,278].

2.6.2. Eterobarb (**ETB**)

N,*N*′-Dimethoxymethylphenobarbital, **ETB**, is a barbiturate that does not enter the brain, but is rapidly *N*-dealkylated to monomethoxymethylphenobarbital (**NMMP**) which is slowly dealkylated to **PB** [33,279]. As a consequence, **ETB** can be considered as a **PB** prodrug [280]. Therefore, no extensive assay method development has been undertaken. A RP-HPLC–UV assay method with isocratic elution is reported for the determination of **ETB**, **NMMP** and **PB** in human serum [281]. The procedure requires a deproteinisation step by means of perchloric acid followed by a LLE with dichloromethane. Another RP-HPLC–UV procedure, which involved a gradient elution is also reported for the determination of **ETB** and its metabolites in human plasma [282]. In addition, analytical methods based on stable isotope labeling in conjunction with GC–MS were also developed for the determination of **ETB**, **NMMP** and **PB** [283]. Deuterium-labeled analogs of the three compounds are synthesized for use as internal standards in measuring the concentrations of the unlabeled drug and metabolites in biological fluids. The methods were applied to the study of the plasma and brain levels of the three compounds in rat after i.v. administration and in man after oral doses. As expected, the GC–MS assay methods were found to be noticeably more sensitive than the HPLC–UV methods.

2.6.3. Primidone (**PRM**)

Like **PB**, **PRM** is an old AED that is still used worldwide in epilepsy therapy. It is rapidly metabolized to the active metabolite phenylethylmalondiamide (**PEMA**) but slowly metabolized to **PB**. The metabolite *p*-hydroxyphenobarbital has been identified in urine [284]. Nowadays, the TDM of unchanged **PRM** is performed mainly by means of high throughput automated analyzers [245]. These systems, which require low sample volumes, are mainly based on FPIA and EMIT techniques. **PB** TDM is often performed to monitor **PRM** therapy because **PB** is supposed to have most of the activity of **PRM**. Chromatographic methods involving GC and HPLC are nevertheless of importance when both the unchanged drug and its metabolites have to be quantified [11,285–287] in plasma or when the simultaneous determination of several AEDs is required (see Section 3). A HPLC–UV procedure involving a SPE was reported to be suitable for the determination of **PRM** and its three metabolites in rat urine [268].

2.7. Oxazolidinediones

Several oxazolidinediones including ethadione, troxidone and paramethadione were investigated. However, no extensive analytical investigations have been published.

2.8. Benzodiazepines

Benzodiazepines such as clobazam (**CBM**), clonazepam (**CNZ**), diazepam (**DZM**), lorazepam (**LRZ**) and nitrazepam (**NTZ**) are used either as first-line or as second-line AEDs. General reviews are published on the determination of this class of drugs in biological samples as well as in the field of forensic and clinical toxicology [269,270,485]. The biological materials included hair [288], liver tissues [290], erythrocytes [290], saliva [289,290], sweat [289], further blood, plasma, serum and urine [290,291]. A series of papers appeared on different aspects for the determination of benzodiazepines in biological fluids: the role of SPE [292], micro-SPE [293]; the application of HPLC column-switching techniques [294–296] and of porous microspherical silica gel HPLC columns [297]; HPLC simultaneous gradient analysis [298]; HPLC–DAD [299,300]; dual GC–NPD–ECD detection [301,302]; positive and negative ion MS [303]; HPLC–MS [304,305] and CE [306]. Methods dedicated to the simultaneous determination of the mentioned benzodiazepines in human serum were reported [307–310].

Serum **CNZ** was shown to be very sensitive to both sun and artificial light [311].

2.9. Miscellaneous classes

2.9.1. Lamotrigine (LTG)

LTG belongs to the class of phenyltriazines. As a consequence, **LTG** is structurally unrelated to any currently marketed AED and has specific features for chromatographic separations. **LTG** is a basic compound with a pK_a value of 5.5 [341]. On the other hand, it has strong chromophores responsible of the two absorption maxima at 200 and 313 nm. **LTG** is metabolized and excreted mainly by glucuronidation [312]. HPLC–MS equipped with a TSP interface has been reported to be a powerful tool in elucidating a number of urinary **LTG** metabolites [313] but a conventional approach has also been reported [314]. Therapeutic concentrations are not yet well established [27].

In Table 3 are summarized analytical methods proposed for the determination of **LTG** in biological fluids. Those include HPLC, CE, GC methods and immunoassays. HPLC separations on RP columns in isocratic mode are widely applied but NP columns have been also used [320,346]. Miscellaneous sample preparations as fast and simple deproteinization using ACN, solvent-demixing extraction, LLE with either EA or chloroform or chloroform–isopropanol mixtures in basic media, SPE on either C_8 or C_{18} DECs, dialysis as well as combinations of those extraction techniques give satisfactory results. However, the LLE extraction yields ranged from ≤60% [328] to 100% [334]. SPE of **LTG** was extensively investigated on several DEC types [338]. Serum should not be obtained from blood collected with phase separating gels because the barrier gel can retain **LTG**, resulting in a falsely low concentration for the specimen [330]. Commercial drug-free serum products were not recommended because they often contain contaminants not present in human serum and because of the lack of essential normal constituents such as lipids and proteins [330]. Abnormal serum matrix effects were investigated using lipemic, hemolyzed and sera with high bilirubin concentrations [325]. Saliva was sampled after stimulation of salivary flow by chewing paraffin wax [346]. The study of interindividual correlation between serum and saliva **LTG** levels is reported [315]. Setting of UV detector to a wavelength of about 310 nm allows a high specificity of detection because all other AEDs and their metabolites are UV-transparent at this wavelength with very few exceptions. As a consequence, short run times can be easily obtained on regular columns. In a typical example, **LTG** can be selectively and accurately determined among other AEDs including **CBZ**, **CBZ-E**, **CNZ**, **ESM**, **PB**, **PHT**, **PRM**, **VPA** as well as caffeine, theophylline and digoxin within 2 min [150]. The serum samples from an external quality assessment scheme [316] were chemically deproteinized with ACN and then separated onto a regular C_{18} column (125 mm× 4.0 mm I.D). An automated HPLC system combining sequential trace enrichment and gradient HPLC has been described. CE has been applied to the determination of **LTG** in serum. The method is faster than the HPLC method selected for comparison. However, the electropherograms exhibit strongly disturbed baselines and only **PHT** was investigated for the specificity of the assay. A GC–NPD assay method requiring no derivatization has been shown to be an alternative to HPLC methods despite the fact that the required run times are longer [326]. **LTG** has been shown not to be prone to thermal degradation [326]. GC–MS has been recently shown to be an alternative to HPLC for the TDM of **LTG** but the method requires a tedious derivatization step of extracts obtained by LLE and a relatively long run time [327]. As for the HPLC methods, the GC–NPD method can be integrated in a comprehensive toxicology screening. Although the chemical structure of **LTG** allows the preparation of suitable haptens, immunoassay development remains still limited. A major difficulty in developing RIA for **LTG** comes from the high concentrations found in some clinical samples, which may require several dilution–assay cycles [345]. In addition, short turnaround times are prevented by the necessary incubation time of 16 to 24 h.

Stability data of **LTG** in biological fluids are reported [150,317,323,329,331,335,339,519].

*2.9.2. Felbamate (**FBM**)*

The role of felbamate, 2-phenyl-l,3-propanediol dicarbamate, in the treatment of epilepsy has been recently reviewed [347]. **FBM** is extensively metabolized in the liver via hydroxylation and conjugation. Three inactive major metabolites have been identified: 2-(4-hydroxy-phenyl)-1,3-propanediol di-

Table 3
Analytical methods for the determination of **LTG** in biological material

Medium	Volume (µl)	Sample preparation	Technique	Column	Detection	LOD/LOQ (µg/ml)	Specificity against	Internal standard	Comments	Year	Ref.
Human blood	1000	LLE	HPLC	RP, C_{18}	UV 306 nm	0.008/0.03	Frequently coadministered AEDs	BW725C78	Linear range 0.1 to 15 µg/ml	2001	[317]
Rat brain	Whole brain	Deproteinization +LLE	HPLC	RP, C_{18}	UV 306 nm	0.02/0.08	Frequently coadministered AEDs	BW725C78	Linear range 0.1 to 5 µg/ml	2001	[317]
Human serum and saliva	200	LLE	HPLC	C_8	ND	ND/0.1	ND	ND	All analyses in duplicate	2000	[315]
Human serum	500	SPE	HPLC	RP, C_{18}	UV 210 nm	ND/0.2	Frequently coadministered AEDs	Butalbital	Column temperature 40°C	2000	[318]
Human plasma	1000	LLE	HPLC	RP, C_{18}	UV 306 nm	0.2/0.6	**CBZ**, **CBZ-E**, **PB**, **PHT**, **PRM**	BW725C		2000	[319]
Human serum	300	LLE	HPLC	NP, silica	UV 280 nm	ND/0.35	**CBZ**, **PB**, **PHT**	Protriptyline		1999	[320]
Human plasma		SPE	HPLC	RP, C_{18} endcapped	UV 310 nm	0.05	Cimetidine, rifampicin			1999	[321]
Human plasma	200	Deproteinization	HPLC	RP, C_8	UV 310 nm	0.6/1.0	**CBZ**, **CBZ-E**, **PHT**, **VGB**, **GBP**, **VPA**, **ESM**, **PB**, ***p*-HPPH**	Diamino-methoxypheny-triazine		1999	[322]
Human plasma/serum		Deproteinization	HPLC	RP, C_{18}	UV 313/210 nm	ND/0.10	**CBZ**, **CBZ-E**, **CBZ-Diol**, **OXC**, **MHD**, **ESM**, **PB**, **PHT**, ***m*-HPPH**, ***p*-HPPH**, **PRM**, **PEMA**, **FBM**, **RCM**, **VPA**	Ethyl-tolyl-barbituric acid	Chromatographic run time <2 min	1998	[150]
Human plasma/urine	500	Deproteinization	HPLC	RP, C_{18}	UV 310 nm	0.03/ND	ND	None	**FBM** was reported to reduce **LTG** recovery, stability data reported	1998	[323]
Human plasma	250	LLE	HPLC	NP, silica	UV 280 nm	0.1/0.5	**CBZ**, **CBZ-E**, **ESM**, **PB**, **PHT** **PRM**, **VPA**		Extraction recoveries ≤75%	1997	[324]
Human serum	500	LLE	HPLC	RP, C_{18}	UV 306 nm	0.5/ND	**CBZ**, **CBZ-E**, **ESM**, **PB**, **PHT** **PRM**, **VPA**	Thiopental	Experimental conditions suitable for concomitant barbiturates assay	1997	[325]
Human serum	1000	LLE	GC	Capillary, HP-5	NPD	ND/0.15	**PB**, **PRM**, **PHT**, **VPA**	Prazepam	No derivatization step	1997	[326]
Human serum	500	LLE+ derivatization	GC	Capillary, methylsilicone	MS (EI 70eV) SIM	0.25/0.5	**CBZ**, **PB**, **PHT**, **VPA**	Oxazepam-d_5	Derivatization required	1997	[327]
Human plasma	200	LLE+ back LLE	HPLC	RP, Diphenyl	UV 265 nm	0.1/0.2	ND	Diamino-methoxypheny-triazine	Extraction recoveries about 60%	1997	[328]
Human plasma dialysates	550	Dialysis on ASTED	HPLC	RP, C_8	UV 270/215 nm	ND/0.04	Metabolites 166C89 and 583C80	None	Automated system with gradient eleution	1997	[329]

Table 3. Continued

Medium	Volume (μl)	Sample preparation	Technique	Column	Detection	LOD/LOQ (μg/ml)	Specificity against	Internal standard	Comments	Year	Ref.
Human serum	200	SPE, disk cartridges	HPLC	NP, CN	UV 214 nm	ND/0.15	**CBZ**, **CBZ-E**, **MHD**, **PHT**, ***p*-HPPH**, **ESM**, **FBM**, **GBP**, **PB**, **PEMA**, **PRM**, **VPA**, **DZM**, **CNZ**, **NTZ**, oxazepam	Cyheptamine	Simultaneous quantification of **CBZ**, **CBZ-E**, **MHD**, **PHT**, ***p*-HPPH**. The mobile phase was recycled for 3 weeks. Column temperature 50°C.	1997	[330]
Human plasma	250	LLE	HPLC	NP, silica	UV 240 nm	0.1/0.4	**CBZ**, **CBZ-E**, **OXC**, **MHD**, **ESM**, **PB**, **PEMA**, ***p*-HPPH**, **PHT**, **PRM**, **VPA**, **VGB**	Nortriptyline	**CBZ-diol** not resolved completely from **LTG**. This interference was considered to be negligible at therapeutic levels.	1997	[331]
Human plasma	1000	Deproteinization	TLC	Silica	UV 306 nm	ND	**CBZ**, **CBZ-E**, **PB**, **PHT**, **CBM**, **CNZ**	Diamino-methoxyphenyl-triazine	The procedure was compared with a HPLC procedure. It was also applied to the determination of **LTG** in tablets.	1996	[332]
Human plasma	1000	Deproteinization	HPLC	RP, C_{18}	UV 306 nm	ND	**CBZ**, **CBZ-E**, **PB**, **PHT**, **CBM**, **CNZ**	Diamino-methoxyphenyl-triazine	The procedure was compared with a TLC procedure. Method also applied to the determination of **LTG** in tablets.	1996	[332]
Human serum	50	Deproteinization	CE	Capillary	UV 214 nm	0.3/0.5	**PHT** and other non AEDs	Tyramine	The method was compared to HPLC	1996	[333]
Human plasma	200	LLE	HPLC	RP, C_{18}	UV 270 nm	0.02/0.1	**CBZ**, **VGB**, **VPA**, **PB**, **PHT**, **ESM**, **NTZ**, **CNZz**	None	Pediatric samples	1996	[334]
Human serum	200	LLE	HPLC	RP,C_8	UV 306 nm	ND	**PHT**, **PB**, **ESM**, **VPA**, **CBZ**, **CBZ-E**	BW 430C78		1995	[335]
Human serum	100	SPE, DECs	HPLC	RP, C_{18}	UV 265 nm	ND/0.2	**ESM**, **PRM**, **CBZ**, **CBZ-E**, **PB**, **ZNS**, **PHT**, **CNZ**	Acetanilide		1995	[336]
Human plasma	100	LLE	HPLC	RP, CN	UV 305 nm	ND	**CBZ**, **ESM**, **PB**, **PHT**, **PRM**, **VPA**	Diamino-methoxyphenyl-triazine	Chromatographic run time <5 min	1995	[337]
Human serum/urine	40/100	Deproteinization +SPE, DECs	HPLC	RP, C_8	UV 306 nm	ND/0.09	ND	Lamotrigine analog	Method also applied to the determination of **LTG** in tablets	1995	[338]
Human serum	300	Deproteinization	HPLC	RP, C_{18}	Dual 220/310 nm	0.2/ND	**PRM**, **OXC**, **VAL**, **ESM**	Hexobarbital		1994	[339]
Serum	500	LLE	HPLC	RP, C_{18}	UV 280 nm	0.1/ND	**CBZ**, **ESM**, **PRM**, **PB**, **PHT**, methsuximide	Oxprenolol	Chromatographic run time <5 min	1994	[340]
Human plasma	100	LLE	HPLC	NP, silica 35°C	UV 313 nm	0.5/ND	**VAL**, **PRM**, **PHT**, **PB**, **ESM**, **CBZ**, **CBZ-E**, **CNZ**, **DZM**, **NTZ**	BW A725C78	Retention times <3 min	1992	[341]
Guinea pig blood/urine	200 to 1000	Solvent-demixing extraction	HPLC	RP, CN	UV 280 nm	0.055/ND	**PB**, **PHT**, **CBZ**, **VAL**, **CNZ**	A725C		1991	[342]
Guinea pig blood/urine	250 (b) 100 (u)	Deproteinization +SPE, DECs (b) or LLE (u)	HPLC	RP, C_8 40°C	UV 277 nm	0.05 (**LTG**) 0.02 (metabolites)	**ESM**, **PB**, **PHT**, **CBZ**, **FBM** and their metabolites, benzodiazepines	BW A725C	Ion pair reagent, simultaneous determination of 2-*N*-glucuronide metabolite, mean recoveries about 70%	1991	[343]
Human plasma	50	Incubation 1 h	Immunofluorometric assay	n/a	Fluorescence	ND	**LTG** metabolites, **PHT**, **PB**, **CBZ**	n/a	Concentration range not declared, correlation with an HPLC assay method reported	1991	[344]
Human plasma	20	Dilution	RIA	n/a	Gamma counter	0.02	**PB**, **PHT**, **VPA**, **CBZ**, **DZP**, **CNZ**, nine **LTG** metabolites	n/a	Inter-assay accuracy >117% at 0.5 and 5.0 μg/ml levels, incubation time 16 to 24 h	1990	[345]
Plasma, urine	200	LLE	HPLC	NP, silica	UV 306 nm	0.5/ND	No specificity study reported	Diamino-methoxyphenyl-triazine	Method applied for the PK study of **LTG** in normal volunteers	1987	[346]

Abbreviations: met. = metabolite; ND = not declared; other abbreviations, see text.

Table 4
Analytical methods for the determination of **FBM** in biological material

Technique	Detection	Analyte	Medium	Volume (μl)	Sample preparation	Separation	Specificity	I.S.	LOD (μg/ml)	Year	Ref.	Remarks
HPLC	UV 210 nm	**FBM**	Pediatric human serum/plasma	100	Deproteinization with ACN	Gradient elution on a short C_{18} RP column	No interference with **CBZ**, **CBZ-E**, **CNZ**, **ESM**, **PB**, **PHT**, **PRM**, **VPA**	2-Methyl-2-phenyl-1,3-propanediol dicarbamate	ND	1997	[350]	
HPLC	UV 214 nm	**FBM**	Human plasma	200	Deproteinization with ACN	Isocratic elution on a C_{18} RP column	No interference with **CBZ**, **CBZ-E**, **CNZ**, **ESM**, **PB**, **PHT**, **PRM**, **VPA**, **PB** coeluted with I.S.	2-Methyl-2-phenyl-1,3-propanediol dicarbamate	10	1996	[351]	Totally automated system for direct-sample analysis of **FBM**
GC	FID	**FBM**	Human serum	500	LLE with dichloromethane	Isothermal, capillary column	No interference observed with 60 commonly encountered drugs	2-Methyl-2-phenyl-1,3-propanediol dicarbamate	<2	1995	[352]	Thermal degradation of carbamates was minimized and reproducible, peak identity was confirmed by GC–MS
HPLC	UV 254 nm	**FBM**	Human serum	500	Either LLE using dichloromethane or SPE on C_{18} disposable cartridges	Isocratic elution on a C_{18} RP column	No interference observed with 13 commonly encountered drugs	2-Methyl-2-phenyl-1,3-propanediol dicarbamate	5	1995	[353]	The method was compared to the GC assay method reported by the same authors in the same paper
GC	NPD	**FBM**	Human serum	500	Either LLE using dichloromethane or SPE on C_{18} disposable cartridges	Capillary column	No interference observed with 13 commonly encountered drugs	2-methyl-2-phenyl-1,3-propanediol dicarbamate	5	1995	[353]	The method was compared to the HPLC assay method reported by the same authors in the same paper
HPLC	UV 205 nm	**FBM**	Human serum	100	Deproteinization with ACN	Isocratic elution on a C_{18} RP column	No interference observed with 17 commonly encountered drugs	Acetoacetanilide	3	1994	[354]	The method was compared to the MECC assay method reported by the same authors in the same paper
MECC	UV 214 nm	**FBM**	Human serum	<<0.1	Direct injection	Capillary column, 16.5 kV	No interference observed with 17 commonly encountered drugs	Acetoacetanilide	4	1994	[354]	The method was compared to the HPLC assay method reported by the same authors in the same paper
GC	FID	**FBM**	Pediatric human serum/plasma	100	LLE with dichloromethane	Wide-bore capillary column	No interference observed with 18 AEDs and eight commonly encountered drugs	5-Methylphenyl-5-phenyl-phenylhydantoin	5	1994	[349]	The stability of **FBM** at 4, −20 and −78°C was investigated

Table 4. Continued

Technique	Detection	Analyte	Medium	Volume (μl)	Sample preparation	Separation	Specificity	I.S.	LOD (μg/ml)	Year	Ref.	Remarks
HPLC	UV 210 nm	**FBM**	Human serum	50	LLE with dichloromethane	Isocratic elution on a C_8 RP column	No interference observed with 33 commonly encountered drugs	Alphenal	ND	1994	[355]	
HPLC	UV 210 nm	**FBM**	Human plasma	200	LLE with dichloromethane	Isocratic elution on a C_{18} RP column	No interference observed with several AEDs with exception of **PRM** used as I.S.	**PRM**	0.1	1994	[356]	Robotic sample preparation by means of a Zymate II robot
HPLC	UV 210 nm	**FBM** and three AEDs and their metabolites	Human plasma	100	LLE with EA MTBE	Isocratic elution at 40–50°C on a C_{18} RP column	No interference observed with several AEDs	Two internal standards	0.4	1994	[357]	The method has been applied to thousands of patient samples
HPLC	UV 210 nm	**FBM** and three metabolites	Human plasma	100	LLE with chloroform–MTBE	Isocratic elution C_{18} RP column	Some interfering compounds identified	2-Methyl-2-phenyl-1,3-propanediol dicarbamate	0.8	1994	[358]	
HPLC	UV 210 nm	**FBM** and three metabolites	Brain and heart tissues of rats	–	Homogenates	Isocratic elution at 50°C on a C_{18} RP column	–	2-Methyl-2-phenyl-1,3-propanediol dicarbamate	0.2 (homogenate)	1993	[359]	The method was applied to the determination of **FBM** and its metabolites in surgical human brain samples
HPLC	UV 210 nm	**FBM** and three metabolites	Rat and dog plasma	100	LLE with chloroform–MTBE	Isocratic elution at 40°C on a C_{18} RP column	Endogenous plasma constituents might interfere	2-Methyl-2-phenyl-1,3-propanediol dicarbamate	0.2	1993	[360]	
HPLC	UV 210 nm	**FBM**	Dog plasma	100	Deproteinization with ACN	Isocratic elution on a C_{18} RP column	–	2-Methyl-2-phenyl-1,3-propanediol dicarbamate	0.15	1992	[361]	The method has been applied to 2700 samples
HPLC	UV 215 nm	**FBM** plus **CBZ**, **PHT** and their metabolites	Human plasma	500	LLE with dichloromethane–EA	Isocratic elution on a C_8 RP column	–	2-Methyl-2-phenyl-1,3-propanediol dicarbamate	0.5	1990	[362]	

Abbreviations: ND = not declared; other abbreviations, see text.

Table 5
Analytical methods for the simultaneous determination of AEDs in a single run

Technique			Sample		Analytes																	Year	Remarks	Ref.
Detection	Column	Elution	Medium, volume	Sample preparation	I.S.	**CBZ**	**CBZ-E**	**MHD**	**VPA**	**LTG**	**PHT**	***p*-HPPH**	***m*-HPPH**	**PRM**	**PB**	**PEMA**	**ESM**	**ZNS**	**PTD**	**FBM**	Other AEDs			
GC methods																								
FID	OV-17	T-program	Plasma, 500 μl	LLE in diethylether, flash methylation, packed column	Heptabarbitone +cyclohexane carboxylic acid +***p*-MPPH**	x			x		x			x	x		x		x		x	1977		[499]
MS	Cross-linked methyl-silicone	T-program	Urine, 10 ml	Acidic hydrolysis, LLE and acetylation	None	x					x			x	x		x				x	1990	General screening procedure with 40 reference MS spectra	[500]
NP-HPLC methods																								
UV $\lambda=214$ nm	CN	Isocratic, 50°C	Serum, 200 μl	SPE C_8	Cyheptamide	x	x			x	x											1997	Comparison study showing accuracy problems related with **LTG** testing	[330]
RP-HPLC methods																								
UV $\lambda=210$ nm	RP, C_{18}	Isocratic	Plasma, 500 μl	Ultrafiltration	None	x	x				x			x	x							1985	Comparison to immunoassay methods	[501]
UV $\lambda=200$ nm	RP, C_{18}	Isocratic, ion pairs	Plasma 500 μl	LLE with EA	Heptabarbital	x					x			x	x		x		x			1980		[502]
UV $\lambda=200$ nm	RP, C_{18}	Isocratic 60°C	Serum, 10–100 μl	Automated column-switching, direct injection	None	x	x	x			x			x	x	x	x					1984	Extensive investigations on column switching parameters	[503]
UV $\lambda=207$ nm	RP, C_8	Gradient, 35°C	Serum, 500 μl	LLE with EA	Hexobarbital	x	x	x			x			x	x	x	x		x		x	1984		[504]
UV $\lambda=205$ nm	RP, C_{18}	Gradient, 70°C	Serum, 50 μl	Column-switching, direct injection	None	x	x	x			x			x	x	x	x				x	1984	Comparison with GC and HPLC following LLE	[505]
UV $\lambda=204$ nm	RP, C_8	Isocratic	Plasma, 500 μl	MeOH precipitation and LLE	Hexobarbitone	x					x			x	x		x				x	1984		[506]
UV $\lambda=214$ nm	RP, C_{18}	Isocratic, 68°C	Plasma, 400 μl	Automated on-line LLE	Hexobarbital	x	x				x			x	x							1984		[507]
UV $\lambda=200$ nm	RP, C_{18}	Isocratic	Plasma, 500 μl	ACN precipitation		x			x		x			x	x							1985		[508]
UV $\lambda=210$ nm	RP, C_{18}		Serum ultrafiltrates, 20 μl	Direct injection	None	x	x				x			x	x							1985		[509]

Table 5. Continued

Technique			Sample		Analytes																	Year	Remarks	Ref.
Detection	Column	Elution	Medium, volume	Sample preparation	I.S.	**CBZ**	**CBZ-E**	**MHD**	**VPA**	**LTG**	**PHT**	***p*-HPPH**	***m*-HPPH**	**PRM**	**PB**	**PEMA**	**ESM**	**ZNS**	**PTD**	**FBM**	Other AEDs			
UV $\lambda=195$ nm	RP, C_{18}	Isocratic	Brain tissue, 300 mg	Dichloromethane extraction	***p*-MPPH**	x					x				x		x					1985		[169]
UV $\lambda=214$ nm	RP, C_{18}	Isocratic	Serum	LLE		x					x			x	x							1985		[121]
UV $\lambda=210$ nm	RP, C_{18}	Isocratic, 40°C	Serum, 100 µl	LLE	***p*-MPPH**	x	x	x			x			x	x						x	1986		[510]
UV $\lambda=204$ nm	RP, C_{18}	Isocratic, 60°C	Serum, 1 µl	Direct injection	None	x	x				x			x	x	x	x				x	1986	Column-switching	[511]
UV $\lambda=214$ nm	RP, C_{18}	Isocratic, 40°C	Serum or plasma, 50 µl	ACN precipitation	***p*-MPPH**	x					x	x			x							1987		[137]
UV $\lambda=205$ nm	RP, C_{18}	Gradient, 60°C	Serum, 500 µl	LLE	Ethyl-tolyl-barbituric acid + methyl propylsuccinimide	x	x	x			x			x	x	x	x	x			x	1987		[203]
UV $\lambda=214$ nm	RP, C_{18}	Isocratic, 40°C	Serum, 50 µl	ACN precipitation	***p*-MPPH**	x					x	x			x							1987		[137]
UV $\lambda=207$ nm	RP, C_{18}	Gradient, 65°C	Brain tissue, 20–100 mg	Acetone extraction and SPE	Ethyl-tolyl-barbituric acid	x	x				x				x						x	1987		[257]
UV $\lambda=208$ nm	RP, C_8	Isocratic	Plasma, 100 µl	LLE	Tolylpheno-barbital	x	x				x			x	x		x				x	1989	Bronchodilators, and hypnotic agents were simultaneously separated	[512]
UV $\lambda=215$ nm	RP, C_{18}	Isocratic	Saliva 100 µl			x	x				x				x							1989		[513]
UV $\lambda=215$ nm	RP, C_8	Isocratic, 40°C	Serum, 500 µl	LLE	10,11-Dihydro-**CBZ** and 2-methyl-2-phenyl-1,3-propanediol dicarbamate + ETLB + cyheptamide	x	x	x			x	x									x	1990		[362]
UV $\lambda=254$ nm	RP, C_{18}	Isocratic	Plasma	ACN precipitation and LLE	Cyclopal	x					x				x							1990		[126]
UV $\lambda=210$ nm	ISRP	Isocratic	Serum	Direct injection	***p*-MPPH**	x					x				x							1990	Comparison to EMIT	[147]
UV $\lambda=205$ nm	CN	Isocratic	Plasma, 200 µl	LLE	4-Methoxy-2-nitroaniline	x				x	x				x						1992		[514]	

Table 5. Continued

Technique			Sample		Analytes																	Year	Remarks	Ref.
Detection	Column	Elution	Medium, volume	Sample preparation	I.S.	**CBZ**	**CBZ-E**	**MHD**	**VPA**	**LTG**	**PHT**	***p*-HPPH**	***m*-HPPH**	**PRM**	**PB**	**PEMA**	**ESM**	**ZNS**	**PTD**	**FBM**	Other AEDs			
UV $\lambda=200$ nm	RP, C_{18}	Isocratic, column 270 mm ×2 mm I.D. containing 3 µm particles, 40°C	Serum, urine, 100 µl Serum ultrafiltrates, 100 µl Saliva, 250 µl	ACN precipitation for plasma and saliva, SPE for urine	**NTZ**	x	x	x			x			x	x						x	1993	DAD at 200, 215, 254, and 285 nm	[515]
UV $\lambda=195$ to 210 nm	RP, C_{18}	Gradient	Plasma, serum, 200 µl	ACN precipitation and derivatization with 4-bromophencyl bromide	Nonanoic acid	x			x					x	x						x	1993		[516]
UV $\lambda=240$ nm	Silica	Isocratic	Serum, 5 µl	Direct injection	None	x					x				x							1993	Syringe-type minicolumn Extrashot-Silica injection system	[517]
UV dual wavelength $\lambda=220$ and 310 nm		Isocratic	Serum 300 µl	ACN precipitation	Hexobarbital	x				x	x											1994		[339]
UV $\lambda=210$ nm	RP, C_{18}	Isocratic, 40–50°C	Plasma 100 µl	LLE	10,11-Dihydro-**CBZ** and 2-methyl-2-phenyl-1,3-propanediol dicarbamate	x	x	x			x	x		x	x					x		1994		[357]
UV $\lambda=210$ nm	RP, C_{18}	Isocratic, 26°C	Serum, 500 µl	SPE	Allobarbital	x	x	x			x				x						x	1994	Separation of chiral compounds via cyclodextrin inclusion complexes by a column switching chromatographic technique	[178]
																						1996		[140]
UV $\lambda=220$ nm	RP, C_{18}	Isocratic	Serum	ACN precipitation	Phenacetin	x					x				x							1996	Comparison to FPIA	[135]
UV $\lambda=214$ nm	CN	Isocratic	Plasma, 200 µl	SPE disk C_8	Cyheptamide	x	x			x	x											1997	Investigations on separation of AEDs on C_{18}, C_8 and CN columns, mobile phase recycling	[330]
UV $\lambda=210$ nm	RP, C_{18}	Isocratic	Serum, 20 µl	Direct injection	None	x									x						x	1997	Syringe-type minicolumn Extrashot-ODS injection system	[518]

Table 5. Continued

Technique			Sample		Analytes																	Year	Remarks	Ref.
Detection	Column	Elution	Medium, volume	Sample preparation	I.S.	**CBZ**	**CBZ-E**	**MHD**	**VPA**	**LTG**	**PHT**	***p*-HPPH**	***m*-HPPH**	**PRM**	**PB**	**PEMA**	**ESM**	**ZNS**	**PTD**	**FBM**	Other AEDs			
UV $\lambda = 254$ nm	RP, C_8	Isocratic	Plasma	LLE	Asarone	x					x				x						x	1999		[524]
UV $\lambda = 220$ nm	RP, C_{18}	Isocratic	Plasma, 100 μl	LLE	9-Hydroxy-methyl-10-carbamyl acridan	x	x	x		x	x			x	x		x					1999		[519]
HPTLC methods																								
UV TLC scanner $\lambda = 210$ nm	Silica plates		Serum,	SPE	Hexobarbital	x	x				x			x	x	x	x				x	1984		[520]
MECC methods																								
On-column UV $\lambda = 210$ nm	Fused-silica capillary	Constant voltage 30 kV 30°C	Plasma, 100 μl	LLE	Hexobarbital	x			x		x			x	x		x					1992		[521]
On-column UV	Fused-silica capillary	Constant voltage 20 kV	Serum, saliva, few nl	Direct injection	None	x					x				x							1996		[522]
On-column UV $\lambda = 220$ nm and 245 nm	Fused-silica capillary	Constant voltage 20 kV, 35°C	Filtered serum, few nl	Direct injection	None									x	x		x					1997	Comparison with FPIA and HPLC	[523]

Abbreviations: see text.

carbamate (*p*OHF), 2-hydroxy-2-phenyl-1,3-propanediol dicarbamate (2OHF) and 2-phenyl-1,3-propanediol monocarbamate (MCF) [348]. Many analytical methods were developed for the determination of **FBM** levels and its metabolites in serum, plasma, urine and tissues from animals and humans. These developments were driven by the unexpected development of aplastic anaemia related to **FBM**, by the possible **FBM**-associated hepatic failure as well as by significant PK interactions with **PHT**, **CBZ** and **VPA** [33]. Chromatographic techniques such as HPLC, GC, CE are suitable for measuring **FBM** levels in the presence of other AEDs and **FBM** metabolites. Those are summarized in Table 4.

Plasma **FBM** was found to be stable for 1 month at 4°C, −20°C and −78°C [349]. Despite this observation, Behnke and Narahari Reddy [350] recommend to store both plasma and serum samples at 4°C no longer than 1 day. An interfering peak was reported when blood specimens were collected in red-top Vacutainer tubes and analyzed according to the procedure given in Ref. [349]. Nevertheless, this problem might be alleviated by modifying the recommended chromatographic parameters.

*2.9.3. Losigamone (**LSG**)*

[(±)-5(*R*,*S*)-α(*S*,*R*)-5-(2-Chlorophenylhydroxymethyl)-4-methoxy-2(5H)-furanone, **LSG**, is a tetronic acid derivative. It is structurally related to a family of naturally occurring substances found in *Piper fadyenii* and *Piper sanctum* [363]. **LSG** exhibits anticonvulsant activity in various in vivo animal seizure models and in vitro epileptiform models [364]. Promising results were obtained in pilot clinical studies [365]. **LSG** is a racemic mixture of two enantiomers. Those were shown to possess different metabolic profiles in human hepatic microsomes [366] and to exhibit enantiomer–enantiomer interaction under the in vitro human metabolic conditions [367]. Since **LSG** is a new candidate drug, only a few analytical methods have been reported. An RP-HPLC procedure is described for the determination of plasma racemic **LSG** followed by separation of the enantiomers on a chiral colunm [368]. A simple enantioselective HPLC assay is reported to be suitable for PK studies in epileptic patients [367]. There was no evidence of metabolites in non-hydrolyzed patient samples. However, after enzymatic hydrolysis, small amounts of both enantiomers could be quantitated [367].

The stability of **LSG** in plasma has been studied [367].

*2.9.4. Topiramate (**TPM**)*

The sulfamate-substituted monosaccharide **TPM** is structurally distinct from other AEDs. It is currently under clinical investigation for other indications than epilepsy. Six trace metabolites of **TPM** have been isolated from human plasma, urine and feces. They were characterized and identified [49,369,370]. GC methods and a FPIA assay have been developed because **TPM** is a UV-transparent compound. The GC methods involve either an SPE followed by GC separation on a capillary column with FID [371] or a double LLE of plasma followed by GC separation on a capillary column with NPD [370,372,373]. The GC methods have been successfully applied in preclinical studies in mice, rats and dogs as well as in PK studies and in several clinical trials. Sample aliquots as small as 50 μl have been analyzed with a suitable sensitivity. An FPIA procedure has been developed for the TDM of **TPM** [374]. The cross-reactivity of the inactive metabolite 9-hydroxytopiramate is ≤15% in the concentration range 4 to 32 μg/ml. Correlation between GC and FPIA techniques was reported by data determined on patient samples [370,374]. The sensitivity of all techniques is suitable for the determination of plasma or serum **TPM** below the μg/ml level. A procedure for the determination of **TPM** in rat brain fluid by means of in vivo microdialysis and HPLC–TSP-MS has been reported [375]. The saturable distribution between erythrocytes and plasma of whole blood using a GC assay method has been recently evaluated [376].

Plasma **TPM** is reported to be stable for 24 h at 2–8°C [374].

*2.9.5. Remacemide (**RCM**)*

(±)-2-Amino-*N*-(1-methyl-1,2-diphenylethyl)-acetamide hydrochloride, FPL 12924AA, **RCM**, can be considered as a prodrug. Its major metabolite, the deglycinated metabolite, FPL 12495 (±), **desRCM**, exhibits a greater efficacy than the parent compound in animal seizure models [377]. **desRCM** has been found in the CSF of dogs and rats, as well as human, dog and rat plasma [377]. Eight other metabolites

found in human and dog urine were also identified and evaluated for potential pharmacological effects [377]. Furthermore, the *S*-(−) stereoisomers of **RCM** and **desRCM** were found to be more potent that the *R*-(+) stereoisomers [378,379].

No stereoselective method has been reported but sensitive and selective methods have been developed for the quantification of racemic **RCM** in rat and dog plasma and urine [380]. The methods employed LLE (urine) or ion-exchange SPE (plasma) followed by HPLC with UV detection. The detection limits for both methods is 10 ng/ml. The plasma method was also applied to measurement of in vitro plasma protein binding of **RCM** in rat, dog and human plasma. A similar HPLC–UV procedure allows the simultaneous determination of **desRCM** [381]. It involves also an ion-exchange SPE. Recently, a HPLC procedure for the simultaneous determination of **RCM** hydrochloride and **desRCM** in brain tissue was reported [382].

When stored at −20°C, **RCM** and **desRCM** were shown to be stable in human plasma for at least 1 year [381].

2.9.6. Rufinamide (RFM)

RFM, CGP 33101 is currently under clinical evaluation for use as AED. It is structurally unrelated to any currently marketed anticonvulsant. Unchanged **RFM** is the major compound in plasma, whereas only traces of the parent compound are recovered in urine. The main metabolite in urine is a carboxylic acid derivative, CGP 47292 [385]. Procedures have been described for the HPLC determination of **RFM** in plasma and for the simultaneous determination of **RFM** and CGP 47292 in urine. The procedures involve an LLE [383] or an automated SPE using either a Zymate II laboratory automation system [384] or a Gilson ASPEC device [385]. The method involving the ASPEC system has been applied to epileptic patients treated with various AEDs including commonly prescribed AEDs and benzodiazepines [385]. In plasma, no interference from the AEDs and their metabolites was found. However, **PB** interfered with the internal standard.

2.9.7. Stiripentol (STP)

(1-[3,4-Methylenedioxyphenyl]-4,4-dimethyl-1-penten-ol), **STP**, is an AED selected from a series of aromatic allylic alcohols having anticonvulsant and hypnotic activity in rodent screens [386]. A notable structural feature of **STP** is the presence of a chiral center at C-3. The stereoselective synthesis of **STP** enantiomers is reported. It includes [3-^{2}H]**STP** and [3-^{18}O]**STP** [387] as well as ^{14}C and ^{3}H labeling of **STP** [388]. The optical purity of **STP** enantiomers was determined by HPLC analysis of their diastereomeric carbamate derivatives formed by reaction with naphthyl isocyanate [389]. The chemical and pharmacological properties as well as PK and toxicology in humans and laboratory animals has been reviewed [390]. The comparative anticonvulsant potency and PK of the *R*(+)- and *S*(−)-enantiomers of **STP** was reported later [389]. Recently, the metabolic chiral inversion of **STP** in rat has been extensively investigated [387,391]. A chiral NP-HPLC method with fluorescence detection was first developed by Zhang et al. [392] for the determination of **STP** enantiomers and then slightly modified later [393]. The procedures require the LLE of **STP** enantiomers from either plasma or brain homogenates by using piperonyl alcohol as internal standard. The extraction is followed by the reaction with phenyl isocyanate to form carbamate derivatives. The separation of the latter derivatives is performed on a Pirkle-type HPLC column containing a *N*-(3,5-dinitrobenzoyl) phenylglycine covalently linked to ψ-aminopropyl silica gel as stationary phase.

Non enantioselective RP-HPLC–UV methods have been also developed for the determination of racemic **STP** in plasma [394–396] and in brain tissues of rats [395,397]. Three metabolites derived from the cleavage of the methylenedioxyphenyl moiety were also analyzed in urine samples by RP-HPLC–UV [396]. The protein binding of **STP** was studied by means of HPLC and by equilibrium dialysis combined with HPLC [396]. Adsorption of **STP** at glass surfaces is reported [393].

Preliminary investigations indicated that **STP** is stable in the frozen state [396].

2.9.8. Valproic acid (VPA) and derivatives

2-Propyl-valeric acid, **VPA**, and its sodium salt, are widely used in the treatment of a variety of seizure types as major AEDs [398,399]. Its amide derivative 2-propyl-valeramide, valpromide (**VPD**) is

also used as anticonvulsant. Valdice, a new diethylcarbonate prodrug of **VPA**, has been recently investigated in humans and dogs. In both species valdice was biotransformed to **VPA**. No valdice was detected in the plasma after oral administration [400].

VPA, a short-chain fatty acid, is metabolized in the body by a combination of mitochondrial, microsomal and cytosolic enzymes to produce at least 20 known metabolites [401,402]. Studies in animals indicate that several of the metabolites contribute to both the therapeutic and toxic effects of the drug. Thus, the unsaturated metabolite 2-ene-**VPA** [403] and 2,3′-diene-**VPA** [404] were found to have significant anticonvulsant activity in rodent models. Other unsaturated metabolites, namely 4-ene-**VPA** and 2,4-diene-**VPA**, are hepatotoxic in the rat [405] and are thought to be responsible for the rare but fatal hepatotoxicity associated with the drug [402]. Metabolic profiling of **VPA** by cDNA-expressed human cytochrome P450 enzymes using GC–MS (NCI) has been recently reported [406]. GC–MS is considered as the analytical method of choice for the simultaneous determination of **VPA** and its metabolites and for metabolism studies. Procedures have been reported allowing the detection of **VPA** and five [407], seven [408], eight [409], 13 [410], 14 [411,412], and 20 metabolites [402]. One of these papers reports the use of an ion trap detector [407]. GC–MS has been shown to be suitable for the study of 2-*n*-propyl-4-pentenoic acid, a putative toxic metabolite of **VPA**, in the perfused rat liver [409] and in the rhesus monkey [402]. Evidence for the formation of chemically reactive intermediates generated by some enzyme systems was demonstrated by the authors. The formation of glutathione, glutathione–glucuronide diconjugates and *N*-acetylcysteine adducts of reactive intermediates derived from **VPA**, which are thought to be responsible for **VPA** hepatotoxicity, have been studied in rat and humans by GC–MS [413] and by LC–MS–MS [413,414]. The separation of the stereoisomers of the **VPA** metabolites 2-*n*-propyl-2-pentenoic acid and 2-*n*-propyl-3-pentenoic acid is reported [407]. GC methods with either MS or FID were obviously widely used for the determination of unchanged **VPA** only or **VPA** plus some unsaturated metabolites or **VPA** in the presence of **FBM** [415]. In serum or plasma, **VPA** and metabolites are analyzed as follows: underivatized [416]; as methyl esters [407]; as methyl esters after extractive methylation [417]; as MTBSTFA derivatives [408,410,418]; as TMS derivatives [419,420]; as a combination of TMS and pentafluorobenzyl derivatives [411,421]; as phenacyl derivatives [422,423] and finally as underivatized drug after SPE [424]. Isotopic dilution GC–MS has been found to be suitable for serum **VPA** [425]. The synthesis of **VPA**-d_{14} is reported [421]. The phenacyl ester derivatives obtained with phenacyl bromide is reported to be suitable for both GC and HPLC–FD analysis. 6,7-Methylenedioxy-1-methyl-2-oxo-1,2-dihydroquinoxaline-3-yl-propionohydrazide was recently found to be suitable for the simple and sensitive HPLC–FD determination of **VPA** in human serum [426]. A solvent flush GC method was reported allowing the assay of sample volumes as low as 1 μl of plasma [427]. In this paper, the plasma is ''sandwiched'' by phosphoric acid phases and directly injected into the GC system. The other biological materials analyzed include: maternal milk [428,429]; rhesus monkey plasma [402]; whole blood [430]; saliva [418]; brain [431]; urine [411]; tears [421]. The adaptation of packed column GC methods used for plasma **VPA** to capillary columns has been reviewed [432]. GC procedures were also reported for the simultaneous determination of **VPA** and VPD in plasma [433,434].

Despite the wide use of FPIA methods in the field of clinical chemistry and of reported GC methods, HPLC assays have been developed. **VPA** has no strong chromophore. However, methods with UV detection at low wavelengths of underivatized **VPA** are reported to be suitable for the determination of therapeutic concentrations of **VPA**, i.e., 40 to 100 μg/ml [435–437]. Either fluorescent labels or UV visualizing reagents allow the sensitive determination of **VPA** in mouse brain [438,439], in rat brain [440], in plasma, serum or blood. These include bromoacetophenone [441], phenacyl bromide [442], 4-bromomethyl-7-methoxycoumarin (BrMMC) [439, 443,444], 3-bromomethyl-6,7-dimethoxy-l-methyl-2(1H)-quinoxalinone (BrDMEQ) [438], 9-aminophenantrene (9-AP) [445], and *O*-*p*-nitrobenzyl-*N*,*N*′-diisopropylisourea [446]. The levels of unchanged **VPA** and those of the acidic metabolites 3-oxo-2-propylpentanoic acid and 2-propyl-2-pentenoic acid are determined simultaneously applying the procedure reported by Wolf et al. [439]. Some procedures require sample volumes as low as 25 μl

[441] to 50 μl [442]. The use of (3,4-dimethoxyphenyl)-4′-trimethylammoniummethylcinnamonitrile methosulfate was proposed as post-column ion-pair reagent [447]. 6,7-Methylenedioxy-1-methyl-2-oxo-1,2-dihydroquinoxalin-3-ylpropionohydrazide has been recently reported as new fluorescent label for the derivatization of **VPA** in aqueous solutions at 37°C. Injected amounts as low as 2.3 fmol on column are reported to be detectable using this reagent [448]. An automated fluoroimmunoassay of **VPA** and theophylline was reported by using a flow-injection analysis HPLC instrument [449].

Monitoring of free **VPA** concentration may be helpful in patient management because **VPA** has an unusual nonlinear protein binding characteristic and a wide interindividual variation [444]. Both HPLC [444] and GC [450,451] procedures are reported for the determination of free **VPA**.

VPA is reported to be stable in blood samples stored at 4°C for 1 to 10 days [430].

*2.9.9. Flunarizine (**FNR**)*

Flunarizine, **FNR**, i.e., 1-[bis(4-fluorophenyl)-methyl]-4-(3-phenyl-2-propenyl)-piperazine is an AED candidate under clinical evaluation [452]. RP-HPLC–UV methods have been recently reported for the determination of **FNR** in human plasma [453] and rat brain tissues [454]. Both methods involve a LLE. A rapid HPLC–ESP-MS procedure is also reported to be suitable for the determination of **FNR** in rat brain [455] and further investigations on HPLC–ESP-MS assay optimization for artificial CSF are also reported [456].

2.9.10. Other antiepileptic drugs

Nowadays, several new AED candidates are under preclinical and clinical development [457,458]. Those include chemical entities closely related to current AEDs such as the **GBP** analog pregabalin [459,460]; SGB-017 (Fig. 1), a molecule structurally related to **CBZ** [112,461]; the 3-*N*-substituted 2,3-benzodiazepine talampanel [462] as well as compounds from new chemical families: protirelin, the smallest known peptide hormone [463]; the thiazolidinone ralitoline [464,465]; the 4-(benzoylamino)-benzopyran derivative carabersat [466, 467]; BW 534U87, a compound selected in a series of [(fluorophenyl)methyl]triazolo[4,5-*c*]pyridines [468]; GP3269, a pyrrolopyrimidine nucleoside [469]; ionotropic glutamate receptors (AMPA) antagonists SYM 2206 from a novel class of substituted 1,2-dihydrophthalazines [470] and SIB-1757, 6-methyl-2-(phenylazo)-3-pyridinol [471]. Analytical methods have been reported recently for the determination of the following drug candidates in biological fluids.

(i) Ganaxolone, a neuroactive steroid from the epalons class [472]. The determination of ganoxolone in rat, monkey, dog and human plasma by means of HPLC–MS–MS has been reported [473].

(ii) Retigabine, *N*-[2-amino-4-(4-fluorobenzylamino)-phenyl]ethylcarbamate [474], a potent anticonvulsant in a variety of animal models. Retigabine is mainly metabolized through glucuronidation and acetylation reactions [475,476]. Glucuronides are detected in incubates with liver microsomes or slices, in plasma, and in bile and feces. They were absent in urine (0–24 h) that contained about 2% of the dose as unchanged retigabine and approximately 29% of the dose in more than 20 metabolites. Plasma profiling and spectroscopic analysis was performed by means of HPLC–MS–MS and NMR [475]. A column switching HPLC procedure with tandem MS detection was recently reported for the determination of retigabine and its acetylated metabolite in biological fluids [477].

(iii) LY 300164, 7-acetyl-5-(4-aminophenyl)-8,9-dihydro-8-methyl-7H-1,3-dioxo(4,5H)-2,3-benzodiazepine, an ionotropic glutamate receptors (AMPA) antagonist [478]. Some combinations of LY 300164 with AEDs were found to be superior to these AEDs alone in terms of their lack of adverse effects [479]. The levels of LY 300164 and its *N*-acetyl metabolite have been measured in mouse, rat, dog and monkey plasma using RP-HPLC–UV. The methods exhibited a large linear range from 50 ng/ml to 50 μg/ml.

The different classes of compounds currently under investigations and the properties of these chemical entities suggest challenging analytical developments in the coming years.

3. Simultaneous determination of antiepileptic drugs

Many AEDs are used in polytherapy including up to three different AEDs. Each of them may have

several metabolites. As a consequence, the fast specific simultaneous determination of a panel of AEDs as large as possible is of paramount importance in TDM of patients under polytherapy [27] as well as in STA. In addition, polytherapy may easily result in drug to drug interactions, changing the metabolism of the drugs and the therapeutic outcomes in the patients [480–482].

Analytical methods have been reported for the monitoring of AEDs in human breast milk to give the necessary information for the prevention of adverse drug events in infants through intake of milk [483]. The usefulness of hair analysis in relation to compliance testing of AEDs is presently under investigation and preliminary data suggest that this approach is indeed fruitful [484]. In Table 5 are summarized the available methods allowing the simultaneous determination of at least three different AEDs in a single run. No chromatographic or electrokinetic technique (including HPLC–UV, HPLC–MS, GC–FID, GC–MS, TLC and CE) has been reported for the simultaneous determination in a single run of the all common AEDs currently in use i.e., **CBZ**, **GBP**, **LTG**, **MHD**, **PB**, **PHT**, **PRM**, **TPM**, **TGB**, **VPA**, **VGB**, and their metabolites. All procedures suffer from possible interference with either potential co-medication or endogenous compounds. Procedures to be developed encounter the following problems.

(i) The variety of chemical properties of the molecules to be determined, i.e., acidic, neutral and basic compounds.

(ii) The lack of either strong chromophores or strong fluorophores or appropriate chemical functions easily detectable by EC detection for drugs such as **VPA** and GABA derivatives.

(iii) The large scale of the therapeutic levels ranging from ng/ml to μg/ml.

(iv) The number of analytes possibly present.

Because the targeted drugs are well known, methods based on HPLC and MS coupling should be available soon for the fast simultaneous determination of a greater number AEDs. However, the cost of such instruments seems to be an obstacle for extensive investigations in this field.

Advantages and limitations of assay systems for chromatographic screening of AEDs and other drugs in STA were recently reviewed [485]. Many AEDs including **CBZ**, **PHT** and **LTG** were readily detectable in HPLC and GC chromatographic screens, but the detection of **VGB** and **VPA** was found to be difficult [485]. A specific technique for the identification and confirmation of some AEDs is reported [500].

3.1. Collection of biological material, storage and stability

In recent years, the practice of ordering multiple chemistry analytes and therapeutic drugs on a single patient blood specimen has become prevalent in clinical medicine, particularly when dealing with pediatric and geriatric patient populations [486]. Therefore, the adequate sampling procedure should be carefully evaluated prior to assay. As an example, blood collected with phase-separating gels results in a serum specimen which is not adequate for the determination of **LTG** because the barrier gel can retain **LTG** resulting in a falsely low value for the specimen [330]. The stability of **CBZ**, **PB** and **PHT** and other common therapeutic drugs has been investigated in serum stored in Vacutainer SST and Corvac serum separator blood collection tubes [486]. A significant decrease was observed for measured concentrations as a function of both time and sample volume when serum was stored in glass Vacutainer SST. Additional investigations showed that concentrations of **CBZ**, **PB** and **PHT** are also reduced in plastic Vacutainer collection tubes with barrier gel [487]. In contrast, measured concentrations of ETH, **PRM**, **VPA** and other drugs such as theophylline and salicylate did not change under identical storage conditions. No significant change was observed in the concentration of **CBZ**, **PB** and **PHT** when serum was stored either in Corvac serum separator blood collection tubes or in standard dry Vacutainer collection tubes without barrier gel. The decreases were shown to be due to absorption of AEDs by the barrier gels. For **PB** and **PHT**, the reduction in total drug concentrations also resulted in a proportional decrease in free drug concentrations and was dependent on the extent of protein binding by the drug. Several other studies report similar problems with the stability of **CBZ**, **PB** and **PHT**, when specimens were stored in Vacutainer SST, although results have varied considerably in relation to differences in the

duration of specimen storage and storage temperature [488–490].

The stability of **CBZ**, **PB**, **PHT** and **VPA** on SST tubes from Becton-Dickinson containing a new gel formulation has been recently evaluated [491]. An unknown peak, which may interfere with **CBZ-E** [150] and **FBM** [349] in RP-HPLC, was detected when standard dry Vacutainer collection tubes and red-top Vacutainer tubes were used for blood collection, respectively. This peak was also present when blood was replaced by water [150].

The stability of **CBZ**, **CNZ**, **PB**, **PHT** and **VPA** has been studied in whole blood after storage at conditions simulating storage and transport [492–494]. The stability of the same compounds was also reported in plasma under a variety of storage conditions [493,495,496,519]. Stability studies led to controversial results. In plasma, **VPA** was found to undergo a statistically significant degradation after 20 days. A 6-month stability study of **PB**, **PHT** and **PRM** suggested that disagreements over the stability of AEDs might be due to the specificity of the analytical methods [494]. The effect of heating human sera at a temperature necessary to deactivate human immunodeficiency virus on measurement of free **CBZ**, free **PHT** and free **VPA** concentrations has been recently reported [498]. Only a few papers report stability data of AEDs despite of the fact that these are integrating part of the analytical methods validation (for **CNZ**, **GBP**, **LTG**, **LSG**, **OXC**, **PB**, **PGB**, **RCM**, **STP**, **TGB**, **TPM**, **VGB**, **VPA** and **ZNS**: see corresponding individual sections).

3.2. Sample preparation

Proteins are mostly removed from serum and plasma by precipitation by using ACN [126,135,137,339,515,516] or MeOH [506]. An LLE step is sometimes required after the deproteinization step [126,506].

LLE was the method of choice for years but the technique suffers from several disadvantages. These are problems of environment concern, difficult automation and sometimes poor extraction recoveries for some specific drugs. Diethylether [121,499,519] or MTBE [524] or EA [203,244,502,504] or solvent mixtures [131,357,362,506,525] were used as extraction solvent. The effects of the nature of the solvents on the drug recovery was studied [514]. An interesting automated online LLE procedure has been proposed [507]. The actual sample preparation throughput for LLE is often considered to be higher than that obtained by means of off-line SPE.

Off-line SPE was used to overcome the disadvantages of LLE. Because most of the UV-absorbing AEDs have no strong basic or acidic functional groups, DECs with either regular C_8 [330,512] or regular C_{18} [178,257,520] packings are used for extraction. SPE using disc technology was also applied [330]. Some reports describe the direct injection of plasma or serum volume ranging from 1 to 500 μl (see Table 5) onto ISRP columns [147] or onto syringe-type minicolumns [517,518] as well as by means of column switching techniques [138,503,511] or automated on-line SPE systems such as Merck OSP-2 [150]. Some of these systems allowed both the direct injection of a large number of samples and the full automation of the analytical system. The on-line SPE by means of a Merck OSP-2 system with automated exchange of stainless steel C_{18} extraction columns previously described [526] allows the determination of eight common AEDs and their metabolites in a single run [150]. By applying this method, neither peak area nor peak height decrease were observed when a single 4 mm×4 mm I.D. C_{18} extraction column was used up to 30 times. Even up to 50 successive injections of 200 μl serum samples on the same cartridge were possible without noticeable peak shape disturbance or peak area decrease. The theoretical potential of such an automated system is more than 2160 samples per run when 72 extraction columns are placed in the Merck OSP-2 carousel and when each of them is used for the extraction of 30 samples. By applying this system, the limitation factors are the capacity of the autosampler and the analytical column lifetime. Such systems (including also the Prospekt device from Spark Holland) may be of great interest for future developments involving very fast analyses by means of various tandem HPLC–MS systems.

For regular column-switching systems, the effects of the important parameters were extensively investigated [503]. Exploratory investigations have been performed using syringe-type minicolumn for sample injection into RP columns [518] but no further application was reported. The column switching

technique was also applied to the separation of chiral compounds via β-cyclodextrin inclusion complexes [178].

Ultrafiltration remains the method of choice for the determination of free AEDs in serum [501,509]. On-line dialysis is reported to be suitable for the routine monitoring of free and total **CBZ**, **PB** and **PHT** in plasma [140]. Microdialysis is reported to provide a simple and inexpensive alternative method for in vitro drug protein binding monitoring and protein binding interaction studies [527]. Numerous AEDs in serum samples were investigated. Optimal probe recovery was based on five variables including plasma volume, time to steady-state recovery, perfusion rate, membrane length and temperature. A sample preparation procedure for the routine measurements of AEDs in saliva collected daily from patients with intractable epilepsy was also reported [513]. The suitability of saliva for TDM of AEDs in children was evaluated [528].

Brain tissues were extracted with acetone [257] or dichloromethane [169].

3.3. Chromatography

The applications of both GC and TLC to the simultaneous determination of AEDs in biological fluids remains of limited use. Very little investigations were performed by means of these techniques to our knowledge. One exception is the detection of AEDs and their metabolites in urine by a ''general screening'' analysis procedure using computerized GC–MS [500]. This paper is dedicated to problems of toxicology concern.

Many AEDs recently developed such as **GBP**, **VGB**, **VPA**, **TPM** are UV-transparent. Therefore, they require a derivatization step prior to HPLC analysis. Despite that fact, HPLC remains the method of choice for the simultaneous determination of AEDs and their metabolites. The main reasons are probably due to:

(i) Its simplicity of use when used with conventional detectors.

(ii) Its widespread use in laboratories.

(iii) Its fast adaptation to specific needs.

(iv) The advances done in column efficiency and availability.

(v) Its relative low cost.

Some of the same reasons make that conventional HPLC–UV or HPLC–DAD or HPLC–FD has not yet been replaced with HPLC–MS systems. Among all UV-absorbing AEDs only **LTG** has alkaline properties. Therefore, most of the HPLC methods are based on RP chromatography on regular C_{18} or C_8 columns (see Table 5). Binary mixtures of ACN [121,138,203,244,257,502,503,512,513] or MeOH [135,506] and water or pH 4 to pH 7 buffered aqueous solutions are used as mobile phase. Ternary mixture of the latter [126,131,137,169,330,339, 357,514,515,519] or even quaternary mixtures [330,510] are also reported. THF was used in ternary mixtures [362]. One of those allows the separation of deuterated and non deuterated analogs of **PHT** and **CBZ** with UV detection [179]. An RP ion-pair HPLC procedure is also reported [502]. CN columns have been used in both reversed-phase mode and normal-phase mode. A systematic evaluation of C_{18}, C_8 and CN columns is reported [330]. Separations on NP mode onto silica columns [517] became less popular because of the availability of efficient lot to lot reproducible RP columns and problems of environmental concern. Isocratic elution was considered to be not suitable for the simultaneous determination of AEDs on RP columns [203]. However, isocratic elution remains the preferred elution mode among others including gradient elution and flow-step gradient modes according to data in Table 5. This fact may be due to improvements in column efficiencies as well as issues related to equilibration time, throughput, easiness of use and availability of suitable pumps. Typical chromatograms obtained by means of gradient elution and isocratic elution are given in Fig. 2 and Fig. 3, respectively.

CZE and MECC represent attractive methods for the determination of drugs and metabolites such as AEDs in body fluids. When compared to HPLC and GC, electrokinetic capillary electrophoresis has distinct advantages [522,529,530]. These include: small sample size; minimal sample preparation; use of very small amounts of organic solvents and inexpensive chemicals; automation; easiness of method development; low cost of capillary columns. However, very few applications have been reported up to now (Table 5) despite calls encouraging newcomers to start using this analytical methodology [522].

UV detection at fixed wavelengths around 210 nm

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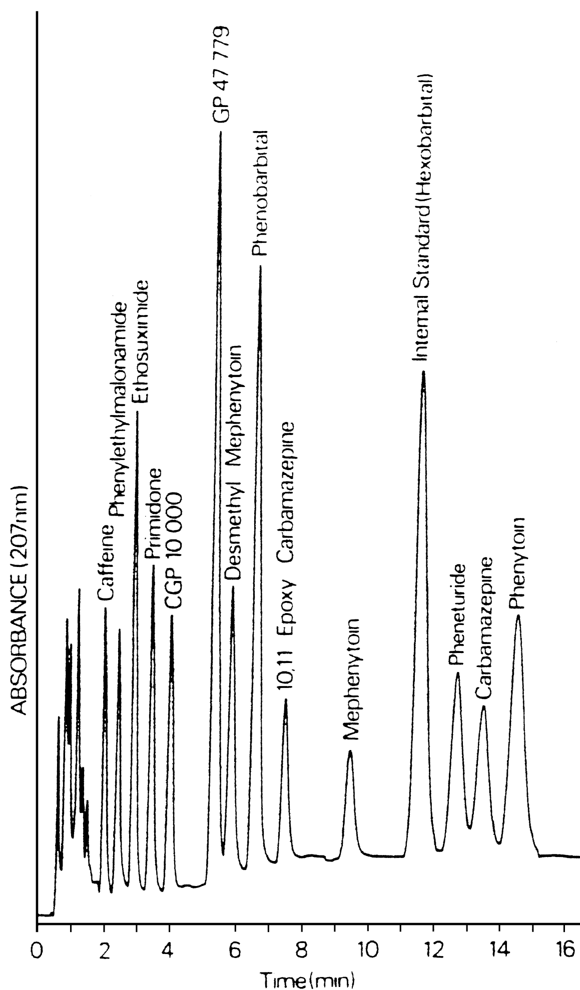

Fig. 2. Determination of AEDs in serum by means of HPLC–UV with gradient elution: typical chromatogram of a spiked drug-free serum extracted as a patient sample. CGP 10000 = **CBZ-diol**, GP 47779 = **MHD**, 10,11-epoxy carbamazepine = **CBZ-E**. From Ref. [122], with permission.

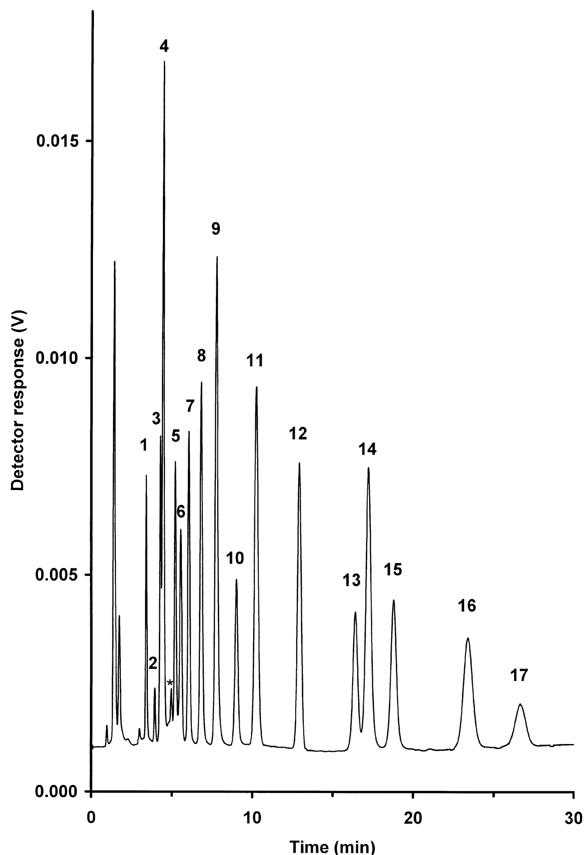

Fig. 3. Determination of AEDs in plasma by means of HPLC–UV with isocratic elution: typical chromatogram of a spiked drug-free plasma deproteinized with ACN [150]. Experimental conditions: column: Nucleosil C_{18} (d_p = 5 μm, 250 mm × 4 mm I.D.); mobile phase: 0.05 *M* (Na^+) phosphate buffer, pH 7.00–ACN–MeOH (70:24:6, v/v); flow-rate = 1.20 ml/min; detection wavelength = 210 nm. Peak identification: 1 = **PEMA**, 2 = **ESM**, 3 = **PRM**, 4 = **CBZ-diol**, 5 = **FBM**, 6 = **MHD**, 7 = **PB**, 8 = ***p*-HPPH**, 9 = ***m*-HPPH**, 10 = **CBZ-E**, 11 = **OXC**, 12 = I.S., 13 = **PTD**, 14 = **PHT**, 15 = **CBZ**, 16 = **NTZ**, 17 = **CNZ**. LAM (not present) is partly co-eluted with ***p*-HPPH**. * By-product from **PTD** reference substance.

was mainly used for HPLC and electrokinetic separations because most of AEDs have no strong chromophore. Some procedures recommend the use of dual wavelength detection [339] or DAD [511,515].

4. Comparison of analytical methods

The advantages and limitations of immunochemical assays and HPLC are compared in several papers [27,326,531,532]. The cost savings of an automated HPLC system over immunochemical methods for AEDs has been evaluated [533]. The precision and accuracy of analytical methods currently in use in

TDM have been extensively evaluated from proficiency test data provided by laboratories participating in an international Healthcontrol external quality assessment scheme for a range of AEDs. These include **CBZ**, **CBZ-E**, **CNZ**, **ESM**, **PB**, **PHT**, **PRM** and **VPA** [532,534]. The evaluation shows that **CBZ-E** cross-reactivity amounted to 49% for some immunoassays commercialized for the determination of **CBZ** [150,535]. A number of papers devoted to the TDM of a selected AED report correlation data obtained in comparing either a GC or a HPLC procedure with a commercialized FPIA or EMIT assays. In most of the cases, the investigations were performed in the course of accuracy study during the analytical method validation. Suitable correlation were reported for selected methods. These cover the assay of **CBZ** [170] in saliva as well as the following AEDs in plasma or serum: **CBZ** [135,147, 501,505,510,536–538]; **ESM** [505,522,523]; **LTG** [345]; **PB** [135,147,501,505,510,522,523,537–539]; free **PHT** [250,259]; total **PHT** [135,147,259,501, 505,510,537,538]; **PRM** [501,505,510,523,537]; **TPM** [370]; **VPA** [259,424,425,444–446,450,539]. A comparison of a GC–MS method with different immunoassays is reported for the screening of benzodiazepines in urine [540]. The performance characteristics of four immunoassays compared with HPLC has been recently reported for free **PHT** [541]. A comparative evaluation of a **RCM**EDI system from Bio-Rad and a conventional HPLC instrument for benzodiazepines in clinical samples is also reported [542]. The data obtained show that such an automated system can be used for rapid qualitative screening of high levels of clinically important benzodiazepines. However, all samples should be further confirmed by conventional HPLC procedure [542].

The comparison of selected HPLC, GC and EMIT assays is reported for **CBZ** [120]. **CBZ** levels were found to be in good agreement, but GC seemed to have a relative poor reliability. Accuracy problems were reported for **LTG** testing by means of HPLC procedures [330]. The serum **PCT** levels measured after the same SPE procedure by means of a HPLC–UV and a GC–FID procedure were found to be in good agreement [100]. A good agreement was also found for serum **VPA** in applying a selected HPLC and a selected GC procedure [437]. The correlation of **ZNS** levels measured by means of a MECC and a HPLC procedure is reported to be excellent ($r=0.981$, $n=25$), although the levels obtained with MECC are slightly lower than those found with HPLC (slope=0.869) [207]. Results obtained in applying the same techniques to serum **LTG** [333] and serum **FBM** [354] were reported to correlate well. In the latter case, a LLE step is performed before the HPLC separation and direct injection of serum in the MECC system is applied.

5. Conclusion

A large number of studies have been performed for the determination of AEDs in biological material. Extensive investigations are still currently in progress. A panel of both chromatographic and electrochromatographic methods are available today for the specific determination of a given AED among several AEDs and their metabolites. However, no procedure has been yet reported to be suitable for the simultaneous determination of all common AEDs currently in use despite the application of powerful techniques. This still remains a challenge that recent hyphenated techniques such as HPLC–MS, CE–MS could solve even if problems of economical concern prevent the widespread use of such systems. The new AED candidates currently under evaluation in early stages of drug development include new chemical entities and classes of compounds that suggest challenging analytical developments in the coming years.

6. Abbreviations

6.1. Standardization of abbreviations for antiepileptic drugs

The review of the papers devoted to the determination of AEDs reveals that a standardization of their abbreviated names would be useful. For most of the recent AEDs, a consensus abbreviation seems to be of widespread use, e.g., **TPM** for topiramate. However, a variety of abbreviations were used for most of the older drugs. Table 6 summarizes a tentative standardization of the abbreviations. The

Table 6
Abbreviations for AEDs and their metabolites

Antiepileptic drug or metabolite	Tentative abbreviation for standardization	Most reported abbreviation	Occurrence (%)	Other abbreviations used ranked by occurrence
Parent drugs				
Acetazolamide	**AZD**	[a]	[a]	[a]
Carbamazepine	**CBZ**	**CBZ**	98	**CM**>**CB**>**CAR**
Clobazam	**CBM**	[c]	[c]	[c]
Clonazepam	**CNZ**	[c]	[c]	[c]
Diazepam	**DZM**	[c]	[c]	[c]
Eterobarb	**ETB**	[a]	[a]	[a]
Ethosuximide	**ESM**	**ESM**	44	**ET**>**ES**≥**ETH**≥**ETHO**
Felbamate	**FBM**	**FBM**	100	
Flunarizine	**FNR**	[a]	[a]	**FN**
Fosphentoin	**FOS**	[a]	[a]	[a]
Gabapentin	**GBP**	**GBP**	92	**GP**
Lamotrigine	**LTG**	**LTG**	92	**LG**
Levetiracetam	**LVT**	[a]	[a]	[a]
Lorazepam	**LRZ**	[c]	[c]	[c]
Losigamone	**LSG**	[a]	[a]	[a]
Nitrazepam	**NTZ**	[c]	[c]	[c]
Oxcarbazepine	**OXC**	**OXC**	46	**OCBZ**>**OX**>**OCB**>**OCBX**
Pheneturide	**PTD**	[a]	[a]	[a]
Phenobarbital	**PB**	**PB**	80	**PHENO**>**PBT**
Phenytoin	**PHT**	**PHT**	68	**PT**>**PY**>**PHT**>**DPH**
Pirecetam	**PCT**	[a]	[a]	[a]
Primidone	**PRM**	**PRM**	42	**PR**>**PRIM**>**PMD**
Progabide	**PGB**	**PGB**	33	**PG**≥**PGB**≥**PRG**
Ralitoline	**RLT**	**RLT**	100	
Remacemide	**RCM**	**RCM**	100	
Rufinamide	**RFM**	[a]	[a]	[a]
Stiripentol	**STP**	**STP**	100	
Tiagabine	**TGB**	**TGB**	100	
Topiramate	**TPM**	**TPM**	100	
Valproic acid	**VPA**	**VPA**	100	
Valpromide	**VPD**	[a]	[a]	[a]
Vigabatrin	**VGB**	**VGB**	67	**GVG**>**VGT**
Zonisamide	**ZNS**	**ZNS**	67	**ZA**
Major metabolites				
10,11-Dihydro-10,11-dihydroxy-*trans*-carbamazepine, CGP 10 000 (**CBZ** and **OXC** metabolite)	**CBZ-diol**	**CBZ-diol**	41	**CBZ-D**,**CBZ-diOH**,**CBZ-TR** **CBZ-2OH**,**DHCBZ**,**DHD**
10-Hydroxy-carbamazepine, GP 47 779 (**OXC** metabolite)	**MHD**	**MHD**	57	**CBZ-OH**[b]>**MH**>**OHC**
5-(3-Hydroxyphenyl)-5-phenylhydantoin (**PHT** metabolite)	***m*-HPPH**	***m*-HPPH**	[a]	**HPPH**
5-(4-Hydroxyphenyl)-5-phenylhydantoin (**PHT** metabolite)	***p*-HPPH**	***p*-HPPH**	[a]	**HPPH**
Carbamazepine-10,11-epoxide (**CBZ** metabolite)	**CBZ-E**	**CBZ-E**	55	**CBZ-EP**≥**CBZ-epo**≥**EPO** ≥**CBZ**-epoxide>**CBZ-EPOX**> **E-CBZ**>**CE**≥**CBZ-E**>**CEP**
Desglycinylremacemide metabolite	**desRCM**	[a]	[a]	[a]
Phenobarbital (**PRM** metabolite)	**PB**	**PB**	80	**PHENO**>**PBT**
Phenylethylmalondiamide (**PRM** metabolite)	**PEMA**	**PEMA**	100	
Progabide acid metabolite	**PGBa**	[a]	[a]	[a]
Rufinamide carboxylic acid metabolite	**RFMa**	[a]	[a]	[a]

[a] Not applicable.
[b] See Section 6.
[c] The benzodiazepines are not considered due to their widespread use in various therapeutic indications.

abbreviations commonly reported as well as their frequency of use are also given.

Some abbreviations should be avoided to prevent confusion. As an example, **MHD** should be preferred to **CBZ-OH** for the pharmacologically active monohydroxylated derivative of **OXC** to prevent confusion with the 2- and 3-hydroxycarbamazepine derivatives of **CBZ** (Fig. 1). Other abbreviations should be avoided when these are commonly used for other drugs. For instance, **VPD** should be used for vaipromide instead of **VPM** because the latter abbreviation is commonly used for the antiarrythmic drug verapamil.

6.2. Nomenclature

ACN	Acetonitrile
ADME	Absorption, distribution, metabolism and excretion
AED(s)	Antiepileptic drug(s)
BrMmC	4-Bromomethyl-7-methoxycoumarin
CE	Capillary electrophoresis
CSF	Cerebrospinal fluid
CZE	Capillary zone electrophoresis
DAD	Diode array detection
DEC(s)	Disposable extraction cartridge(s)
Dns-Cl	Dansyl chloride
EA	Ethyl acetate
EC	Electrochemical
ECD	Electron-capture detection
El	Electron impact
EMIT	Enzyme-multiplied immunoassay technique
ESP	Electrospray
FD	Fluorometric detection
FID	Flame ionization detection
FPIA	Fluorescence polarization immunoassay
GABA	γ-Amino-*n*-butyric acid
GC	Gas chromatography
HFBA	Heptafluorobutyric acid
HPFA	High-performance frontal analysis
HPLC	High-performance liquid chromatography
I.S.	Internal standard
LLE	Liquid–liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
MECC	Micellar electrokinetic capillary chromatography
MeOH	Methanol
MLC	Micellar liquid chromatography
MS	Mass spectrometry
MTBE	Methyl-*tert*.-butyl ether
MTBSTFA	*N*-(*tert*.-Butyldimethylsilyl-*N*-methyl-trifluoroacetamide
NMDA	*N*-Methyl-D-aspartate
NMMP	Monomethoxymethylphenobarbital
NP	Normal phase
NPD	Nitrogen–phosphorus detection
OPA	*O*-Phtaldialdehyde
PK	Pharmacokinetics
RP	Reversed phase
SIM	Selected ion monitoring
SPE	Solid-phase extraction
SST	Serum separator tube
STA	Systematic toxicological analysis
TFAA	Trifluoroacetic acid
TID	Thermionic specific detection
TSP	Thermospray
UV	Ultraviolet

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