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REVIEW

DRUG LEVEL MONITORING: SEDATIVE HYPNOTICS

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1. INTRODUCTION

Anxiety and sleep disorders are among the most common complaints in modern medical practice. There is a clinical need and very lucrative market for drugs which provide symptomatic relief of anxiety and induce sleep. Drugs like barbiturates act as anxiolytic agents (sedatives) when given in small doses and act to induce and maintain sleep (hypnotics) when given in large doses. Still higher doses lead to anaesthesia (coma). Since the early 1970s, there has been a campaign to restrict the use of barbiturates for sleep disorders because of deaths associated with barbiturate overdose [1,2]. However, barbiturates are still being extensively prescribed and used in excessive amounts.

Analytical procedures for the determination of barbiturates and other traditional non-barbiturate sedative hypnotics are reviewed in this chapter. Analytical procedures for benzodiazepines or histamines which are now commonly used for the treatment of anxiety and sleep disorders have been reviewed elsewhere.

2. BARBITURATES

2.1. General remarks

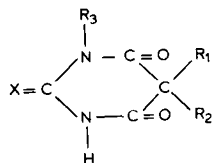
Barbituric acid was synthesized in 1864 by condensation of malonic acid and urea. Hundreds of derivatives of barbituric acid have been prepared by substituting alkyl or aryl groups at the carbon atom in position 5, by substitution of one imide hydrogen by alkyl groups or by replacement of one of the oxygen atoms by sulphur. However, only a few of these derivatives are used clinically. The pharmacologic properties of these derivatives depend upon their acidic nature and their lipid solubility. In general, any change in the structure of a barbiturate which increases its lipid solubility leads to a decrease in duration of action and an increase in potency for hypnosis and rapidity of onset of action. Barbiturates have been classified as ultrashort acting, short to intermediate acting or long acting depending upon the rate of onset of hypnotic activity. The structural formulae of some of the barbiturates and their classifications on the basis of their onset of action are summarized in Table 1.

Barbiturates were primarily introduced as hypnotics. Barbiturates are also being used therapeutically as anticonvulsants, anaesthetics or enzyme inducers. They are also being evaluated for brain resucitation in metabolic or toxic encephalopathy [3]. Barbiturates are also used as industrial chemicals and laboratory reagents.

There are diverse reasons for the analysis of biological samples for barbiturates. Emergency determination of barbiturates for the diagnosis of poisoning is carried out by most laboratories in metropolitan hospitals. Monitoring of

TABLE 1

STRUCTURES OF BARBITURATES



Barbiturate	Onset of action*	R_1	R_2	R_3	X
1. Allobarbital	2	Allyl	Allyl	H	O
2. Amobarbital	2	Ethyl	Isoamyl	H	O
3. Barbital	3	Ethyl	Ethyl	H	O
4. Butabarbital	2	Ethyl	*sec.*-Butyl	H	O
5. Cyclobarbital	2	Ethyl	Cyclohexynyl	H	O
6. Heptabarbital	2	Ethyl	1-Cyclohepten-1-yl	H	O
7. Hexobarbital	1	Methyl	Cyclohexenyl	CH_3	O
8. Mephobarbital	3	Ethyl	Phenyl	CH_3	O
9. Methohexital	1	Allyl	1-Methyl-2-pentynyl	CH_3	O
10. Pentobarbital	2	Ethyl	1-Methylbutyl	H	O
11. Phenobarbital	3	Ethyl	Phenyl	H	O
12. Secobarbital	2	Allyl	1-Methylbutyl	H	O
13. Thiamylal	1	Allyl	1-Methylbutyl	H	S
14. Thiopental	1	Ethyl	1-Methylbutyl	H	S
15. Vinbarbital	2	Ethyl	1-Methyl-1-butenyl	H	O

*1 = Ultrashort acting; 2 = short to medium acting; 3 = long acting.

therapeutic concentrations of phenobarbital used as an anticonvulsant is now routinely performed. A few laboratories are providing assays of pentobarbital and thiopental when these barbiturates are used aggressively for the treatment of head injury or Reye's syndrome [4,5]. Barbiturates are among the drugs tested for in autopsy samples in suspected cases of homicide or suicide. Detection of barbiturates also forms a part of urinary drug screen carried out for the diagnosis of drug abuse or addiction. In some cases samples of meat are analyzed for residual barbiturate when the animals were tranquilized with a barbiturate before slaughter.

Every possible technique for the estimation of organic compounds in biological samples has been applied for the determination of barbiturates. Identification of barbiturates by chromatographic procedures has recently been reviewed [6].

2.2. *Sample preparation*

For quantitative analysis plasma or serum is most commonly used. Use of saliva as a possible specimen for drug monitoring is still being investigated [7]. In general, blood is collected in commercially available evacuated tubes and in some cases serum separators are used for ease of separation of blood plasma from cells. These devices should be checked for possible interferences and extraction efficiency with the selected analytical technique [8]. There does not

appear to have been a systematic study to determine the effect of different anticoagulants or other additives in blood collection tubes on barbiturate concentration in plasma or serum. Biological samples containing barbiturates stored at 4°C for three months show a decrease of barbiturate concentration by 25% [9] which for forensic purposes is not considered significant.

Barbiturates are analyzed by immunoassays without prior extraction and concentration. Thiopental has been analyzed by liquid chromatography (LC) by injecting diluted plasma directly [10]. The analytical column was protected by a precolumn. However, the life of the precolumn is short and this approach cannot be applied to other barbiturates without risk of interference as they are monitored at wavelengths where many plasma constituents show high absorbance. In a number of LC procedures for the determination of barbiturates plasma proteins have been precipitated with organic solvents, e.g. acetonitrile [11], 96% ethanol [12], methanol [13] and acetone [14] and the supernatant had been injected directly into the liquid chromatograph. This approach has been quite popular because of its simplicity. However, it also suffers from reduced column life as in some cases proteins may not be completely precipitated. Further, this non-selective sample preparation is subject to more interferences than selective sample preparation by liquid—liquid extraction [15]. Preparation of protein-free filtrate is also not compatible with fast LC procedures using short columns [16]. Extraction of barbiturates from biological samples with water-immiscible organic solvents remains the most popular technique. Recently a number of organic solvents have been evaluated for their efficiency in extracting barbiturates from plasma, and it was concluded that diethyl ether is the most efficient solvent [17]. Further, diethyl ether being a low-boiling solvent can be evaporated easily. However, in some laboratories the use of diethyl ether is discouraged because of the potential danger of explosions due to peroxide formation. A number of other solvents not included in the above mentioned extraction efficiency study, e.g. methyl *tert.*-butyl ether [18], dichloromethane [19] and a mixture of diethyl ether—hexane (1:1) [20] have been used. Thus there is no agreement about the most appropriate solvent for the extraction of barbiturates.

A wide range of pH (1—7.5) has been used for the isolation of barbiturates. At pH below 5 acids and neutral compounds are co-extracted with barbiturates but basic compounds are only minimally extracted. In some applications, the organic extract is washed with pH 7.4 buffer to remove acids [21]. On the other hand when the extraction is carried out at neutral pH, acids are not extracted and washing of the extract with pH 7.4 buffer is omitted [22]. However, neutral and basic compounds are present in the extract. This initial organic extract has been manipulated in a number of alternative ways for the application of a specific analytical technique. An aliquot of the extract is directly injected into a gas chromatograph particularly when a nitrogen-selective detector is used and derivatization is not carried out [23]. In another approach the organic layer containing barbiturates is back-extracted with aqueous alkali. The aqueous layer now contains primarily acidic compounds and can be analyzed directly spectrophotometrically [22], and by reversed-phase LC [21, 24] or is re-extracted into a suitable solvent after adjusting the pH of the aqueous layer to <7 [25]. This multi-step extraction enhances the selectivity

of the analytical procedure. However, this approach is no longer popular as it is considered tedious and slow. In a number of gas—liquid chromatographic (GLC) procedures, the first organic extract is back-extracted into methanolic phenyltrimethyl ammonium hydroxide [26] or methanolic tetramethyl ammonium hydroxide [27]. Aliquots of these methanolic extracts are directly injected into the gas chromatograph where they are derivatized "on-column" to their methyl derivatives. In these procedures any degradation of barbiturate by strong alkali-like phenyltrimethyl ammonium hydroxide or tetramethyl ammonium hydroxide is compensated for by the use of an appropriate internal standard which also undergoes similar degradation.

It appears that the most commonly used technique is either to evaporate the initial solvent extract and dissolve the residue in a small volume of mobile phase for analysis by LC [20,28,29], or to dissolve the residue in a suitable solvent for analysis by gas chromatography (GC) without derivatization [30] or to dissolve the residue in derivatizing reagents [31].

In another approach, barbiturates are extracted in anionic form as an ion pair with a quaternary ammonium ion into an organic solvent containing the alkylating agent, e.g. ethyl iodide [32] or pentafluorobenzyl bromide [33]. The organic phase from this extractive alkylation contains excess reagent as well as traces of counter ion. Special clean up steps are required if electron-capture detection (ECD) or nitrogen—phosphorus detection (NPD) or gas chromatographic—mass spectrometric (GC—MS) analysis is used.

Despite its wide use, extraction of biological samples with immiscible organic liquids has many problems, e.g. formation of emulsions, use of relatively large volume of solvents compared to specimen volume, variable recovery of different barbiturates, and in some cases the need to dry the extracts with desiccants prior to evaporation. To overcome these problems, a number of solid supports have been used for the isolation of barbiturates. Thus barbiturates present in plasma have been adsorbed onto charcoal which is then eluted with a small volume of dichloromethane [34]. Recently phenobarbital has been isolated in 99% yield by passing plasma through a small column packed with graphitized carbon black (Carbopack B) and eluting phenobarbital with methanol after suitable washes of the column [35].

In a number of procedures drugs of abuse including barbiturates have been isolated with the use of disposable columns packed with non-ionic Amberlite XAD-2 resin which is a hydrophobic polystyrene-divinylbenzene copolymer developed by Rohm and Haas [36]. Recently these types of columns have been used with a DuPont Prep 1 automated sample processor* for the isolation of barbiturates from post-mortem specimens [37] with excellent results.

In the past few years disposable reversed-phase columns packed with C_{18} or CN bonded silica of 40—50 μm particle size have become very popular for isolation of a variety of drugs from biological specimens. The sample at an appropriate pH is passed through the column, washed with water or water—alcohol mixtures and then eluted with a small volume of methanol [16,38]. The eluate can be analyzed spectrophotometrically, by reversed-phase LC, GC or thin-layer chromatography (TLC) directly without prior evaporation. However, the eluate

*This instrument has been temporarily withdrawn from the market.

can be evaporated for further concentration, clean-up or derivatization [38]. The use of glassware is minimal and the yields of barbiturates are satisfactory. For economy, columns can be packed in the laboratory [38].

There are other types of extraction columns which are quite different from the solid-phase extraction columns discussed so far. Columns packed with kieselguhr are marketed by E. Merck under the trade name Extrelut columns. These columns hold the liquid matrix and the desired compounds can be eluted with water-immiscible solvents only [39]. Another column of this type marketed by Analytichem International under the trade name of ClinElut has been used for the isolation of sedative-hypnotic drugs [40].

2.3. Spectrophotometry

There is no simple screening procedure for the detection of barbiturates when a reagent could be added directly to an aliquot of biological fluid to produce a specific colour. However, colorimetric procedures have been proposed when biological samples are extracted with water-immiscible solvents and the extracts are treated with mercury—dithizone reagent [41,42] or the dried residue of the extracts is treated with cobalt nitrate—pyrrolidine reagent [43]. It seems that these procedures are not commonly used in clinical laboratories because of poor specificity [44]. On the other hand, the spectrophotometric procedure of Goldbaum or its modified version [45] is the most widely used technique for the identification and determination of barbiturates as a group. In these procedures barbiturate is selectively extracted from plasma or blood and the difference in absorbance at 260 nm at pH 13 and pH 10 is determined. The UV scan is characteristic and false positive results are rare. Presence of some compounds may distort the barbiturate spectrum [46]. To improve selectivity a differential scan of barbiturate at pH 13 versus pH 10 is obtained [22]. The major drawback of the spectrophotometric procedure is lacking selectivity in that the barbiturate is not identified since the toxicity of a barbiturate is dependent on its structure. To improve the clinical usefulness of the spectrophotometric procedure, the barbiturates have been differentiated into short or long acting by the determination of partition ratio of barbiturates in aqueous and chloroform phases at pH 9.45 [47].

2.4. Gas chromatography

Gas chromatography is the most widely used technique for the determination of barbiturates. Different types of detectors have been used for improved sensitivity and selectivity, a variety of liquid phases have been used in an attempt to separate barbiturates as free acids without prior derivatization, and numerous techniques have been tried to prepare stable derivatives rapidly. Pillai and Dilli [48] have reviewed the literature for GC determination of barbiturates upto 1980.

2.4.1. Choice of detector

The flame ionization detector provides adequate sensitivity for the determination of therapeutic or toxic concentrations of barbiturates. However, alter-

native detectors are being evaluated for improved sensitivity and selectivity. Underivatized barbituric acids show reasonable response with the electron-capture detector. However, this response is virtually lost when dialkyl derivatives of barbituric acids are prepared to reduce tailing of barbiturate peaks [49]. The use of ECD for the determination of barbiturates in biological samples without prior derivatization has not been reported. However, a number of procedures have been described for the determination of barbiturates using the electron-capture detector after the preparation of their pentafluorobenzyl derivatives [33,50,51]. The performance of the photoionization detector for the determination of barbiturates has been compared with that of the flame ionization detector and the photoionization detector was eight to sixteen times more sensitive than the flame ionization detector [52]. However, it is clear from the lack of publications that the photoionization detector has not been accepted for general use. On the other hand a large number of publications indicate that the nitrogen—phosphorus detector is the detector of choice for the analysis of barbiturates and other drugs as it shows high response to either underivatized or derivatized barbiturates. The newly designed nitrogen—phosphorus detectors are relatively easy to use as they have electrically heated rubidium silicate beads and hydrogen flow-rate is precisely controlled by a fine metering system [53, 54]. The ratio of NPD/flame ionization detection (FID) response of an unknown drug to the NPD/FID response of caffeine has been used as an additional parameter for the identification of unknown drugs [55]. Despite their widespread use the nitrogen—phosphorus detector also has its shortcomings. These detectors show high response to phosphorus containing compounds, e.g. plasticizers which are abundantly present in many types of stoppers. Special precautions have to be taken to minimize the contamination of the sample with plasticizers. Furthermore, the alkali beads lose sensitivity with use and it is costly to replace them.

Another detector, the Hall electrolytic conductivity detector, has been proposed for the detection of barbiturates [56]. This detector, when used in the reductive mode, is specially suitable for barbiturates as they produce ammonia without the need of a catalyst and excellent selectivity was achieved for barbiturates without the use of a $Sr(OH)_2$ scrubber. However, this detector has failed to gain popularity as compared to the success of a nitrogen—phosphorus detector.

The mass spectrometer remains the most sensitive and specific GC detector for both derivatized and underivatized barbiturates. It allows the use of isotopically labelled analogues to be used as internal standard [57,58]. It also provides an additional parameter for the identification of unknown barbiturates. However, because of high capital and maintenance cost, mass spectrometers are being used only in a few laboratories.

2.4.2. Separation of barbiturates as free acids

2.4.2.1. Packed columns. It was realized in the early 1960s that barbiturates have a strong tendency for adsorption onto GC columns. A number of alternative approaches have been proposed to reduce adsorption such that barbiturates may be chromatographed without derivatization. In one approach the stationary support has been benzoylated prior to its coating with the liquid phase

[59]. In another approach the glass column and glass wool are carefully silylated and the packed column is repeatedly treated with tetraethylorthosilicate [60]. It has been claimed that the use of particular liquid phases allows the separation of barbiturates without any special deactivation steps [61]. Tailing and adsorption of barbiturates has been reduced by saturating the carrier gas with formic acid vapour [62,63]. However, use of formic acid is toxic, potentially hazardous and corrosive and its use in routine clinical laboratories is discouraged. A safe device for the introduction of formic acid vapour has recently been described [64]. To avoid the hazards of formic acid, solid supports coated with methyl—phenyl or phenyl silicones and glass wool have been deactivated with phosphoric acid (Supelco, Bellefonte, PA, U.S.A.). The Supelco catalogue lists the packings 3% SP-2250-DA for the assay of barbiturates and 3% SP-2510-DA for the determination of antiepileptic drugs without derivatization. A number of reports have described the use of these packings [30,65—68]. One of the drawbacks of this kind of packing is bleeding of phosphoric acid which interferes with the performance of the nitrogen—phosphorus detector. Further, these packings have limited life as compared to unmodified silicone phases. As an alternative to diatomite support (Supelcoport) used in these packings, use of graphitized carbon black (Carbopack C) as a stationary support phase for GC determination of barbiturates has been proposed [35]. Glass column and the support were coated with trimesic acid in place of phosphoric acid. It is likely that this acid may be more compatible with the nitrogen—phosphorus detector than is phosphoric acid, although the flame ionization detector was used in this report [35]. This packing also has a limited life as compared to packings uncoated with acids. A number of reports indicate that barbiturates have been separated on unmodified silicone phases coated on diatomite solid supports [69—72] with minimal tailing. These are general-purpose column packings and these can be used for all types of drugs. It appears that extensive purification and deactivation of the diatomite support has made it possible to separate underivatized barbiturates on silicone columns. It is also true that the adsorption of polar barbiturates, e.g. phenobarbital, is not completely eliminated. Mono-N-alkyl barbiturates, e.g. hexobarbital and methohexital, have less tendency for adsorption and have been determined with the use of silicone liquid phases coated on unmodified diatomite supports without derivatization [73—75].

2.4.2.2. Capillary columns. In the last few years rapid advances have been made in the production of capillary columns which allow the separation of underivatized polar barbiturates without loading the column with acids. Highly flexible capillary columns with thin walls are drawn from fused silica or fused quartz. These columns are break-resistant and can be installed without the need for column end straightening. The liquid phases are chemically bonded to the deactivated capillary surface [76]. Excellent separations of underivatized barbiturates on a polar methylsilicone [23,40,77] and on semipolar 5% phenyl methylsilicone [40] column have been demonstrated. Recently a new fused-silica capillary column chemically bonded with trifluoropropyl methylsilicone has been introduced specially for acidic drugs (J & W. Scientific, Rancho Cordova, CA, U.S.A.). Chromatographic data for the determination of underivatized barbiturates are summarized in Table 2.

2.4.3. Derivatization of barbiturates

While one school of chromatographers emphasized the need for improved packings for GC columns to minimize adsorption of barbiturates, the other group of workers devoted their efforts to develop suitable procedures for the derivatization of barbiturates prior to their separation by GC. A majority of the publications since 1975 describe the GC determination of barbiturates after derivatization. In general, derivatization leads to improved peak shape and sensitivity. Further, derivatized barbiturates can be separated on general-purpose silicone liquid phases and the columns have a long life. However, derivatization may lose its present popularity if, and when, capillary columns become popular.

It was recognized early that silyl derivatives are not suitable for the quantitative determination of barbiturates [78]. Preparation of 1,3-dimethyl derivatives appears to be the most commonly used approach for the determination of barbiturates. In some cases, however, more than one barbiturate, e.g. phenobarbital and mephobarbital, produce the same dimethyl derivative. In such cases, alkyl groups other than methyl are introduced to prepare non-polar derivatives of barbiturates. Advantages and problems of the different reagents commonly used for the preparation of dialkyl derivatives of barbiturates are discussed briefly.

2.4.3.1. Diazoalkanes. Diazomethane and diazoethane are among the versatile reagents available for the preparation of alkyl derivatives of acidic compounds. They are particularly suitable for the preparation of derivatives for GC analysis as the excess reagent can be readily and conveniently removed. The derivatization is carried out in volatile organic solvents and further extraction of derivatized products from the reaction mixture is not required. Both dimethyl and diethyl derivatives of barbiturates and other acidic drugs have been prepared for analysis by GC—MS [79]. However, diazoalkanes are both toxic and explosive and have to be prepared when required. Further there is a tendency for the production of multiple products particularly when barbiturates are treated with diazoethane [80]. Therefore diazoalkanes are rarely used for the preparation of dialkyl derivatives of barbiturates.

2.4.3.2. Dimethyl sulphate. Barbiturates have been methylated with dimethyl sulphate in the presence of aqueous alkali [81,82]. Extensive degradation of the methyl derivatives of barbiturates has been observed if the time for the reaction is not rigidly controlled [83]. Extraction of the reaction mixture by an organic solvent is required. Hexane provides clean extracts though the efficiency of extraction is improved when a more polar solvent like benzene is used [82]. There are no peaks owing to derivatizing reagents as the excess reagent is completely destroyed. This approach is particularly suitable with the use of the nitrogen—phosphorus detector as the reagents do not contain nitrogen. In an extension of this procedure derivatization has been carried out with bis(2-chloroethyl) sulphate in the presence of triethylamine in a non-aqueous medium. The resulting N,N-dichloroethyl derivatives of barbiturates show high response to the electron-capture detector. Excess reagents and halogenated reaction by-products are removed by evaporation and washing of the reaction mixture with water [84].

2.4.3.3. Iodoalkanes in acetone and potassium carbonate. Another conve-

TABLE 2

GAS CHROMATOGRAPHY OF UNDERIVATIZED BARBITURATES

Drug	Retention time (min)	Sample[a] (ml)	Extraction	Column	Oven temp. (°C)	Detector	Reference
Allobarbital	2.20	3	Dichloromethane	Stainless steel	220	Flame ionization	118
Amobarbital	2.68			1.8 m × 3 mm	12/min		
Butabarbital	2.44			6% SE-30 + 4% XE-60	240		
Heptabarbital	7.22			Chromosorb W			
Hexobarbital	2.84			80—100 mesh			
Pentobarbital	2.80						
Phenobarbital	3.16						
Secobarbital	6.84						
Barbital	0.5	0.1	Chloroform	Glass	220	Nitrogen—phosphorus	69
Hexobarbital	1.5			1.8 m × 2 mm			
Mephobarbital[b]	1.8			3% SP-2250			
Pentobarbital	0.9			Chromosorb W			
Secobarbital	1.1			100—120 mesh			
Allobarbital	4.7	0.05	Chloroform[c]	Glass	200	Flame ionization	61
Amobarbital	6.4			0.9 m × 4 mm[d]			
Barbital	3.1			3% Poly A103			
Butabarbital	5.5			Chromosorb W			
Cyclobarbital	23.4			80—100 mesh			
Heptabarbital	34.3						
Hexobarbital	5.3						
Pentobarbital	7.3						
Phenobarbital	36.4						
Secobarbital	9.0						
Allobarbital	4.6	2	Ethylene dichloride	Glass	170	Flame ionization	60
Barbital	4		→ sodium phosphate	1.5 m × 2.8 mm	8/min →		
Cyclobarbital	12.4		→ pH 1	10% Dexil 300 GC[e]	260		
Pentobarbital	8.6		→ ethylene dichloride	Chromosorb W			
Phenobarbital	12.4			80—100 mesh			
Secobarbital	9.2						
Amobarbital	5.2	0.5	Dichloromethane	Fused silica	130 for 3 min	Nitrogen—phosphorus	77
Barbital	3.2			15 m × 0.25 mm	12.8/min →		
Butabarbital	2			DB-5 (0.25 μm)	290		
Mephobarbital	7.1						
Pentobarbital	5.6						
Phenobarbital	7.8						

Allobarbital Amobarbital Barbital Butabarbital Cyclobarbital Heptabarbital Hexobarbital Mephobarbital Pentobarbital Phenobarbital Vinbarbital	6.5 7 5.5 6.8 8.5 9 7.3 7.7 7.1 8.9 7.5	0.4	Diisopropyl ether	Fused silica[f] 12 m Methylsilicone	100 for 1 min 20/min → 155 for 2 min 20/min → 240	Nitrogen—phosphorus	23
Hexobarbital[b] Methohexital	2.5 1.5	1	Light petroleum— diethyl ether— propanol (50:50:2)	Glass 1.8 m × 2 mm 3% OV-17 Gas Chrom Q 100—120 mesh	220	Nitrogen- phosphorus	75
Hexobarbital Methohexital[b]	3.5 2.2	2	Light petroleum— amyl alcohol (100:2)	Glass 1.8 m × 4 mm Gas Chrom Q 60—80 mesh	230	Nitrogen—phosphorus	73
Cyclobarbital[b] Thiopental	3.8 1.8	1	Chloroform[g]	Glass 1.8 m × 2 mm GP—2% SP 2510 DA Supelcoport 100—120 mesh	210	Flame ionization	68
Thiopental	3	0.5	Dichloromethane	Glass 1.8 m × 2 mm 2% SP 2110— 1% SP 2510 DA Supelcoport 100—120 mesh	205	Flame ionization	30

[a] Sample is blood, plasma or serum.
[b] Used as internal standard.
[c] Small volume of solvent is used for extraction which is injected directly.
[d] A second column 1.8 m long packed with 3% CDMS is also used simultaneously.
[e] Another column packed with 3% OV-17 was also used. The columns were deactivated by repeated injections of tetraethyl orthosilicate.
[f] Deactivated with Carbowax 20 M (Hewlett-Packard Part No. 19091-600101).
[g] Extraction is repeated twice.

nient technique is to reflux barbiturates in acetone with methyl iodide in the presence of potassium carbonate. The reaction can be applied on a micro-scale and the reaction mixture can be injected directly with the use of the flame ionization detector [85]. This procedure has been extended for the preparation of dialkyl derivatives other than methyl [86]. It has been claimed that a mixture of acetone and methanol leads to better reaction rates than when acetone alone is used owing to the higher polarity of the mixed solvent [87]. The reagents used in this technique do not contain nitrogen, hence do not produce any interfering peaks when the reaction mixture is chromatographed using the nitrogen—phosphorus detector [88]. A number of barbiturates have been separated on a capillary column after preparation of ethyl derivatives with ethyl iodide in acetone in the presence of potassium carbonate [34]. A similar technique has been used to prepare the pentafluorobenzyl derivative of pentobarbital by refluxing it with pentafluorobenzyl bromide in alcohol in the presence of potassium carbonate. The product shows high response to the electron-capture detector [51]. However, the reaction is very slow as the reaction mixture has to be refluxed for 4 h. In another approach triethylamine is used as a base instead of potassium carbonate to avoid hydrolysis of barbiturate on prolonged contact with alkali [50]. Concentration of triethylamine is critical for the formation of dipentafluorobenzyl derivatives of barbiturates.

2.4.3.4. Extractive alkylation. Extractive alkylation with a quaternary ammonium ion as the counter ion in the presence of methyl, ethyl or pentafluorobenzyl halides leads to the facile formation of dialkyl derivatives [89,32,33]. However, the extracts not only contain excess alkylhalides but some of the quaternary salts are also co-extracted. These extracts produce large solvent peaks with NPD and ECD. Furthermore decomposition products of the quaternary salt produce peaks which may interfere wih the analysis of barbiturates [89]. These interferences can be decreased by evaporation of the extract and subsequent re-extraction of the residue with non-polar solvents like hexane [32].

2.4.3.5. Pyrolytic alkylation. Pyrolytic alkylation remains the most commonly used approach for alkylation of barbiturates where a barbiturate dissolved in a selected quaternary ammonium hydroxide is directly injected onto a GC column [90]. Phenyltrimethyl ammonium hydroxide (PTMAH), marketed under the trade name of MethElute, is considered a better methylating agent than tetramethyl ammonium hydroxide (TMAH) for "on-column" methylation. In a number of procedures these reagents have been incorporated in the solvent used for extraction to obtain clean extracts containing acidic drugs from biological fluids [26,27]. These reagents are used in adequate concentrations so that the solvent—reagent peak is not excessively large with the use of either FID [26,27] or NPD [91]. Dialkyl derivatives other than dimethyl, e.g. diethyl, dibutyl [92] and dihexyl derivatives [93], have been prepared by using tetraethyl ammonium, tetrabutyl ammonium or tetrahexyl ammonium hydroxide, respectively. These quaternary ammonium hydroxides are either commercially available or can be readily prepared by treating methanolic solutions of the corresponding quaternary iodide with silver oxide. This pyrolytic technique for the preparation of dialkyl derivatives of barbiturates has a serious drawback. Barbiturates, particularly phenobarbital, produce

multiple products owing to thermal decomposition of the dialkyl derivative in the presence of excess strongly alkaline reagent [94]. The products of decomposition of phenobarbital during pyrolytic methylation have been identified by MS [94,95]. A number of attempts have been made to overcome this problem of decomposition to enable this simple derivatization technique to remain useful. In some approaches phenobarbital is determined as a sum of the major peaks [96]. In another approach, the decomposition of the dimethyl derivative was deliberately forced by using a much stronger solution of PTMAH and the "early phenobarbital" peak was used for quantitation of phenobarbital [97]. It has been claimed that the decomposition of phenobarbital can be prevented if the pyrolytic reaction is carried out in the test tube at 100°C instead of in the injection port of the gas chromatograph at 220—250°C [98]. This approach is particularly useful with the use of the nitrogen—phosphorus detector as volatile nitrogenous by-products of pyrolysis are lost and the resultant solvent peak is of reasonable size. This approach was used for the simultaneous determination of the major antiepileptic drugs [99]. The nearly neutral reaction product is also desirable for the long column life. It has also been suggested that a low concentration of the derivatizing reagent be used and that GC be carried out immediately after mixing the reagent with the barbiturate to minimize the formation of the "early phenobarbital" by-product [25,100,101].

There have been many other suggestions to minimize the decomposition of phenobarbital. Thus, it has been observed that flash heater ethylation leads to insignificant decomposition of phenobarbital [102]. Use of viscous polyhydric alcohols as co-solvents leads to significant decrease in the decomposition of phenobarbital [103]. The decomposition of phenobarbital has also been decreased by buffering the derivatizing reagent with phosphate buffer (pH 4) [104]. However, such a system may not be applicable with the nitrogen—phosphorus detector due to bleed of phosphorus-containing compounds. Further, the effect of buffer salts on column life is not clear. Tetramethyl ammonium acetate and phenyltrimethyl ammonium acetate have been proposed [105] to reduce the decomposition of barbiturates during pyrolytic methylation because of their low alkalinity. Finally, it has been suggested that the decomposition of phenobarbital is influenced by the presence of moisture in the derivatizing agent. Drying of the reagent with anhydrous sodium sulphate inhibits the decomposition of dimethylphenobarbital [106].

2.4.3.6. Iodoalkanes in N,N-dimethylacetamide and quaternary ammonium hydroxides. An alternative procedure for alkylation of barbiturates has been proposed to avoid the problems of pyrolytic alkylation [107]. In this procedure barbiturates dissolved in dimethylacetamide form soluble tetramethyl ammonium salts with a methanolic solution of tetramethyl ammonium hydroxide which in turn rapidly react with an alkyl halide to produce the corresponding dialkyl derivative at room temperature. Excess tetramethyl ammonium hydroxide is precipitated as iodide. The supernatant of the reaction mixture is directly applied to a system equipped with a flame ionization detector. However, this reaction mixture is not suitable with NPD. In the latter case the reaction mixture can be extracted with cyclohexane—methylene chloride (95 : 5) and the extract is evaporated to remove dimethylacetamide. The residue

TABLE 3

GAS CHROMATOGRAPHY OF DERIVATIZED BARBITURATES

Drug	Retention time (min)	Sample[a] (ml)	Extraction	Column	Oven temp. (°C)	Detector	Derivatization	Reference
Allobarbital	5.4	1	Chloroform → sodium hydroxide → pH 2 → chloroform	Glass 20 m × 0.5 mm SE-30	135 8/min → 245	Flame ionization	PTMAH[b]	101
Amobarbital	7.1							
Barbital	4.2							
Butabarbital	6.4							
Cyclobarbital	10.95							
Hexobarbital	10.35							
Mephobarbital	10.85							
Pentobarbital	7.5							
Phenobarbital	10.85							
Secobarbital[c]	8.15							
Vinbarbital	7.65							
Allobarbital	5.98	2	Chloroform	Glass 1.8 m × 2 mm 2% OV-17 Chromosorb W 80—100 mesh	170 for 4 min 7.5/min → 260	Flame ionization	N,N-Dimethylacetamide—TMAH—1-iodobutane	107
Amobarbital	7.28							
Barbital	4.52							
Butabarbital	7.00							
Heptabarbital	13.6							
Hexobarbital	9.18							
Mephobarbital	9.72							
Pentobarbital	8.02							
Phenobarbital	11.96							
Secobarbital	8.58							
Vinbarbital	8.58							
Allobarbital	9	0.5	Charcoal[d]	Glass 43 m × 0.5 mm SE-30	170 for 4 min 4/min → 260	Flame ionization	Iodoethane—tetrabutylhydrogen sulphate[d]	32
Amobarbital	11							
Heptabarbital	19							
Hexobarbital	15.2							
Pentobarbital	12							
Phenobarbital	16.2							
Secobarbital	13.5							
Vinbarbital	12.5							
Amobarbital	25	1	Diethyl ether—hexane (1:1) → carbonate buffer → pH 1 → diethyl ether—hexane	Glass 3 m × 3 mm 3% OV-17[e] Chromosorb W 100—120 mesh	150 for 12 min 1/min → 280	Flame ionization	PTMAH[b]	25
Butabarbital	22							
Cyclobarbital	59							
Heptabarbital	69							
Hexobarbital	57.5							
Methohexital[c]	42							
Pentobarbital	30							
Secobarbital	35							
Vinbarbital	35.5							

Amobarbital Heptabarbital Secobarbital	7.5 12.5 8	0.02	Acetone— diethyl ether (1:1)	Glass 0.65 m X 2 mm 3% OV-225 Chromosorb W 120—140 mesh	100 for 2 min 81 min → 240	Flame ionization	Acetone—iodomethane— potassium carbonate	85
Pentobarbital Secobarbital[c]	3.1 3.8	0.1	Diethyl ether	Glass 1.22 m X 2 mm 2% OV-101 Chromosorb W 100—120 mesh	140	Nitrogen— phosphorus	Acetone—iodomethane— sodium carbonate	88
Mephobarbital[c] Pentobarbital Heptabarbital	8.5 5.1 13	1	Toluene → TMAH	Glass 1.8 m X 4 mm 3% OV-17 Gas Chrom Q 100—120 mesh	215	Flame ionization	TMAH—N,N-dimethyl- acetamide—1-iodobutane	112
Allobarbital Amobarbital Barbital Cyclobarbital Hexobarbital Heptabarbital Pentobarbital Phenobarbital Secobarbital Vinbarbital	4.73 5.73 3.88 8.06 7.30 8.90 6.07 7.81 6.47 6.23	0.5	Charcoal → dichloromethane	Fused silica 25 m X 0.2 mm SP-2100[f]	110 10/min → 230	Nitrogen— phosphorus	Acetone—iodoethane— potassium carbonate	34
Phenobarbital[c] Thiopental	1.3 1.9	1	Hexane— 2-propanol (4:1) → sodium hydroxide → pH 2 → hexane	Glass 1.2 m X 0.2 mm 3% OV-17 100—120 mesh	200 32/min → 240	Nitrogen— phosphorus	Acetone—iodomethane— potassium carbonate	119

[a] Sample is whole blood, plasma or serum.
[b] The reagent is mixed with the extract just prior to injection in the syringe.
[c] Used as internal standard.
[d] Followed by extractive alkylation in the presence of tetrabutyl hydrogen sulphate and ethyl iodide.
[e] Another column packed with 3% OV-101 is also used.
[f] Deactivated with Carbowax 20 M (Hewlett-Packard Part No. 19091-60025).

is dissolved in ethyl acetate which is quite suitable for GC with NPD [108]. This approach has been quite popular for the preparation of different types of dialkyl derivatives of barbiturates [109,110]. In a modification of this technique, the initial organic extract was back-extracted with TMAH instead of evaporating the extract to dryness. Derivatization was carried out with the TMAH extract [111,112]. In another modification [113] dimethylacetamide was replaced by acetonitrile and alkylation carried out at 60°C for 1 h rather than at room temperature so that the phenolic group of hydroxyphenobarbital would also be alkylated. The alcohol group of the metabolite of hexobarbital cannot be alkylated by this procedure. After alkylation, the residue is silylated prior to chromatography [114].

2.4.3.7. Dialkylacetals in N,N-dimethylformamide. Another convenient derivatization technique is to inject the barbiturate dissolved in a mixture of dimethylformamide and dimethyl acetal used as the derivatizing reagent [115]. It has been shown that the derivatives formed are methoxyacetals rather than di-N-alkyl derivatives produced by other alkylation reactions [116]. In another report a number of different alkyl groups have been introduced by using different dialkyl acetals [117]. However, these derivatives have been referred to as N,N-dialkyl derivatives [117] in contradiction to the earlier conclusion about the structure of barbiturate derivatives prepared by treatment with dimethylformamide—dialkylacetal [116]. Despite its simplicity and lack of decomposition of the derivatized products, application of this technique to the analysis of clinical samples has not been fully investigated.

Chromatographic data for the determination of barbiturates after derivatization are summarized in Table 3.

2.5. Liquid column chromatography

There has been a phenomenal growth in the use of liquid chromatography for the analysis of endogenous or foreign compounds in body fluids in the last ten to fifteen years. Various quality control surveys for therapeutic drug monitoring indicate that LC is being used by more laboratories to monitor therapeutic concentrations of phenobarbital and other anticonvulsants than GC. A number of attempts are being made to develop general schemes for the identification and determination of hypnotic sedatives by LC [120—122]. In the pharmaceutical industry LC remains the method of choice for routine quality control. This technique is particularly preferred to GC for the determination of polar compounds, which are either difficult to volatilize, tend to get adsorbed on the column or are thermally labile. Recent developments in the detection and column technology for LC as applied for the determination of barbiturates are discussed in the following sections.

2.5.1. Detection

There is as yet no detector for LC which could be compared to either the flame ionization detector or the nitrogen—phosphorus detector used in GC. In the absence of such universal detectors, the UV absorbance detector is the only detector that has been used (with rare exceptions) for the detection of barbiturates. Most of the early LC detectors were single-wavelength detectors, most

often operated at 254 nm. Barbiturates in their unionized form show an extremely weak absorbance at 254 nm and cannot be detected in the extracts of biological samples particularly if present in therapeutic concentrations. A number of alternatives have been suggested in order to improve sensitivity of detection.

2.5.1.1. Detection at low wavelengths. Barbiturates are monitored at 210 nm [122] or at 216 nm [123] in order to improve their detection limit. However, these wavelengths are non-specific and are subject to a greater number of interferences [18,124] than the measurement at 254 nm. Barbiturates can be monitored simultaneously at two different wavelengths to improve selectivity either by connecting two detectors in series [125] or by using dual-wavelength detectors [126]. A number of rapid scanning linear photodiode array spectrophotometers specially designed as LC detectors are currently marketed. These instruments not only allow simultaneous measurement of absorbance of the column eluate at two or more wavelengths but can also provide a complete spectrum of the components in a chromatogram or in a single peak from the stored spectral data [127]. These detectors are potentially very useful for the identification of unknown drugs [127].

2.5.1.2. Detection at alkaline pH. The barbiturates, except thiobarbiturates, have very weak UV absorption properties as free acids. The barbiturates ionize to monoanions at a pH of approximately 10 and barbiturates without N-substitution ionize to dianions at pH >12. The UV absorption properties of the monoanion show an intense band in the 240-nm area. Thus the sensitivity and selectivity of barbiturate detection can be significantly improved by monitoring the absorbance of the LC column eluate at pH > 9. In one approach [128], this was achieved by the post-column infusion of a borate buffer solution (pH 10) into the column eluate stream. There was a twenty-fold increase in the detection limit of barbiturates and no peak broadening was observed. It had been suggested that barbiturates can be adequately separated on reversed-phase silica columns only as free acids and the pH of the mobile phase was adjusted so as to suppress their ionization [123,128]. However, barbiturates have now been separated adequately as monoanions with silica columns, and with a mobile phase of pH $\geqslant 8.5$ [20,126]. This allows optimal detection of barbiturates without having to use additional equipment to achieve post-column change in pH. To avoid the dissolution of costly silica-based analytical columns, guard columns packed with silica are used [126,129]. In another approach barbiturates have been separated on a non-silica PRP-1 column (Hamilton, Reno, NV, U.S.A.) with the use of an alkaline mobile phase [21]. This column does not require a pre-column as the macroporous poly(styrene—divinylbenzene) adsorbent of this column is compatible with mobile phases over the pH range 1—13. In an earlier report barbiturates in pharmaceutical preparations have been separated with the use of a strong anion-exchange resin and an alkaline borate buffer [130]. The absolute retention of the compounds could be varied without changing their elution order by changing the concentration of the anionic competitor, sodium nitrate, in the mobile phase.

Thiobarbiturates are detected with excellent sensitivity at 290 nm at acidic or neutral pH.

2.5.1.3. Derivatization. Pre-column off-line derivatization is a useful

approach to improve the detection limit in LC [131]. Dns derivatives of barbiturates have been prepared for sensitive fluorescence detection [132]. Later, the same group of investigators has advocated the preparation of 4-bromomethyl-7-methoxycoumaryl derivatives of barbiturates for their fluorescence detection in the picomole concentration range prior to LC [133]. More recently, 2-naphthacyl derivatives of barbiturates have been prepared by reacting the barbiturate solution in acetone with 2-naphthacyl bromide in the presence of caesium carbonate at 30°C. The naphthacyl derivatives are stable and absorb strongly at 249 nm, allowing the detection of 1 ng of the derivative. The naphthacyl derivatives of a number of commonly used derivatives have been separated on a μBondapak C_{18} column with 80% methanol [134]. However, the advantages, if any, of naphthacyl derivatives of barbiturates over the commonly prepared phenacyl derivatives of acidic compounds are not clear. It is expected that the separation of naphthacyl derivatives by reversed-phase LC will require a higher concentration of methanol in the mobile phase than the concentration of methanol required for the separation of phenacyl derivatives.

It appears that pre-column derivatization of barbiturates to improve detection has failed to gain popularity for their determination in biological samples. Reagents of appropriate purity and conditions of reaction for derivatization have to be carefully selected so as to produce stable derivatives in reproducibly optimal yields. Furthermore derivatization leads to a decrease of selectivity as any compound other than barbiturate tagged with a chromophore will absorb just as a derivative of barbiturate does. Introduction of common bulky groups may decrease the separation of closely related barbiturates. Separation of barbiturates by GC after derivatization is preferred to separation by LC after derivatization.

2.5.2. Choice of stationary phase

It is estimated that more than 75% of LC separations are now carried out with bonded-phase columns in the reversed-phase mode [135]. It is particularly true for the analysis of biological samples for the determination of drugs. Reversed-phase LC is most commonly performed with columns packed with porous silica with *n*-alkyl groups chemically bonded to its surface. The mobile phase is invariably a mixture of water and an organic modifier which is usually methanol, acetonitrile, or tetrahydrofuran. Unlike GC, the choice of stationary phase for LC is limited, the C_{18} silica column is practically the only column that has been used for the separation of barbiturates. The performance of this type of column has been evaluated for the separation of drugs [136] and it was concluded that the pH of the mobile phase should be carefully selected such that compounds to be separated remain unionized. Use of an ion-pairing reagent, heptane sulphonic acid, in the mobile phase had no effect on the separation of barbiturates [137]. However, as was pointed out earlier, barbiturates are being separated as partially ionized for improved detection [20, 126] or for improved separation [138] without loss of peak symmetry. The macroporous poly(styrene—divinylbenzene), a non-ionic resin adsorbent, also affects LC separations in the reversed-phase mode with the claimed advantage of compatibility of this material from pH 1 to 13 [139]. However, this material has failed to gain popularity as only a few publications have appeared describing its use for the determination of drugs.

TABLE 4

LIQUID COLUMN CHROMATOGRAPHY OF BARBITURATES

Drug	Retention time (min)	Sample[a] (ml)	Extraction	Column	Mobile phase	Flow-rate (ml/min)	UV detection (nm)	Reference
Amobarbital	7	1	Hexane—diethyl ether—*n*-propanol (49:49:2) → 0.01 *M* Na_3PO_4 → pH 2 → organic layer	10 cm × 2.8 mm[b] Methyl silica 5 μm	Methanol—water (35:65)	NA[c]	205, 220	140
Cyclobarbital	3.8							
Heptabarbital	6							
Hexobarbital	4.8							
Phenobarbital	2							
Secobarbital	9.5							
Allobarbitone	1.33[d]	0.1	Hexane—diethyl ether[e] (50:50)	10 cm × 5 mm ODS-Hypersil 5 μm	0.1 *M* NaH_2PO_4—methanol (60:40) (pH = 8.5)	2.0	240	20
Amobarbitone	7.05							
Barbital	0.63							
Butabarbital	3.42							
Cyclobarbital	2.61							
Heptabarbital	4.93							
Hexobarbital	5.67							
Pentobarbital	8.07							
Phenobarbital	1.23							
Secobarbital	11.47							
Thiopentone	9.20							
Amobarbital	23	0.2	Acetonitrile[f]	15 cm × 4.6 mm[g] Ultrasphere-ODS 5 μm	Phosphate buffer, pH 3.2—acetonitrile[h]	3.0	210	122
Butabarbital	15							
Hexobarbital	20.6							
Pentobarbital	22.4							
Secobarbital	25.8							
Allobarbital	5.8	Tissue	Ethyl acetate[i]	30 cm × 4 mm[b] μBondapak C_{18} 10 μm	Methanol—water (1:1)	1.0	210	141
Amobarbital	11.5							
Barbital	4.5							
Cyclobarbital	7.8							
Pentobarbital	11.5							
Phenobarbital	6.1							
Allobarbitone	2.3	Pure standards	—	12.5 cm × 4.6 mm ODS-silica 3—7 μm	0.1% Aqueous $(NH_4)_2CO_3$—methanol (60:40)	1.5	220, 240	125
Amobarbital	5.8							
Barbital	1.8							
Butabarbital	3.7							
Heptabarbital	4.3							
Hexobarbital	5.8							
Pentobarbital	6.8							
Phenobarbital	2.1							
Secobarbital	8.4							
Thiopental	6.1							

(Continued on p. 158)

TABLE 4 (continued)

Drug	Retention time (min)	Sample[a] (ml)	Extraction	Column	Mobile phase	Flow-rate (ml/min)	UV detection (nm)	Reference
Vinbarbital	2.9							
Allobarbital	1.8	0.5	Chloroform → 0.05 *M* sodium hydroxide	15 cm × 4.1 mm RP-1[j] 10 μm	Methanol—acetonitrile—0.02 *M* sodium hydroxide (10:3:87)	1.5	240	21
Amobarbital	6.7							
Barbital	1.1							
Butabarbital	3.1							
5-Ethyl-5-tolyl-barbituric acid[k]	5.5							
Hexobarbital	7.4							
Mephobarbital	10.6							
Pentobarbital	7.5							
Phenobarbital	2.5							
Secobarbital	12.9							
Thiopental	17.5							
Methohexital[k]	1.5	1	Diethyl ether	15 cm × 3 mm Spherisorb ODS NA	Methanol—water (50:50)	—[l]	254	142
Thiopental	0.8							
Thiopental[m]	4.22[d]	0.1	Ethyl acetate	15 cm × 4 mm LiChrosorb RP 18 5 μm	Methanol—water (60:40)	1.0	290	143
Barbital[k]	1	2	*n*-Butyl chloride	12.5 cm × 4.6 mm ODS-silica 5 μm	0.16 *M* Phosphate buffer, pH 6.6—tetrahydrofuran (86:14)	2.0	240	24
Pentobarbital	2							
Thiopental	4							
Thiamylal[k]	5							
Pentobarbital[k]	9	0.2	Chloroform	30 cm × 3.9 mm μBondapak C_{18} 10 μm	0.01 *M* Phosphate buffer, pH 7.8—acetonitrile—tetrahydrofuran (78:22:4)	2.0	254	144
Thiopental	11							
Hexobarbital	7.6	0.2	Dichloromethane	25 cm × 4 mm Nucleosil C_{18} 10 μm	3.5 m*M* NaH_2PO_4—acetonitrile (67:33) → pH 2.7	1.9	238	19
Phenobarbital[k]	4.4							
3-Hydroxy-pentobarbital	6.2	0.1	Ethyl acetate	25 cm × 4.6 mm[g] Partisil ODS-2 10 μm	Tetrahydrofuran—water (5:95)	2.5	215	145
Thiopental[n]	9.7	0.05	Acetonitrile[o]	20 cm × 4.6 mm[b] Spherisorb C_6 5 μm	0.01 *M* Acetate buffer, pH 3.6—acetonitrile (70:30)	1.5	280	146
Thiopental[p]	2.8	0.2	50% Acetonitrile in methanol[q]	30 cm × 3.9 mm μBondapak C_{18} 10 μm	Phosphate buffer, pH 7.9—methanol (22:30)	2.0	280	147

Thiopental[r]	6	0.5	Dichloromethane	25 cm × 4 mm RP-8 10 μm	Methanol—water (60:40)	2.0	290	29
Barbital Hexobarbital Methohexital Pentobarbital Phenobarbital Thiopental	2.9 4.8 7.3 4.5 3.6 6.8	1	Toluene	25 cm × 4 mm LiChroCart RP-18[b] 7 μm	0.05 *M* NaH_2PO_4, pH 4.6—acetonitrile (1:1)	1.0	195	28

[a] Sample is blood, plasma or serum unless noted otherwise.
[b] A precolumn was used.
[c] Not available.
[d] Capacity factors.
[e] Extraction carried out at pH 7.5.
[f] Protein precipitation with acetonitrile—plasma in 1:1 ratio.
[g] Column at 50°C.
[h] Gradient elution.
[i] Homogenized tissue is extracted with ethanol. The residue of ethanol extract is extracted with ethyl acetate at acidic pH.
[j] Poly(styrene—divinylbenzene) resin.
[k] Used as internal standard.
[l] The pump is operated at a constant pressure of 700 kPa.
[m] Carbamazepine (capacity factor = 2.66) used as internal standard.
[n] Flunitrezepam (retention time, t_R = 11.1 min) used as internal standard.
[o] Protein precipitation with acetonitrile in 1:4 ratio.
[p] Flufenamic acid (t_R = 3.8 min) used as internal standard.
[q] Protein precipitation with acetonitrile—methanol in 2:5 ratio.
[r] Phenolphthalein (t_R = 3 min) used as internal standard.

Normal-phase LC with polar silica as stationary phase and a mixture of organic solvents of relatively low polarity as mobile phase is practically outdated. However, LC in this mode has been used as an additional parameter to confirm the identification of an unknown drug made by reversed-phase LC [120,123].

The data concerning LC separation of barbiturates as published in some recent papers are summarized in Table 4.

2.6. *Thin-layer chromatography*

Thin-layer chromatography (TLC) is also a commonly used technique for the detection of drugs [148]. This technique differs from GC or LC in that no special instrument is required if the objective is only a qualitative identification of compounds. In both GC and LC, injection, separation and detection are integrated and the next sample can be analyzed when the previous sample has eluted completely. In TLC these three operations are separate and a number of samples can be processed simultaneously. Furthermore, there is no danger of a ruined column due to the injection of improper sample as the TLC plate is disposable. A number of TLC systems have been evaluated for the separation of barbiturates [6,148]. With rare exceptions, silica gel plates are used as the stationary phase. The commonly used solvents for the development of TLC plates consist of mixtures of semipolar solvents, e.g. chloroform and acetone [138,148]. In some applications general-purpose solvents are used for the separation of drugs of abuse which also include narcotics and non-barbiturate hypnotics besides barbiturates. These solvents are relatively more polar than chloroform—acetone and contain ammonium hydroxide [149,150]. Reversed-phase silica gel plates have also been evaluated for the separation of barbiturates [151]. The plate, after spotting with the reference standards, was dipped in a mixture of mineral oil—light petroleum (1:10). The plate was then developed in water—methanol—ammonium hydroxide (80 : 20 : 2). The migration order of barbiturates is reversed to that observed in normal-phase TLC making the reversed-phase system a useful tool for confirmation.

Visualization of barbiturates on TLC plates is most commonly done with mercury salts—diphenyl carbazone sprays [138]. In some systems the plate is dipped in these reagents. However, there is a trend to avoid the use of mercury salts in clinical laboratories. Increase in the intensity of fluorescence quenching after the plate has been exposed to strong ammonia vapours can provide specific detection of barbiturates [152]. In another approach a 0.1% solution of 2,6-dichlorobenzoquinone-4-chloroimide is sprayed or poured on the dried TLC plate which is then sprayed with a colour developer. This developer is a solution of potassium acetate in aqueous dimethyl sulphoxide [153]. It is claimed that this visualization system provides sensitive and selective detection of barbiturates.

TLC is also a useful technique for the quantitative determination of known drugs. Commercially available TLC scanners or densitometers allow rapid quantitation of separated spots on TLC plates by measuring their reflectance or transmittance in the UV or fluorescence mode. A number of reports for the quantitation of phenobarbital and other drugs have been described [154—156].

Despite its simplicity TLC is not a popular technique for the quantitation of known barbiturates or for detection and identification of possible barbiturate overdose. Spectrophotometric UV procedures are more informative for emergency screening of barbiturates than TLC is. Any positive result suspected by TLC screening requires confirmation. There is no single TLC system which allows the separation of even major barbiturates and the detection systems lack sensitivity, selectivity and are potentially toxic. However, TLC remains the most widely used qualitative technique for the detection of chronic abuse of barbiturates [150].

2.7. *Immunoassays*

Progress in chromatographic techniques gave an impetus for increased demand for drug analysis in clinical laboratories. However, many laboratories could not utilize chromatographic procedures for the determination of drugs because of lack of skilled personnel. Furthermore, chromatographic procedures are slow and in general not amenable to rapid turn around time in cases of emergency. Reagents for immunoassays of drugs including barbiturates have been developed to meet these requirements and are being marketed by a number of suppliers. In most cases these analyses are simple to perform and the results can be produced rapidly.

Reagent kits for barbiturates are available in a number of options. There are reagent kits which allow the detection of barbiturates as a group and the results are qualitative or at best semi-quantitative as the antibody has widely different reactivity with different barbiturates. One of the earliest reagent kits was marketed by Hoffmann-La Roche under the trade name of Barbiturate Abuscreen. This assay utilizes radioiodinated tracer and is applicable to all types of samples including urine, blood or plasma. Recently a modified radioimmunoassay for barbiturates in blood and urine has been developed in a noncommercial laboratory [157].

To avoid the use of radioisotopes, an enzyme immunoassay kit under the trade name Emit Barbiturate DAU is marketed by Syva. Unlike Abuscreen, Emit DAU cannot be applied to the analysis of blood samples. Performance of both of these reagent kits has been compared with TLC [158,159]. More recently another reagent kit under the trade name Emit Barbiturate-tox has been marketed by Syva for emergency detection of barbiturates in blood serum. The use of this reagent kit for the detection of barbiturates in urine is not recommended. Chromatographic procedures are used to identify and quantitate the barbiturates in the sample which gives positive result by Emit screening procedure. In a number of reports known barbiturates have been precisely quantitated with Emit-tox reagents by analyzing plasma standards of a given barbiturate [160—162]. However, if a mixture of barbiturates is present, as is often the case, this approach is less than accurate.

Reagents have also been developed for the specific determination of phenobarbital. For a number of years the Emit Phenobarbital kit remained the most popular technique for therapeutic monitoring of phenobarbital. This assay is quite specific and can be applied for the determination of phenobarbital in the presence of other barbiturates with the exception of mephobarbital. More

TABLE 5

GAS CHROMATOGRAPHY OF NON-BARBITURATE HYPNOTIC-SEDATIVES

Drug	Retention time (min)	Sample[a] (ml)	Extraction	Column	Oven temp. (°C)	Detector	Reference
α-Bromoisovateryl urea[b] α-Bromocaproyl urea[c]	2 3.8	0.1	Diethyl ether—ethanol (5:1)	1 m × 3 mm 5% PEG 20 M Chromosorb G 60—80 mesh	200	Electron-capture	167
Chloral hydrate Trichloroethanol[d]	1 2.5	1	Head space vapours	Glass 1.8 m × 3 mm Gas Chrom Q 80—100 mesh	125	Electron-capture	168
Trichloroethanol[d]	0.8[e]	0.1	Ethanol	Glass 1.5 m × 6 mm 3% Carbowax 6000 Diatomite CQ 85—100 mesh	130	Electron-capture	169
Trichloroethanol[d] Ethchlorvynol Chloromethiazole	2.08 2.8 5.1	0.05	Chloroform	Glass 1.5 mm × 4 mm 2% Carbowax 20 M + 5% KOH Chromosorb W 80—100 mesh	140	Flame ionization	170
Trichloroacetic acid[f]	1.2	0.10	Toluene[g]	Glass 1.8 m × 4 mm 3% OV-17 Gas Chrom Q	80	Electron-capture	171
Chloromethiazole 5-Acetyl-4-methylthiazole[h] 5-(1-Hydroxyethyl)-4-methylthiazole[b]	2.4 1.8 3.7	1	Diethyl ether	Glass 1.5 m × 2 mm 3% OV-225 Gas Chrom Q 100—120 mesh	130	Mass spectrometer	172
5-Acetyl-4-methylthiazole[h] 5-(1-Hydroxyethyl)-4-methylthiazole[b]	4 4.6	0.05—0.5	Diethyl ether → hydrochloric acid → pH 12 → diethyl ether	Glass 1.5 m × 3 mm 5% OV-7 Gas Chrom Q 100—120 mesh	145	Nitrogen—phosphorus	173
Chloromethiazole	2			Glass 1.6 m × 2 mm 1% SP 1000 Supelcoport 100—120 mesh	95	Nitrogen—phosphorus	174

Ethchlorvynol	2.5	5	*n*-Hexane	Glass 1.8 m × 2 mm 3% OV-17 Chromosorb G 80—100 mesh	125	Electrochemical	175
Ethchlorvynol	1.9	2	Chloroform	Glass 1.8 m × 4 mm 3% OV-1 Gas Chrom W 80—100 mesh		Flame ionization	176
Glutethemide	10	1	Chloroform	Steel 1.8 × 4 mm 3% OV-17 Gas Chrom Q	180 for 6 min → 24/min → 190	Flame ionization	177
Glutethemide[j]	5	2	Diethyl ether	Glass 1.8 × 4 mm 8% SE-30 Supelcoport 80—100 mesh	215	Flame ionization	178
Glutethemide[j]	1.8	10	Chloroform	Glass 2 m × 4 mm 3% SP 2250 Chromosorb W 80—100 mesh	210	Flame ionization	179
Glutethemide[k]	2.9	0.1—2	Diethyl ether	Glass 1.5 m × 2 mm 3% OV-1 Supelcoport 80—100 mesh	220	Mass spectrometer	180
Meprobamate	7.5	1	Cellulose column → chloroform	Glass 1.8 m × 2 mm 0.3% PDEAS + 3% DC560 Gas Chrom P 100—120 mesh	225[l]	Flame ionization	181
Meprobamate[m]	5.5	1	Diethyl ether	Glass 1.5 m × 6 mm 3% SE-30 Chromosorb W 80—100 mesh	115	Flame ionization	182

(Continued on p. 164)

TABLE 5 (continued)

Drug	Retention time (min)	Sample[a] (ml)	Extraction	Column	Oven temp. (°C)	Detector	Reference
Meprobamate[n]	3.8	0.5	Dichloromethane	Glass 1.8 m × 4 mm 3% OV-17 Gas Chrom Q 100—120 mesh	260	Flame ionization	183
Methaqualone[o]	2.5	Urine 0.5 ml	Ethanol—chloroform (1:4)	Glass 1.8 m × 2 mm 3% SE-30 Gas Chrom Q 80—100 mesh	255	Flame ionization, nitrogen—phosphorus	184
Methaqualone	2.5	1	*n*-Butylchloride	Glass 1.2 m × 2 mm 1% SP-1000 Gas Chrom Q 100—120 mesh	220 for 4 min → 8/min → 240	Nitrogen—phosphorus	185
Methaqualone	1.6	5	Celite column → dichloromethane	Glass 1.2 m × 2 mm 3% OV-1 Chromosorb W 80—100 mesh	225	Nitrogen—phosphorus	186
Methylprylon	7.3	0.5	Chloroform	Glass 1.8 m × 2 mm 3% OV-17 Gas Chrom Q 80—100 mesh	140 for 2 min → 16/min → 260	Flame ionization	187
Methyprylon	6	2	Chloroform	Glass 50 m × 0.5 mm Superox 4[p]	200	Flame ionization	188
Paraldehyde[q]	1.1	0.1	Head space vapour	Steel 1.8 m × 2 mm 0.4% Carbowax Carbopack A	130	Flame ionization	189
Paraldehyde	12.8	0.3	Head space vapour	Steel 1.8 m × 2 mm 0.2% Carbowax 1500 Carbopak C	120	Flame ionization	190

[a] Sample is blood, plasma or serum unless stated otherwise.
[b] Analogue of carbromal.
[c] Used as internal standard.
[d] Active metabolite of chloral hydrate.
[e] Retention time relative to that of dibromobenzene used as internal standard.
[f] Toxic metabolite of chloral hydrate.
[g] Toluene extract was methylated with boron trifluoride in methanol.
[h] Active metabolite of chloromethiazole.
[i] Extract injected directly without evaporation to avoid losses of ethchlorvynol.
[j] Hydroxymetabolite of glutithemide also determined after acetylation.
[k] Hydroxymetabolites have been determined after preparing trifluoroacetyl derivatives.
[l] The injector is maintained at a lower temperature (155° C) to minimize decomposition of meprobamate.
[m] Meprobamate is hydrolyzed with alkali, re-extracted with diethyl ether and silylated with trimethylsilyl-acetamide.
[n] Mepromate is hydrolyzed with alkali, converted to benzoyl derivative by Schotten-Baumann reaction and re-extracted with dichloromethane.
[o] Heptafluorobutyryl derivatives of metabolites were prepared.
[p] Equivalent to Carbowax 20 M.
[q] Retention time of acetaldehyde formed by hydrolysis of paraldehyde with sulphuric acid.

TABLE 6

LIQUID CHROMATOGRAPHY OF NON-BARBITURATE HYPNOTIC-SEDATIVES

Drug	Retention time (min)	Sample[a] (ml)	Extraction	Column	Mobile phase	Flow-rate (ml/min)	UV detection (nm)	Reference
Bromisoval Carbromal	2.5 5	0.5	Charcoal → dichloromethane—isopropanol—diethyl ether (65:10:25)	25 cm X 3.1 mm LiChrosorb RP-18 10 μm	Acetonitrile—water (26:74)	2	210	191
Bromisoval Carbromal	7 11	5	Chloroform—ethyl acetate (1:1)	30 cm X 3.9 mm μBondapak C_{18} 10 μm	Methanol—water (1:1)	1.2	205	192
Carbromal	NA[b]	0.5	Dichloromethane	15 cm X 3 mm Partisil Si 5 μm	Tetrahydrofuran—methanol—dichloromethane (6:0.2:93.8)	1	254	193
Chlormethiazole	9	0.5	Methanol[c]	30 cm X 3.9 mm μBondapak C_{18} 10 μm	Methanol—water (45:55)	1.8	254	194
Chlormethiazole	4.2	0.1	Phosphotungstic acid	25 cm X 4.6 mm Ultrasphere C_{18} 5 μm	Acetonitrile—0.025 *M* potassium phosphate, pH 4.6 (45:55)	2	254	195
Ethchlorvynol[d]	5	NA	*n*-Heptane	30 cm X 4 mm μBondapak C_{18} 10 μm	Methanol—water (60:40)	1	280	196
Meprobamate[e]	6	0.5	Dichloromethane	15 cm X 4.6 mm Ultrasphere RP-18 5 μm	Acetonitrile—methanol—water (60:30:30)	2.5	230	183
Methaqualone	6	0.5	Direct injection[f]	25 cm X 4.6 mm LiChrosorb RP-8 10 μm	Acetonitrile—citrate buffer, pH 5.45 (32:68)	3	265	197

[a] Sample is blood, plasma or serum unless stated otherwise.
[b] Not available.
[c] Protein precipitation with an equal volume of methanol.
[d] Ethchlorvynol is hydrolyzed and derivatized with semi-carbazide.
[e] Meprobamate is hydrolyzed with alkali, converted to benzoyl derivative by Schotten-Baumann reaction and re-extracted with dichloromethane.
[f] The sample is purified on-line with extraction columns filled with XAD-2 resin.

recently fluorescence polarization immunoassay reagents for therapeutic monitoring of phenobarbital have been marketed by Abbot Labs. under the trade name of TDX-Phenobarbital. This technique is gaining popularity because of ease of performance of the assay. The instrument required for this assay is fully automatic. However, the reagents for phenobarbital are not specific and show variable reactivity for other barbiturates [163]. In fact these reagents have been adopted for the determination of pentobarbital [164].

3. NON-BARBITURATE HYPNOTIC SEDATIVES

This group of drugs consists of a large number of compounds with diverse chemical and pharmacological properties (see Tables 5 and 6). These compounds are used only as hypnotic sedatives and unlike barbiturates are used rarely for alternative therapeutic purposes. These compounds are being prescribed for the treatment of insomnia to a much lesser extent than the benzodiazepines or the barbiturates. Therefore the need for the determination of these compounds is also limited. Furthermore, there is no agreement about the usefulness of analytical results for these compounds in the management of a poisoned patient. Procedures for the determination of these compounds in biological specimens have been reviewed [165].

There is no single rapid screening procedure for these drugs as a group. Different colorimetric or spectrophotometric procedures are available for individual drugs [165]. It appears that many laboratories use such procedures to monitor occasional poisonings with these agents [166]. Both GC and LC methods have been described for sensitive and specific determination of these drugs. In some GC procedures [11,34,40,55,61] or LC procedures [120—122] these drugs are detected as a general screen for drugs. Some of the GC procedures for the determination of these drugs are summarized in Table 5 and the LC procedures are summarized in Table 6. For some drugs active metabolites are also monitored. In case of poisoning with chloral hydrate only the active metabolite trichloroethanol is monitored in blood as the parent compound chloral hydrate is present only in trace amounts.

4. CONCLUSIONS

Barbiturates are still being used extensively as hypnotic sedatives and for other therapeutic indications despite the introduction of relatively less toxic agents for the treatment of insomnia. The techniques for the determination of barbiturates in biological samples are undergoing rapid changes.

It appears that therapeutic concentrations of known barbiturates can be determined without derivatization with the use of fused-silica columns and a nitrogen-specific detector. Simple pyrolytic methylation of barbiturates with phenyltrimethyl ammonium hydroxide can be conveniently used when only packed columns are available. Decomposition of methylated products can be avoided by completing the pyrolysis in the test tube rather than in the injection port.

Liquid column chromatography is particularly suited for monitoring thiopental as it can be detected with required sensitivity at any pH. Other barbitu-

rates can be monitored with the use of an alkaline mobile phase provided that adequate precautions are taken to protect the silica-based analytical column.

The immunoassay technique, Emit, is a very popular and a reliable technique for therapeutic monitoring of phenobarbital. Fluorescence polarization immunoassay, TDX, is also a convenient and reliable technique for monitoring phenobarbital provided other barbiturates are not present.

The spectrophotometric procedure is still a commonly used screening procedure for the diagnosis of barbiturate poisoning. In some laboratories screening for barbiturates is carried out by Emit reagents. The positive samples are then analyzed by GC or LC for further identification and precise quantitation of barbiturates.

It appears that the use of dual-capillary columns and a flame ionization and nitrogen—phosphorus detector can be useful for general screening of unknown drugs. Most drugs can be chromatographed on modern silica capillary columns without derivatization and retention times are reproducible. Both types of detectors are nearly universal detectors and the ratio of response factors of the two detectors can provide an additional parameter for the identification of unknown drugs.

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6. SUMMARY

Barbiturates and other traditional non-barbiturate sedative hypnotics are still extensively prescribed for the treatment of insomnia. There are a number of situations where identification or quantitative determination of these agents in biological fluids is required. Gas-chromatography offers highly sensitive and specific procedures for the determination of these compounds. The use of a nitrogen-specific detector allows a relatively simple sample preparation for sensitive detection and the use of capillary columns with bonded liquid phase allows separation of barbiturates without derivatization. In recent years liquid chromatography has also been extensively applied to the determination of these compounds. Sensitivity and selectivity of detection of barbiturates have been improved with the use of an alkaline mobile phase. Immunoassays for the determination of therapeutic concentrations of phenobarbital are very popular as the assays can be rapidly performed automatically. Use of these techniques has been extended for emergency detection of barbiturate overdose and for monitoring high-dose pentobarbital therapy.

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