Journal of Chromatography, 220 (1981) 253–274
Chromatographic Reviews
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHREV. 151

ANALYSIS OF BARBITURATES BY GAS CHROMATOGRAPHY

DILRANJAN N. PILLAI
Department of Clinical Chemistry, Prince Henry Hospital, Sydney, N.S.W. (Australia)
and
SERGIO DILLI*
Department of Analytical Chemistry, School of Chemistry, University of N.S.W., P.O. Box 1, Kensington, N.S.W. (Australia)

(Received July 14th, 1981)

CONTENTS

1. INTRODUCTION

Barbiturates are derivatives of malonyl urea, formed by replacement of both hydrogen atoms on the carbon at position 5 by alkyl, aryl or alicyclic groups. The synthesis of barbituric acid (or malonyl urea) [2,4,6-(1H,3H,5H)pyrimidinetrione] was reported by Von Baeyer[1] as early as 1864, but it was only the subsequent discovery of the hypnotic properties of barbital, by Fischer and Von Mering[2] in 1903, which led to the extensive development of the barbituric acid class of drugs. They are most frequently used as sedative hypnotics and anticonvulsants but can also be employed intravenously to effect surgical anaesthesia. Their action on the central nervous system (CNS), and the extent of CNS depression, is dependent on the particular barbiturate. Although variations in the pharmacological properties of the various barbiturates depend on the nature of the 5-substituted entities, the chemical groups of major importance are the imino hydrogens. A definitive publication by Doran[3] in 1959, appears to be the only monograph providing useful information including details of syntheses, chemical and physical properties, reactions, as well as pharmacology.

0378-4355/81/0000–0000/$05.50

Despite the numerous publications that describe the gas chromatography (GC) of barbiturates, there are few major reviews devoted solely to the subject. The literature to 1966 has been reviewed by Brochmann-Hanssen[4], otherwise the subject appears to have been treated only in reviews on general methods[5–7] or in applications of GC in toxicology[8]. The use of paper chromatography, thin-layer chromatography (TLC) and GC in barbiturate analysis have been reviewed by Melzacka[5] in 1971, and an assessment of methods available in 1972 was made by Kananen *et al.*[6]. Selected methods for the screening and identification of barbiturates from biological fluids were again reviewed by Jain and Cravey in 1974[7].

Apart from GC, numerous other methods for the determination of barbiturates include ultraviolet (UV)[9], infrared (IR)[10], nuclear magnetic resonance (NMR)[11,12] and mass spectroscopy (MS)[13,14]; TLC[15,16] and high-pressure liquid chromatography[17], as well as enzyme-[18] and radioimmunoassay[19,20] methods. This variety implies as many different types of problems and shows an historical development also.

In this review, an attempt has been made to comprehensively survey the advances in the analytical chemistry of the barbiturates studied by the GC technique.

2. ANALYSIS OF FREE BARBITURIC ACIDS

GC methods for the analysis of barbiturates were first reported in 1960 by Janak[21]. His method involved heating the sample to 800°C, followed by chromatographic separation of the pyrolytic products. The characteristic pyrolysis pattern facilitated quantification as well as identification. In 1962, a similar procedure[22] gave unique patterns for 22 barbiturates in which the most significant pyrolytic products were identified as nitriles. While these methods appeared to be satisfactory for the analysis of single barbiturates, pyrolysis of mixtures were not considered and would probably have given complicated patterns incapable of resolution. The lack of further reports on pyrolysis methods for barbiturates is indicative of their limited usefulness.

Significant early work on the GC of barbiturates in biological fluids, was done by Baerheim Svendsen and Brochmann-Hanssen[23], Parker and co-workers[24,25] and Anders[26], and the use of two columns was commonly recommended[23,27,28] for barbiturate mixtures which could not be separated on a single column. Of particular relevance are the problems with tailing and adsorption noted in much of the early work. To overcome these, Cieplinski[29] incorporated high-molecular-weight organic acids into the stationary phase to neutralize active sites in the column and reduce peak tailing. McMartin and Street[30,31] obtained similar results with tristearin.

After Bohemen *et al.*[32] showed that absorptive inertness was conferred on diatomaceous-earth supports by silylation, this technique found wide application in the GC of barbiturates. Another approach introduced to minimise adsorptive losses was the saturation of the active sites of the column by the injection of large amounts of barbituric acids[26,33–36] onto the column. However, deactivation here was probably only temporary due to slow elution of the barbiturate from the column, causing re-exposure of the active sites. Predictably, variations in the retention times of barbituric acids were observed in cases where adsorption was suspected[33,34,37].

Studies in 1963 showed that addition of formic acid vapour to the carrier gas, improved the chromatographic properties of several fatty acids[38]. This technique when

first applied to the barbiturates in 1970[39], improved the resolution of 6 barbiturates on an Apiezon L column, yielding good peak symmetry from nanogram quantities. Ioannides *et al.*[40] explained the improvement by postulating that adsorption could result from hydrogen bonding of the imino-protons of a barbiturate with Si–OH and Si–O–Si groups in the diatomite support. The former groups, of course, were deactivated by silanization but the latter acted as proton acceptors to form hydrogen bonds with barbiturates, as had been demonstrated for barbiturates with adenine derivatives[41]. Because of its strong tendency to form hydrogen bonds, it was postulated that formic acid occupied all Si–O–Si sites, thereby preventing adsorption of the barbiturates.

In an application of this technique to the analysis of pharmaceuticals, Greenwood *et al.*[42] demonstrated the on-column liberation of the free acids following direct injection of barbiturates as their sodium salts. Barbiturates extracted from blood[43,44] were well resolved on SE-30 columns with such a system, despite the fact that decreased column life and increased noise were noted[45]. Although several methods for the saturation of carrier gas with formic acid are known, Woo and Lindsay[46] have recently described a simple device, claimed to be safe and effective, for barbiturate and fatty acid analysis.

The use of a wide variety of stationary phases are a feature of the literature on the GC of barbiturates. For example, in an attempt to identify a *single* column capable of specific and reliable identification, a critical examination of 12 different columns was made by Berry[47] who found a moderately polar 4% CDMS column most satisfactory, with 3% OV-225 as the second choice. Mixed liquid phases in columns, have been investigated too in attempts to optimize the separation of barbiturate mixtures. Phases investigated were SE-30–Carbowax[48,49], Apiezon L–NPGA[50], SE-30–XE-60[51] and Apiezon L–SE-30–Tristearin[43]. Of these, the last appeared to provide the best resolution.

Solid injection techniques which produce solvent-free chromatograms have useful application in the GC of barbiturates[52–55]. Optimal conditions for their determination were investigated by Rasmussen *et al.*[52] who found no difference in analytical precision with either liquid or solid injection at flash-heater temperatures over 230°C and injection times of 30 sec. Micropacked[45] and support-coated, open-tubular columns[56] in conjunction with solid injection, have also been used. For the latter procedure, columns with high plate numbers, sensitivity of the order of 10^{-10} g, and high precision were claimed.

Many methods have been proposed for the determination of barbiturates in the presence of other drugs[57–64] and specific procedures for the estimation of barbiturates in pharmaceutical preparations also exist[65–67]. Progressively, with increases in the sensitivity of GC methods, pharmacokinetic studies became possible and levels of amobarbital[68–74], pentobarbital[73,75,76] and phenobarbital[77] were determined in both humans and animals. In most cases, pharmacokinetic parameters were evaluated from concentration–time plots. In another case, the primary metabolite of amobarbital, 3′-hydroxyamobarbital was estimated in blood[54,69,78] and urine[68,69,74,78]. Here, use of the polar phase FFAP permitted the determination of 2 μg of this metabolite in either plasma or urine[78]. More recently, the use of an even more polar phase WG11 for estimation of 3′-hydroxyamobarbital, was reported by Kinsella *et al.*[74]. However, Garrett *et al.*[70] in a detailed study of the pharmacokinetics of amobarbital,

pre-saturated the cyanoalkylsilicone stationary phase (GE-XE-60) with repetitive injections of the metabolite to obtain high precision analysis at the 0.1 μg/ml level, in plasma. In contrast to the use of these polar phases, the principal metabolite of hexobarbital, [3,5-dimethyl-5-(3-oxocyclohexenyl)barbituric acid] has been determined in 0.5 ml of blood with 10% UCC W-982 in a stainless-steel column[79].

Following the advent of the nitrogen-specific detector[80], which exhibits a reduced response to many co-extractable interferents in serum, determinations have been reported of nanogram amounts of thiopental[81–84], pentobarbital[83] and hexobarbital[85] in blood, and trace amounts of phenobarbital in plasma[86] and brain tissue[87]. However, adsorption of barbituric acids by the column appear to limit a further lowering of detection limits. Thus, Dvorchik[88] found that at least 10 ng of barbituric acid had to be injected onto the column before adsorption effects were negligible.

Perhaps the most novel application[89,90] to barbiturate analysis is that of the electrolytic conductivity detector. Up to 0.1 μg/ml of barbiturate in serum or urine was determined without sample clean-up, although column resolution deteriorated gradually with injection of direct extracts.

3. PRIOR DERIVATIZATION OF BARBITURIC ACIDS

Despite increases in sensitivity and selectivity made possible by improvements in column technology and detector specificity, GC methods involving underivatized barbiturates are clearly limited by column adsorption. Since 1975, the authors of over 75% of important publications on the GC of barbiturates, have utilised derivatization procedures prior to analysis.

During the GC separation of many drugs, compounds capable of hydrogen bonding appear to adsorb strongly and, for the barbituric acids adsorption of submicrogram quantities on chromatographic columns is common. The consequences of adsorption are the loss of material, column contamination and unsatisfactory peak profiles, with tailing increasing in severity as sample size or concentration is reduced.

Reduction in polarity of the free acid has been the primary objective in the derivatization of barbituric acids and is virtually limited to alkylation. These derivatives are far less polar than the free acids due to conversion from secondary to tertiary amides so that stationary phases suitable for derivatized barbiturates are also generally less polar than those employed for the free acids. SE-30 and OV-17 now appear to be the phases of choice.

3.1 Methyl derivatives

The GC of barbiturate derivatives was first reported by Cook *et al.*[91] in 1961. Here, overnight methylation with diazomethane was followed by chromatographic separation of 11 barbiturates as their 1,3-dimethyl derivatives. Unfortunately, inadequate resolution of mixtures of barbiturate derivatives still necessitated the use of two columns. Later, in 1966, Stuckey's method[92] for alkylation with dimethyl sulphate was adapted by Martin and Driscoll[93] for the microscale methylation of several barbiturates. In this method, the free acid extracted from 2 ml of serum was heated briefly with the alkylating agent, then after acidification and reextraction, the extract was chromatographed. Another method requiring only 15 min was reported by Stewart *et al.*[94] who subjected the barbituric acids in serum or biological tissue to

direct methylation. Good recoveries of phenobarbital (78–98%)[95] and other barbiturates (96–104%)[96] were also reported for methylations with dimethyl sulphate. Subsequently, successful use of this reagent for derivatization purposes has been described by other workers[97–99].

Since the amide functionality of the barbiturate pyrimidinetrione ring can tautomerise to the lactim form, methylation can result in the formation of either N-methylated and/or O-methylated derivatives. N,O′-methylated derivatives are also feasible. Although NMR studies by Neville[100] indicated that N-methylation was exclusive with dimethyl sulphate, and predominant with diazomethane, evidence exists for formation of small amounts of N,O′-dimethyl[101] and N,O′-diethyl derivatives[102,103] when barbiturates are alkylated by the respective dialkyl sulphate.

The Claisen synthesis of allyl-phenyl ethers was adapted by Dünges and Bergheim-Irps[104] in 1973 for the methylation of barbiturates. This was achieved by refluxing an acetone solution of the barbituric acid with the alkylating agent (methyl iodide) and a condensing agent (potassium carbonate) and resulted in a yield of 98% ± 6% (standard deviation, SD). The procedure was later extended by Dünges, to alkylations involving ethyl, allyl, methoxymethyl and benzyl derivatives[105,106]. Features of this technique were the direct injection of the reaction mixture into the GC and a micro-refluxer for handling microlitre[106,107] or millilitre[101] amounts of reactants. During the methylation of barbiturates with alkaline methyl iodide, Wu and Pearson[108] found, in a variation of this procedure, that improved reaction rates were obtained with a mixed solvent system of acetone–methanol than with acetone alone. The improvement was attributed to the enhanced polarity of the mixed solvents. Recently, Dünges *et al.*[109] reported the determination of several barbiturates as the allyl, alkyl or benzyl derivatives with glass capillary columns, obtaining good resolution after extraction from blood. Also in 1979, Sun and Hoffman[110] utilised the method of Dünges and Bergheim-Irps[104] to estimate several barbiturates in serum, using nitrogen-specific detection to successfully improve selection and sensitivity.

Methylation of hydroxylated barbiturate metabolites results in alkylation of the imino protons but not necessarily of the hydroxyl group attached to substituents at C-5. To avoid confusion over the identity of the products when barbiturate metabolites were alkylated, Horning *et al.*[111] silylated the hydroxyl group *after* the methylation procedure. The derivatized products were presumably, identical to those identified in later publications from the same laboratory[112,113]. Here, the methylation-silylation procedures resulted in conversion of the imino protons of the barbituric acids and their metabolites, to N-methylated groups. Additionally, any aromatic hydroxyl groups formed a mixture of methyl and trimethylsilyl ethers whereas non-aromatic hydroxyl groups were converted to trimethylsilyl ethers. Identification of these products was made by GC–MS and the technique utilised for the detection of the epoxide metabolites of some barbiturates in rat urine.

Methylation of barbituric acids by extractive alkylation was first reported by Ehrsson[114]. In this reaction, pentobarbital and phenobarbital were extracted as ion pairs from an aqueous phase into an organic phase having a weak solvating capability, resulting in enhanced susceptibility of the barbiturates to the nucleophilic displacement reaction with methyl iodide.

3.2 Other alkyl derivatives

Since the technique of methylation was incapable of distinguishing between mephobarbital and phenobarbital because the methylated products were identical, it is not surprising that to overcome this limitation, and also improve the resolution of other barbiturates, derivatization to form higher alkyl homologues was examined. Several different reactions were employed to achieve derivatization.

Extractive alkylation with ethyl iodide and tetrabutylammonium hydrogen sulphate was employed for the formation of ethylated barbiturate derivatives[115]. Here, satisfactory separation of 15 ethylated barbiturates on an SE-30 support-coated, open-tubular column was reported, although mephobarbital was not included. The Claisen type reaction for preparation of propyl derivatives was described by IJdenberg[116] who reported successful resolution of mephobarbital and phenobarbital, as well as several other anticonvulsants, on a 3.8% SE-30 column which was temperature-programmed. Propylation was effected by heating for 1 h in a sealed tube containing nitrogen.

Butylation of several barbiturates was reported by Greeley[117] in 1974. Derivatization depended on formation of a soluble tetramethylammonium salt of the barbiturate in a highly polar solvent system, followed by a fast S_N2 reaction of the anion of the salt with iodobutane. Separation of 14 barbiturates was obtained, although overlap with some uncommon barbiturates occurred. More recently in 1979, the butylation of several barbiturates, amongst other drugs, was described by Roseboom and Hulshoff[118]. After extraction from acidified plasma and back extraction into tetramethylammonium hydroxide, the drugs were reacted with N,N'-dimethylacetamide and *n*-butyliodide prior to GC. Mephobarbital, phenobarbital and heptabarbital were satisfactorily resolved from each other on a 3% OV-17 column.

Menez *et al.*[119] made a systematic study of the GC behaviour of several barbiturates after N-alkylation with straight-chain alkyl groups from C_1 to C_6, using the technique of Greeley[117]. GC on OV-101, Dexsil 300 GC, SP-2250 and OV-7 columns showed that the smallest change in retention time was observed between methyl and ethyl derivatives, so that separation of methylated and ethylated barbiturates was not always achieved. Propylated derivatives were considered to exhibit the most desirable chromatographic properties and optimum separation was obtained by temperature programming the Dexsil 300 GC column at 4°C/min, after an initial pause at 140°C for 15 min.

The use of dimethylformamide dimethylacetal for the derivatization of barbiturates has also been investigated[120]. Decomposition of this reagent during the reaction with barbiturates, results in the formation of both CH_3^+ and OCH_3^- species and, thus, either N-methylation or acetal-formation is possible, depending on whether carbonyl polarization is preferred to proton abstraction. In fact, acetal-formation was predominant, and quantitative recoveries of several barbiturates was reported.

An attempt to permethylate barbiturates with methyl iodide and the methylsulphinylmethide carbanion, resulted in the formation of mixtures of three permethylated products for each barbiturate[121]. Efforts to obtain only one derivative for each barbiturate were unsuccessful with the exception of secobarbital. It was concluded that the derivatization, later shown to be useful for estimation of polar glucuronide metabolites[122], was of limited value for analysis of free barbituric acids.

3.3 Electron-capture detection of barbiturates

Although the response of the electron-capture detector (ECD) to free barbituric acids was examined as early as 1965[33], derivatization with a suitable electrophore was only reported recently. Pentafluorobenzyl bromide (PFBB) was employed by Walle[123] in 1975 to alkylate several barbituric acids in which triethylamine was used as the base catalyst in preference to potassium carbonate, since the latter caused hydrolysis of the barbituric acids. Response at picogram levels was obtained and, despite a large increase in molecular weight upon derivatizaton, only a 3 to 4-fold increase in retention times (ranging from 5 min for barbital to 21 min for phenobarbital) was observed on 3% OV-17 at 210°C.

Pentafluorobenzylation of a barbiturate extracted from a biological matrix was first accomplished by Gyllenhaal *et al.*[124]. Here, extractive alkylation with tetrabutylammonium ion and PFBB enabled the determination of 60 ng of phenobarbital in 100 μl of saliva to be made, with a precision of 1.9% (S.D.), after a recovery of 93%. However, the procedure required a pre-column venting system for the removal of excess PFBB from the column to avoid the pronounced detector response which would otherwise make quantification impossible. This method may also be unsuitable for barbiturates with retention times smaller than that of phenobarbital, due to two large unidentified peaks seen in the chromatogram of the extracted saliva sample. This limitation would exclude most barbiturates.

Pentafluorobenzylation of pentobarbital prior to EC detection has been reported by Sun and Chun[125]. The barbiturate extracted from serum was reacted with PFBB and sodium carbonate without apparent interference from excess reagent or from interfering peaks. However, the extraction procedure was time consuming (1 h) and prolonged heating of the reaction mixture (4 h) was required. In addition, further washing and concentration steps were necessary prior to GC. Values for the recovery in the derivatization were not given.

Dilli and Pillai[126] recently described the chloroethylation of several barbiturates, prior to electron-capture detection. After quantitative extraction from saliva, the barbiturate was reacted with triethylamine and bis(chloroethyl) sulphate. Chromatography was effected after washing and concentration steps, the entire procedure taking 2 h for duplicate samples of saliva. Amobarbital, pentobarbital and phenobarbital were determined at levels of 0.10–1.0 μg/ml in saliva. A pharmacokinetic study also enabled the estimation of the *in vivo* biological half lives of amobarbital and pentobarbital to be made.

4. ON-COLUMN DERIVATIZATION OF BARBITURIC ACIDS

The on-column derivatization technique involving *in situ* formation of derivatives in the injection port of the gas chromatograph, was established principally by Robb and Westbrook[127]. The technique is considered by many to be the method of choice for routine analysis of barbiturates and related drugs, due to its rapidity and simplicity.

4.1 Trimethylsilyl derivatives

The estimation of 3′-hydroxyamobarbital by on-column silylation with TMCS and HMDS, was reported by Kamm and Van Loon[128] as early as 1966. Extracted

from urine, the metabolite was converted to a "silyl ether" whose structure was not further specified. In 1969, several barbiturates were derivatized by Street[129] with BSA. Again, the resultant structures were unspecified, although, it was postulated that the barbiturates were monosilylated, at either of the nitrogen atoms. In 1971, 3'-hydroxyamobarbital, extracted from rat-liver homogenate, was silylated with BSTFA and TMCS[130]. Here, GC–MS studies showed a peak at *m/e* 458, indicating formation of the tris(trimethylsilyl)-derivative. It was observed[78,132], however, that the relative instability of N-trimethylsilylated barbiturates caused unspecified interference during GC. Variations in the recoveries of silylated barbiturates led Street[131] to recommend trimethylsilylation for qualitative purposes only.

4.2 Methyl derivatives

The on-column methylation of barbituric acids with tetramethylammonium hydroxide (TMAH), was first attempted by Stevenson[133] who injected solutions of the barbituric acids in methanolic TMAH onto a temperature-programmed 5% SE-30 column. Most of the 18 barbiturates investigated, were adequately resolved, however, the presence of an "early peak", with retention time smaller than that of the N,N'-dimethyl derivative, was observed for barbiturates with a phenyl substituent at C-5. Similar results were also observed by Parker *et al.*[134]. With other barbiturates, the appearance of multiple peaks has also been noted[132,135] during on-column alkylation with TMAH. Pippenger and Kutt[136] observed barbiturate decomposition by the alkaline TMAH reagent, even at room temperature. Despite these observations, TMAH has been widely used for derivatization of phenobarbital[137–141] and secondary peak formation has either been absent or, if present, been ignored.

In earlier efforts to find an alternative alkylating agent, Brochmann-Hanssen and Oke[132] noted that a quaternary ammonium base producing a better leaving group than trimethylamine was desirable so that shorter reaction times, and milder reaction conditions conducive to thermal stability, could be used. Such a base, trimethylphenylammonium hydroxide (TMPAH), was claimed to be superior to TMAH.

Quantitative studies on the methylation of barbiturates with TMPAH were first conducted in 1970 to determine[131,142,143] therapeutic amounts of phenobarbital in plasma. On-column methylation with TMPAH was extended to other barbiturates[6,144] in 1972. For these studies, TMPAH was prepared by reaction of trimethylphenylammonium iodide[132,142–144] with silver oxide[6]. Fortunately, during 1973 TMPAH became available commercially, and its time-consuming synthesis was then unnecessary. Now it is probably the most widely used alkylating agent for the determination of barbiturates and anticonvulsant drugs[108,145–175] although difficulties have also been encountered with this reagent. An early study of the degradation of barbiturates showed their decomposition by both TMPAH and TMAH, however, degradation of phenobarbital with TMPAH was not as rapid as with TMAH[136]. It may be noted that of the common barbiturates, it is the least stable to aqueous alkali at room temperature[176]. Subsequent studies of barbiturate degradation with TMPAH have been confined to phenobarbital because of its extensive use as an anticonvulsant.

On-column methylation of phenobarbital with TMPAH results primarily in the formation of the N,N'-dimethylated compound[132], however, an additional peak with a much shorter retention time has also been reported for alkylations with

TMPAH[6,146,149,177] and TMAH[133,140]. The compound responsible was termed "early phenobarbital"[6] and, on the basis of retention times[177], was thought to be 2-ethyl-2-phenylmalondiamide[136], until GC–MS studies by Wu[178] established that the compound in question was actually N-methyl-2-phenylbutyramide (MPB). This was confirmed by Osiewicz *et al.*[151] following synthesis and chemical ionization MS studies of MPB.

There has been considerable interest in the mechanism of the high-temperature reaction of phenobarbital with TMPAH, in the injection port. This originates from the suggestion of Kelly *et al.*[165] that the monomethylated derivative, formed by reaction of phenobarbital with TMPAH, was the principal precursor of MPB. Their proposal was supported by the observation that N-methylphenobarbital was dramatically more prone to ring cleavage than was phenobarbital, when subjected to alkaline hydrolysis[179]. In addition, several steps in the suggested pathway were similar to known decomposition reactions of barbiturates or structurally related compounds. At about the same time, Callery and Leslie[180,181] concluded that MPB was produced during the extensive degradation of N,N′-dimethyl phenobarbital by TMPAH in the injection port. These studies indicated that MPB formation occurred from decomposition of either the monomethyl or dimethyl derivatives of phenobarbital. More recently, Kurata *et al.*[174] reported that MPB formation occurred from the injection-port hydrolysis of phenobarbital itself and was caused by water in the sample or reagents. Thus, with this uncertainty it appears that final clarification of the mechanism of MPB formation must still await further studies.

Several approaches to the problems caused by alkaline degradation of barbiturates by TMPAH include the estimation of phenobarbital by measurement of the degradation products of the on-column reaction. Thus, Perchalski *et al.*[146] determined phenobarbital using the combined peak areas of N,N′-dimethylphenobarbital and two decomposition products. Again, Osiewicz *et al.*[151], using a high concentration of TMPAH, showed that the amount of MPB formed was a reproducible, linear function of the amount of phenobarbital injected onto the column. This method was, however, not entirely satisfactory due to the close proximity of the MPB and solvent peaks and the requirement that the extract be slowly and reproducibly injected to obtain reliable results. Surprisingly, the claim that the decomposition product was a reproducible measure of the phenobarbital present, could not be substantiated by Serfontein and De Villiers[168]. Despite this, Kurata *et al.*[174] recently proposed that the sum of the methylated phenobarbital and MPB was an accurate measure of the amount of phenobarbital present.

In another approach aimed at reducing the decomposition product MPB, reduction or elimination of the interfering peak was reported when a solution of TMPAH was neutralised with buffer, prior to on-column alkylation[132]. Here, back extraction of the drug with an aqueous solution of the reagent, was followed by adjustment to pH 8–10, prior to GC. The idea of reducing the alkalinity was further developed by Mraz and Sedivec[182,183] who used a neutral quaternary ammonium salt (trimethylphenylammonium acetate) as the on-column alkylating agent, and obtained only peaks of N,N′-dimethyl derivatives. Similar results were produced with tetramethylammonium acetate. Important here was the fact that the reaction was unaffected by variations in injection port temperatures (200–300°C), or by excess alkylating agent (5–500-fold excess), but no estimate of percentage conversion was indicated. A fur-

ther refinement of this procedure is illustrated by the work of Vincent *et al.*[175] who recently described on-column methylation of 11 barbiturates with TMPAH. Here, degradation was avoided by using a dilute solution of TMPAH (0.2 *M*) and allowing minimal contact of barbiturate with TMPAH before GC. Thus, to achieve this, TMPAH and internal standards were drawn into the syringe *before* the barbiturate (in carbon disulphide) and the contents immediately injected onto the column.

The role of the solvent in the injection-port degradation of phenobarbital in TMPAH was investigated by Kelly *et al.*[165] who found that MPB interference was inhibited by viscous polyhydric alcohols whereas certain aprotic solvents appeared to promote the decomposition reaction. In the former, an inhibitory effect exerted by the solvent on the activity of the hydroxide ion appears responsible. The formation of anisole as a by-product in on-column methylations involving TMPAH is also known[184], due possibly as the result of nucleophilic attack by the solvent on the strongly alkaline TMPAH reagent in the injection port.

In yet another approach, several authors[164,185,186] have initiated the methylation reaction by pre-heating the reaction mixture at 85–100°C for 5–10 min prior to GC. Reproducible results, with no interfering peaks, were claimed with these methods which are not strictly on-column methods. However, prolonged contact (>10 min) between phenobarbital and TMPAH can result in decomposition of phenobarbital[156].

Finally, reference is made to a report by Wong *et al.*[158] of the presence of an endogenous methylating agent in serum. It was observed that, whereas urine from phenobarbital-treated patients usually contained only phenobarbital, corresponding serum samples extracted at pH 7 with dichloromethane invariably contained small amounts of N-methylphenobarbital. After ruling out the possibility of *in vivo* methylation, lecithin was implicated in the thermally-induced methylation of phenobarbital in the injection port. A deuterated analogue of TMPAH was also recommended for quantification of phenobarbital and mephobarbital in the serum of patients prescribed both drugs, since patients[187] receiving mephobarbital have higher plasma levels of phenobarbital than the parent drug.

4.3 Other alkyl derivatives

MacGee[135] first reported the on-column ethylation of barbiturates with tetraethylammonium hydroxide. No interfering peaks were observed, and separation of mephobarbital from phenobarbital was obtained on 0.05% OV-101. A slow injection technique (10 sec) appeared to markedly reduce tailing of the solvent peak, however, the high injection-port temperature of 360°C may have been responsible for column bleeding and contributed to loss of resolution observed after prolonged use. Using this procedure poor separation of mephobarbital and phenobarbital was obtained on conventional 3% SE-30 or 2.5% OV-17 columns[188].

Ethylation with tetraethylphenylammonium hydroxide has been reported[189] and although phenobarbital was successfully determined, this reagent was unsuitable for quantification of mephobarbital because of the high level of transethylation of the latter (*ca.* 20%) to form N,N'-diethylphenobarbital. Again, separation of the ethyl derivative of phenobarbital and mephobarbital on 3% OV-1 was very poor, but better reproducibility was achieved with a rapid injection technique, in contrast to the slow injection method of MacGee[135].

On-column butylation with tetrabutylammonium hydroxide[190] resulted in a difference of 2 min in the retention times of phenobarbital and mephobarbital on 3% OV-17. Although secondary peaks were absent, solvent peak tailing was far more pronounced when compared to an on-column methylation procedure with TMPAH. Degradation during on-column butylation was observed by Hooper *et al.*[191] with mephobarbital and phenobarbital, each giving two peaks when injected with tetrabutylammonium hydroxide. These barbiturates were quantified only after selection of chromatographic conditions led to elution of the interfering peak at short retention time, with the solvent.

A comparison was made recently between a pre-column and an on-column butylation procedure for barbiturates extracted from plasma with toluene–methanol[192]. The latter method involved treatment of the toluene layer with tetrabutylammonium hydroxide in methanol–water solution, while the former technique involved back-extraction of the toluene layer with TMAH, followed by treatment with dimethylacetamide and iodobutane. The back extraction was found to improve the extraction efficiency of several barbiturates and also led to cleaner chromatograms, whereas the on-column procedure though quicker, resulted in some decomposition of barbiturate in the injection port.

On-column derivatizations have been extended to the higher alkyl homologues of TMAH[153]. Thus, with phenobarbital, minor secondary peaks were noted with tetrapropyl, tetrabutyl and tetrapentylammonium hydroxides. Although the use of trialkylphenylammonium hydroxides with better leaving groups was considered, steric hindrance prevented synthesis of such bases with alkyl moieties longer than the ethyl group. Reports of on-column alkylations with tetrahexyl-[193,194] and tetraheptylammonium[194] hydroxides allowed identification and quantification of 12 out of 17 barbiturates on an OV-17 column. Alkaline degradation was not apparent, and, although phenobarbital could not be resolved from cyclobarbital, very good resolution of phenobarbital and mephobarbital was produced with either alkylation procedure.

It may be concluded that on-column derivatization of barbiturates is greatly advantageous in many situations due to its rapidity and simplicity, however, results of quantitative estimation of barbiturates, especially phenobarbital, should be treated with caution. Since most on-column techniques are prone to interferences from minor-peak formation to an extent which is unpredictable and probably promoted by the alkaline derivatizing agents, the use of neutral on-column alkylating reagents appears desirable. Another factor influencing the choice of the reagent is the pronounced tailing of the solvent peak, presumably related to it, which may interfere with the peaks of barbiturates having relatively short retention times.

5. GC–MS STUDIES

Reports on the analytical application of GC–MS to the detection of barbiturates first appeared in 1970, when Bonnichsen *et al.*[195] identified several barbituric acids in biological samples. In the same year, Gilbert *et al.*[196] utilised the technique, in metabolic studies of barbiturates. As in conventional GC, widespread recognition of the value of derivatization prior to analysis with GC–MS has not only overcome problems such as tailing and adsorption but, in addition, ion-source contamination is avoided when compounds are converted to more volatile derivatives. Consequently,

GC–MS STUDIES OF BARBITURATE METABOLITES IN URINE

ns = Not specified

Barbiturate	*Metabolite*	*Dose excreted (%)*	*Notes*	*Reference and (year)*
Phenobarbital	(a) 5-Ethyl-5-(4-hydroxyphenyl) barbituric acid	ns	(a) is the major metabolite	200 (1971)
	(b) 5-(3,4-Dihydroxyphenyl)-5-ethyl barbituric acid	ns	(b) and (c) are also metabolites of	201 (1972)
	(c) 5-(3,4-Dihydroxy-1,5-cyclohexadien-1-yl)-5-ethyl barbituric acid	ns	mephobarbital	201 (1972)
	(d) 5-(1-Hydroxyethyl)-5-phenyl barbituric acid	ns		201 (1972)
Pentobarbital	5-Ethyl-5-(3-hydroxy-1-methylbutyl) barbituric acid	ns	Metabolite formed by in-vivo incubation	130 (1971)
(Several)	Intact glucuronide conjugates of mephobarbital, phenobarbital and hexobarbital were detected	ns	Glucuronides of secobarbital and butalbital were not found	122 (1973)
Secobarbital	(a) 5-(3-Hydroxy-1-methylbutyl)-5-(2-propenyl) barbituric acid	ns		207 (1973)
	(b) 5-(2,3-Dihydroxypropyl)-5-(1-methylbutyl) barbituric acid	ns		
Nealbarbital	5-(2,3-Dihydroxypropyl)-5-(2,2-dimethylpropyl) barbituric acid	30–40	Metabolite isolated for the first time	211 (1973)
Heptabarbital	(a) 5-Ethyl-5-(3-hydroxycyclohepten-1-yl) barbituric acid	18–21	Metabolites (a) and (c) were new; the free acid was absent in urine; (c) was isomeric with (a)	212 (1973) and 213 (1974)
	(b) 5-Ethyl-5-(3-oxocyclohepten-1-yl) barbituric acid	4–8		
	(c) An unidentified hydroxy derivative	10–17		
Butobarbital	(a) 5-Ethyl-5-(3-hydroxybutyl) barbituric acid	22–27	(a), (b) and (c) were isolated for the first time in humans	214 (1974)
	(b) 5-Ethyl-5-(3-oxobutyl) barbituric acid	14–18		
	(c) 5-(3-Carboxypropyl)-5-ethyl barbituric acid	4–8		
	(d) Unchanged butabarbital	7–9		
Mephobarbital	(a) 5-Ethyl-5-phenyl barbituric acid	ns	(2,4,5-^{13}C) phenobarbital was used	215 and 216
	(b) Unchanged mephobarbital	ns	as a homologue, to quantify empho-barbital, and as a stable isotope analogue to quantify phenobarbital	(1974)
Amobarbital	(a) 5-(3-Hydroxy-3-methylbutyl)-5-ethyl barbituric acid	ns	(b) was the first example of an N-hydroxylated metabolite of any barbiturate	209 (1975)
	(b) N-Hydroxy-5-ethyl-5-(3-methylbutyl) barbituric acid	ns		
	(a) and (b) as above	ns	An on-column methylation method for assay of the two metabolites	217 (1977)
	[illegible]	ns	A new metabolite of amobarbital	218 (1977)

analytical studies of barbiturates with GC–MS, have usually involved the derivatized species.

Skinner *et al.*[197] described GC–MS studies of barbiturates derivatized by on-column alkylation with TMPAH. However, TMPAH did not react reproducibly with the 3′-hydroxylated metabolites of several barbiturates[198]. Diazomethane now appears to be the derivatizing agent of choice as it gives a rapid and quantitative reaction, and leaves no solid residue after derivatization, effected simply by mixing at room temperature for 15 min. Yet, with diazomethane, methylation results in the formation of the N,N′-dimethylated derivatives, together with the N,O′- and O,O′-dimethylated isomers that can account for 10–15% of the total yield[199]. In the particular case of urine where hydroxylated metabolites are present, derivatization by methylation is often followed by silylation. Predictably, this procedure results in the formation of several different derivatives from a single barbiturate, as shown during the analysis of urinary metabolites of phenobarbital[200,201].

In efforts to detect microgram amounts of various drugs in human biological specimens, computer-assisted GC–MS identification procedures have been invaluable. The first of such programmes, described by Finkle and Taylor[202] in 1972, involved the compilation of a MS data system for 11 barbiturates and over a hundred other drugs extracted and presented to the GC–MS instrument in a form comparable with that encountered in toxicological practice. Also in 1972, Bonnichsen *et al.*[203] described the use of a computer to evaluate and process the MS data for several barbiturates, recorded on a digital tape, off-line system. The barbiturates were isolated from the blood or liver of suicide cases, prior to analysis by GC–MS.

Since these developments, several additional computer-assisted GC–MS systems suitable for a variety of needs, have been described[204–206]. In a recent report[206], relative intensities of fragment ions from barbiturates methylated with diazomethane, showed some differences to those of authentic N,N′-dimethyl barbiturate derivatives. These differences, possibly due to formation of small amounts of the N,O′- and O,O′-dimethyl derivatives, were obviated by storage and processing of both spectra in the data system.

Use of stable isotopes for the quantification of barbiturates with GC–MS was introduced in 1973[207]. Internal standards labelled with stable isotopes were added to the biological fluid containing the barbiturate. Extraction and derivatization was followed by selective monitoring of ions corresponding to base peaks of sample and internal standard, followed by computer measurement of peak-height ratios. In this way, [2,4,5-^{13}C]pentobarbital, was used to quantify amobarbital, secobarbital and phenobarbital in plasma[207]. Increasing availability of stable, isotope-labelled barbituric acids has led to the determination of many other barbiturates[208–210].

GC–MS procedures have facilitated the identification of urinary metabolites of several barbiturates for the first time. Thereafter, structural identity of the metabolite has usually been confirmed by synthesis and subsequent characterization. Table I lists some important contributions to studies in barbiturate metabolism by GC–MS methods.

GC–MS procedures employing chemical ionization mass spectroscopy (CI-MS), were demonstrated by Horning *et al.*[200] as early as 1971. The CI-MS mode was preferred to the conventional electron impact (EI-MS) mode because of reduced fragmentation and the marked reduction in the probability of fragment ions from

other compounds contributing to the intensity of the ion being monitored. Again, CI-MS spectra of diazomethane-methylated barbiturates are less liable to misinterpretation than corresponding EI-MS spectra, since all isomeric N,N′-, N,O′- and O,O′-dimethylated barbiturates would be expected to give a single peak for the corresponding M + 1 ion. In fact, both N,N′- and N,O′-isomers of phenobarbital gave virtually identical CI-MS spectra[200]. More recently, the use of low-resolution field desorption and field ionization mass spectroscopy in GC–MS methods has been documented[219]. Relatively small samples gave good field desorption spectra, and 1–10 μg of underivatized barbiturate and less of the methylated compounds were required for satisfactory field ionization spectra.

GC–MS methods have been particularly valuable in pharmacokinetic studies due to the specificity of detection, with metabolites being readily distinguished from the parent barbiturate. Furthermore, because of its inherent sensitivity, only small samples are necessary so that repetitive sampling from humans has been facile. An example of this work is the investigation of the kinetics of hydroxylation of amobarbital in liver tissue, where amobarbital was measured in an incubation derived from less than 3 mg of liver tissue obtained by needle biopsy[220]. A total sample weight of 29 mg of liver tissue was sufficient for the determination of the kinetic parameters K_M (Michaelis constant) and $V_{max.}$ (maximal velocity) for the hydroxylation reaction.

In another study of barbiturate levels in the breast milk of nursing mothers[221] it was shown that, while the short acting barbiturates were present in low concentrations, the long acting barbiturate, phenobarbital, reached high levels. Despite interferences from large quantities of free fatty acids present in breast milk, a limit of detection of 0.4–0.5 ng was obtained with the GC–MS system used.

GC–MS methods have enabled the determination of *in vivo* plasma half-lives of amobarbital and 3′-hydroxyamobarbital after ingestion of therapeutic doses[198,222]. Mephobarbital half-lives were estimated similarly, by computer assisted GC–MS[223,224]. Several kinetic parameters for *in vitro* metabolism of secobarbital in rat-liver homogenate, were evaluated in the same study. Again, GC–MS methods have enabled the study of amobarbital, both as a probe drug for hepatic oxidation[210] as well as for an investigation of the influence of genetic factors on drug elimination[225].

A valuable application of the GC–MS–computer method, was demonstrated by Horning *et al.*[226], who utilised it as a reference procedure for some other methods used in a clinical chemistry laboratory. Concentrations of phenobarbital in saliva and plasma measured by enzyme immunoassay, were 10–15% higher than those obtained with a GC–MS system, suggesting that metabolites as well as parent drug were being measured by the immunoassay procedure used.

6. INTERFERENCES IN GC ANALYSIS OF BARBITURATES

Problems encountered during the analysis of barbiturates have arisen primarily from endogenous artifacts or as a result of manipulative procedures, prior to the actual GC. An example of the latter is the adsorption of barbiturates on glassware which may explain the anomalous losses of these polar molecules during analytical procedures. Pronounced losses at the 0.75 μg/ml level, with complete loss at 0.50 μg/ml are known[239]. Such losses can be prevented by silylation of glassware with silylating reagents applied in solution[89] or the vapour phase[239].

Multiple solvent extractions after acidification of the sample form the basis of most methods for the extraction of barbiturates and their metabolites from biological fluids.

6.1 Endogenous substances

There has not been an extensive investigation of endogenous sources of interference in the analysis of barbiturates by GC, although Niyogi and Rieders[228] have described a number of endogenous compounds that could be mistaken for barbiturates after direct extraction from blood with chloroform. Indicative of the need for a greater understanding of interference by artifacts are prominent, unidentified peaks in chromatograms obtained during the analysis of barbiturate extracts from blood[45,56,78,96,159,162,169,188]. Reported for the first time by Cook[229] in 1963, fatty acids present in blood constitute a primary and predictable source of interference due to their co-extraction in significant amounts by most organic solvents. Extractions with non-polar solvents such as isooctane[111] and cyclohexane[148] have been reported. Although fatty acids were largely removed by these procedures, some concomitant loss of barbiturate was also observed[111].

Selective alkylation of barbiturates in the presence of fatty acids has been reported by Kumps and Mardens[188] who observed the fatty acid alkyl ester peaks in the chromatograms of phenobarbital extracted from blood and subjected to on-column alkylation with methanolic TMAH or aqueous tetraethylammonium hydroxide (TEAH). When methanolic TEAH was used, the reaction was not observed and, furthermore, no reason for this behaviour was given. In another instance[118] of the analysis of barbiturates and other acidic drugs, use of TMAH in a back-extraction of the organic phase obtained after extraction of an acidified plasma was found to reduce substantially the interference by fatty acids. Here, a recovery study with palmitic acid showed that only 0.1% was extracted from toluene with TMAH.

A different approach to overcome the problem of fatty acids was introduced by Mraz and Sedivec[183] who exploited the relative insolubility of the barium salts of fatty acids in diethyl ether in an effort to separate them from barbituric acids in serum. A back-extraction of the organic phase with barium hydroxide also had the advantage of minimising the alkaline degradation of barbiturates, an aspect which appears to have been largely overlooked in most analytical procedures utilising a back-extraction step with strong bases such as sodium hydroxide. Another direct procedure has been reported[230] recently for removing large amounts of free fatty acids co-extracted with barbiturates from autopsy liver and blood samples. Its success depends upon the selective alkylation of the carboxylic acids, under anhydrous conditions, with methanol–HCl. Barbituric acids were then removed and converted to dimethyl derivatives for GC.

As stated earlier, lecithin is responsible for on-column methylation of barbiturates but has also been implicated[158] in the methylation of several fatty acids. This second reaction has been confirmed by GC–MS studies of serum extracts which showed that methyl esters of palmitic, stearic and oleic acids were formed by alkylation in the injection port. Again, extraction of serum with a non-polar solvent may eliminate interference by fatty acids as well as lecithin but there remains the likelihood of some loss of barbiturate[111].

Finally, reference is made to the removal of lipophilic components from serum

by means of a microprocessor-controlled, automatic centrifugal extractor[231]. Lipophilic components were extracted by means of a lipophilic resin (a polystyrene–divinylbenzene copolymer) contained in a compact cartridge, and the recovered drug(s) presented as a dry extract for subsequent analysis. Phenobarbital and other anticonvulsants were determined after on-column methylation and the use of a nitrogen-specific detector.

Interference from cholesterol has also been noted. Although its retention time is much greater than barbiturates, its removal is desirable to prevent column contamination and its slow elution during subsequent analyses. It may also produce a large negative peak, as observed during the GC of indomethacin[232] with the ECD. Cholesterol has been removed from serum with digitonin[45,56,145] but the amount of digitonin added may be critical. Thus, cholesterol was incompletely removed with insufficient amounts of digitonin but gel-formation, with attendant inclusion of drug in the gel, resulted when an excess of digitonin was used[56]. A superior approach appears to be the use of a 4-cm pre-column of 3% SP-2250, as in the separation of cholesterol from primidone, and this also improved resolution of phenobarbital from carbamazepine[223,224] when analyzed on a 2% SP-2510 column.

Proteins can interfere indirectly in the analysis of barbiturates in blood during the extraction step and formation of a protein precipitate often presents difficulty although the use of an acidic precipitant for the determination of protein-bound barbituric acids is well known[78,98,131]. During the analysis of normal plasma or serum, emulsions have usually and simply been resolved by centrifugation. In clinical studies where abnormal plasma is often encountered and intractable emulsions are frequently obtained, Horning *et al.*[111], utilised the salting-out technique involving high concentrations of an inorganic salt to promote transfer of drug from aqueous to organic phase. In this case, diluted plasma containing a small volume of isopropanol was saturated with potassium carbonate and centrifuged then the isopropanol layer containing drug and drug metabolites separated as the upper phase. Since its initial description[227], the salting-out procedure has found wide application in the GC analysis of barbiturates, extracted not only from abnormal plasma but from a range of biological fluids obtained in both healthy and diseased states. Salting-out with ammonium carbonate is preferred to potassium carbonate due to the reduced basicity of its solutions. Ammonium sulphate has also been widely used.

The use of element-selective detectors in situations where endogenous interferences have been encountered, has been of considerable advantage and has led to simplified extraction procedures. Sample volumes as low as 25[107] or 100 μl[92] of whole blood have sufficed for such analyses. However, the use of some solvents may not be compatible with certain element-selective detectors. The disturbing influence on an alkali flame ionization detector of methyl iodide and acetone used in the methylation of barbiturates, was eliminated by column-switching modules[235] which removed most of the solvent peak components prior to elution of the barbiturates[107]. Solvent-related problems have also been encountered with the electrolytic conductivity detector[89,90] during barbiturate analysis. Although halogen-, sulphur- or nitrogen-containing solvents interfered, hydrocarbon solvents were satisfactory. Extraction of barbiturates with diisopropyl ether enabled levels of approximately 2 μg/ml, to be determined both in serum and urine[89].

It would seem that despite the obvious advantages of selective detectors, the

problems of interfering substances in biological fluids cannot be disregarded, as many endogenous compounds contain nitrogen or sulphur. Furthermore, gradual accumulation of co-extracted endogenous artifacts on the column as a result of insufficient clean-up, would ultimately lead to rapid column contamination and loss of performance.

6.2 Miscellaneous sources

Notable among the few examples of interference by exogenous compounds is the oxidation of thiopental during manipulative procedures prior to GC. This reaction was prevented by direct gel chromatography of the haemolyzed blood on Sephadex G-10[81,82]. Similarly, benzene has been recommended for the extraction of thiopental[84] to avoid its degradation by impurities in solvents such as peroxides in diethyl ether. A better-known source of interference is that of plasticizers from butyl-rubber stoppers and bags used for blood collection. Tri-2-butoxyethyl phosphate, in particular was responsible for interfering peaks observed during the analysis of barbiturates in blood by GC[158,161,236].

Another example concerns the compound 5-ethyl-5-*p*-tolylbarbituric acid (EPTB) which has been suggested as an internal standard for on-column methylation of phenobarbital with TMPAH because both barbiturates decompose in a reproducible manner under identical conditions[237]. Unfortunately, co-elution of theophylline (methylated to caffeine) with EPTB on a 3% OV-17 column produced misleadingly low values for phenobarbital in serum[238].

Perhaps because there are fewer references in the literature to the extraction of barbiturates from urine than blood, the more important indicator of tissue barbiturate levels, there is less evidence of interference problems. Since relatively small amounts of most barbiturates are excreted in urine, it is useful nevertheless and certainly the biological fluid of interest in studies of their metabolites. In dealing with this fluid, extraction of barbiturates has been facilitated by the development of adsorptive columns consisting of the weakly basic anion-exchange polymer DEAE-Sephadex[240], and were described[200,201,207] during the early seventies. Again, despite high recoveries of most barbiturates [241–246] there has only been a relatively limited application of the Amberlite XAD-2 resin to barbiturate analysis by GC. In this respect, spurious responses[247] observed with some column eluates may have been more widespread than was thought and interference peaks have been attributed[247] either to impurities in the resin or to incomplete removal of endogenous compounds. The use of XAD-2 columns in the treatment of urine has, however, been widespread in drug screening programmes utilising TLC procedures[241,242,248].

More recently, the use of extraction columns (JETUBES) containing purified cotton fibres that function as an adsorptive matrix was shown to give high recoveries of several drugs, including 90–97% phenobarbital, when extracted from small volumes (15 ml) of urine[249]. A comparison of recoveries with an XAD-2 column and radiolabelled drugs claimed the superiority of the JETUBE both in extraction efficiency and working time. In another device, the removal of endogenous carboxylic acids from urine was demonstrated with pre-packed Kieselguhr columns (Merck Extrelut), prior to analysis by GC[250]. Recoveries of barbituric acids were similar to those obtained by conventional liquid–liquid extraction procedures.

7. SUMMARY

This review surveys the evolution of gas chromatographic procedures for the quantification of barbiturates as either the free acids or their derivatives obtained by direct and on-column reactions. Among the aspects discussed, some emphasis is placed on recognized and other sources of interference encountered during analyses.

REFERENCES

1 A. von Baeyer, *Ann.*, 130 (1864) 129.
2 E. Fischer and J. R. von Mering, *Therap. Gegenwart.*, 44 (1903) 97.
3 W. J. Doran, *Medicinal Chemistry, Vol IV, Barbituric Acid Hypnotics*, Wiley, New York,1959.
4 E. Brochmann-Hanssen, *Theory Appl. Gas Chromatogr. Ind. Med.*, Hahnemann Symp., Grune and Stratton, New York, 1st ed. 1966, pp. 182–193.
5 M. Melzacka, *Wiadomosci Chemiczne*, 25 (1971) 613.
6 G. Kananen, R. Osiewicz and I. Sunshine, *J. Chromatogr. Sci.*, 10 (1972) 283.
7 N. C. Jain and R. H. Cravey, *J. Chromatogr. Sci.*, 12 (1974) 228.
8 M. W. Anders and C. J. Mannering, *Progr. Chem. Toxicol.*, 3 (1967) 180.
9 G. B. Schumann, K. Lauenstein, D. Le Fever and J. Bernard, *Amer. J. Clin. Path.*, 66 (1976) 823.
10 S. Goenechea, *Z. Anal. Chem.*, 218 (1966) 416.
11 H. W. Avdovich and G. A. Neville, *Can. J. Pharm. Sci.*, 4 (1969) 51.
12 H. Lackner and G. Doring, *Arch. Toxicol.*, 26 (1970) 220.
13 W. Arnold and H. Grutzmacher, *Z. Anal. Chem.*, 247 (1969) 179.
14 E. F. Abdel-Bary, G. Bohn and G. Rucker, *Z. Anal. Chem.*, 266 (1973) 361.
15 H. V. Street and C. McMartin, *Nature (London)*, 199 (1963) 456.
16 J. A. Vinson, J. E. Hooyman, H. Koharcheck and M. M. Holmes, *J. Chromatogr.*, 140 (1977) 71.
17 W. Dünges, G. Naundorf and N. Seiler, *J. Chromatogr. Sci.*, 12 (1974) 655.
18 H. E. Booker and B. A. Darcey, *Clin. Chem.*, 21 (1975) 1766.
19 E. J. Flynn and S. Spector, *J. Pharmacol. Exp. Ther.*, 181 (1972) 547.
20 R. Cleeland, R. Davis, J. Hevernan and E. Grunberg, *J. Forens. Sci.*, 20 (1975) 45.
21 J. Janak, *Nature (London)*, 185 (1960) 684.
22 D. F. Nelson and P. L. Kirk, *Anal. Chem.*, 34 (1962) 899.
23 A. Baerheim Svendsen and E. Brochmann-Hanssen, *J. Pharm. Sci.*, 51 (1962) 494.
24 K. D. Parker and P. L. Kirk, *Anal. Chem.*, 33 (1961) 1378.
25 K. D. Parker, C. R. Fontan and P. L. Kirk, *Anal. Chem.*, 35 (1963) 356.
26 M. W. Anders, *Anal. Chem.*, 38 (1966) 1945.
27 E. Brochmann-Hanssen and A. B. Svendsen, *J. Pharm. Sci.*, 51 (1962) 318.
28 L. Kazyak and E. C. Knoblock, *Anal. Chem.*, 35 (1963) 1448.
29 E. W. Cieplinski, *Anal. Chem.*, 35 (1963) 256.
30 C. McMartin and H. V. Street, *J. Chromatogr.*, 23 (1966) 232.
31 C. McMartin and H. V. Street, *J. Chromatogr.*, 22 (1966) 274.
32 J. Bohemen, S. H. Langer, R. H. Perrett and J. H. Purnell, *J. Chem. Soc.*, 494 (1960) 2444.
33 B. J. Gudzinowicz and S. J. Clark, *J. Gas Chromatogr.*, 3 (1965) 147.
34 D. Blackmore, *Perkin-Elmer Anal. News*, 3 (1968) 1.
35 R. C. Driscoll, F. S. Barr, B. J. Gragg and G. W. Moore, *J. Pharm. Sci.*, 60 (1971) 1492.
36 T. Inaba and W. Kalow, *J. Chromatogr.*, 69 (1972) 377.
37 L. I. Braddock and N. Marec, *J. Gas Chromatogr.*, 3 (1965) 274.
38 R. G. Ackman and R. D. Burgher, *Anal. Chem.*, 35 (1963) 647.
39 B. Welton, *Chromatographia*, 3 (1970) 211.
40 C. Ioannides, J. Chakraborty and D. V. Parke, *Chromatographia*, 7 (1974) 351.
41 V. Kyogoku, R. C. Lord and A. L. Rich, *Nature (London)*, 218 (1968) 69.
42 N. D. Greenwood, I. W. Guppy and H. P. Simmons, *J. Chromatogr. Sci.*, 13 (1975) 349.
43 A. J. Williams, T. W. G. Jones and J. D. H. Copper, *Clin. Chim. Acta*, 43 (1973) 327.
44 A. S. Papadopoulos, E. M. Baylis and D. E. Fry, *Clin. Chim. Acta*, 48 (1973) 135.
45 O. Driessen and A. Emonds, *Proc. K. Ned. Akad. Wet. C.*, 77 (1974) 171.
46 A. H. Woo and R. C. Lindsay, *J. Chromatogr. Sci.*, 18 (1980) 273.

47 D. J. Berry, *J. Chromatogr.*, 86 (1973) 89.
48 K. D. Parker, C. R. Fontan and P. L. Kirk, *Anal. Chem.*, 35 (1963) 418.
49 N. C. Jain, C. R. Fontan and P. L. Kirk, *Microchem. J.*, 8 (1964) 28.
50 P. H. Scott, *Ann. Clin. Biochem.*, 8 (1971) 105.
51 S. L. Levy and T. Schwartz, *Clin. Chim. Acta*, 54 (1974) 19.
52 K. E. Rasmussen, S. Rasmussen and A. Baerheim Svendsen, *Pharm. Weekbl.*, 107 (1972) 821.
53 H. Leach, P. A. Toseland, *Clin. Chim. Acta*, 20 (1968) 195.
54 K. Balasubramaniam, G. E. Mawer and E. M. Rodgers, *Brit. J. Pharmacol*, 37 (1969) 546.
55 K. E. Rasmussen, S. Rasmussen and A. Baerheim Svendsen, *J. Chromatogr.*, 66 (1972) 136.
56 C. A. Cramers, E. A. Vermeer, L. G. van Kuik, J. A. Hulsman and C. A. Meijers, *Clin. Cheim. Acta*, 73 (1976) 97.
57 H. L. Thompson and W. J. Decker, *Amer. J. Clin. Path.*, 49 (1968) 103.
58 H. A. Bloomer, R. K. Maddock, J. B. Sheehe and E. J. Adams, *Ann. Internal. Med.*, 72 (1970) 223.
59 H. F. Proelss and H. J. Lohmann, *Clin. Chem.*, 17 (1971) 222.
60 R. J. Flanagan and G. Withers, *J. Clin. Path.*, 25 (1972) 899.
61 J. R. Shipe and J. Savory, *Ann. Clin. Lab. Sci.*, 5 (1975) 57.
62 F. P. Abramson, *Clin. Chem.*, 22 (1976) 1906.
63 E. L. Stern and G. P. Caron, *Amer. J. Med. Technol*, 43 (1977) 834.
64 R. J. Flanagan and D. J. Berry, *J. Chromatogr.*, 131 (1977) 131.
65 J. I. Allen, *J. Ass. Offic. Anal. Chem.*, 51 (1968) 619.
66 J. L. Sibert and F. L. Fricke, *J. Ass. Offic. Anal. Chem.*, 51 (1968) 1326.
67 N. D. Greenwood, *Pharm. J.*, 210 (1973) 431.
68 K. Balasubramaniam, S. B. Lucas, G. E. Mawer and P. J. Simons, *Brit. J. Pharmac.*, 39 (1970) 564.
69 J. Grove and P. A. Toseland, *J. Pharm. Pharmac.*, 23 (1971) 936.
70 E. R. Garrett, J. Bres, K. Schnelle and L. L. Rolf, *J. Pharmacokin. Biopharm.*, 2 (1974) 43.
71 D. Kadar, T. Inaba, L. Endrenyi, G. E. Johnson and W. Kalow, *Clin. Pharmacol. Ther.*, 14 (1973) 552.
72 T. Inaba and W. Kalow, *Clin. Pharmacol. Ther.*, 18 (1975) 558.
73 T. D. Yih and J. M. van Rossum, *J. Pharmacol. Exp. Ther.*, 203 (1977) 184.
74 H. C. Kinsella, S. Smith, H. J. Rogers and P. A. Toseland, *J. Pharm. Pharmacol*, 31 (1979) 153.
75 R. B. Smith, L. W. Dittert, W. O. Griffen and J. T. Doluisio, *J. Pharmacokin. Biopharm.*, 1 (1973) 5.
76 M. Ehrnebo, *J. Pharm. Sci.*, 63 (1974) 1114.
77 S. Kojima, R. B. Smith and T. J. Doluisio, *J. Pharm. Sci.*, 60 (1971) 1639.
78 J. Grove and P. A. Toseland, *Clin. Chim. Acta*, 29 (1970) 253.
79 H. Steinbach and W. Gielen, *J. Chromatogr.*, 139 (1977) 191.
80 M. Donike, L. Jaenicke, D. Stratmann and W. Hollmann, *J. Chromatogr.*, 52 (1970) 237.
81 A. Heyndrickx and P. Schepens, *Int. Symp. VI. Chromatographie-Electroforese*, Presses Academiques Europeennes, Bruxelles, 1970, p. 268; *C.A.*, 78 (1973) 79513m.
82 P. Schepens and A. Heyndrickx, *Eur. J. Toxicol.*, 8 (1975) 87.
83 M. Reidmann, *Naturwissenschaften*, 59 (1972) 306.
84 L. T. Sennello and F. E. Kohn, *Anal. Chem.*, 46 (1974) 752.
85 D. D. Breimer and J. M. van Rossum, *J. Chromatogr.*, 88 (1974) 235.
86 P. A. Toseland, M. Albani and F. D. Gauchel, *Clin. Chem.*, 21 (1975) 98.
87 G. W. Houghton, A. Richens, P. A. Toseland, S. Davidson and M. A. Falconer, *Europ. J. Clin. Pharmacol.*, 9 (1975) 73.
88 B. H. Dvorchik, *J. Chromatogr.*, 105 (1975) 49.
89 R. C. Hall, C. A. Risk, *J. Chromatogr. Sci.*, 13 (1975) 519.
90 B. E. Pape, *Clin. Chem.*, 22 (1976) 739.
91 J. G. H. Cook, C. Riley, R. F. Nunn and D. E. Budgen, *J. Chromatogr.*, 6 (1961) 182.
92 R. E. Stuckey, *Quart. J. Pharm. Pharmacol.*, 14 (1941) 217.
93 H. F. Martin and J. L. Driscoll, *Anal. Chem.*, 38 (1966) 345.
94 J. T. Stewart, G. B. Duke and J. E. Willcox, *Anal. Lett.*, 2 (1969) 449.
95 E. M. Baylis, D. E. Fry and V. Marks, *Clin. Chim. Acta*, 30 (1970) 93.
96 E. A. Fiereck and N. W. Tietz, *Clin. Chem.*, 17 (1971) 1024.
97 R. C. Baselt and L. J. Casarett, *J. Chromatogr.*, 57 (1971) 139.
98 A. Premel-Cabic and P. Allain, *Therapie*, 28 (1973) 951.
99 A. Premel-Cabid and P. Allain, *Therapie*, 28 (1973) 969.

100 G. A. Neville, *Anal. Chem.*, 42 (1970) 347.
101 W. Dünges, *Chromatographia*, 9 (1976) 571.
102 D. Bursil, *M. Chem. Thesis*, University of N.S.W., Kensington, 1975.
103 S. Dilli and D. Pillai, unpublished results.
104 W. Dünges and E. Bergheim-Irps, *Anal. Lett.*, 6 (1973) 185.
105 W. Dünges, *Chromatographia*, 6 (1973) 196.
106 W. Dünges, *Anal. Chem.*, 45 (1973) 963.
107 W. Dünges, E. Bergheim-Irps, H. Straub and R. E. Kaiser, *J. Chromatogr.*, 145 (1978) 265.
108 A. Wu and M. L. Pearson, *Anal. Lett.*, 10 (1977) 381.
109 W. Dünges, R. Langlais and R. Schlenkermann, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 2 (1979) 361.
110 S. Sun and D. J. Hoffman, *J. Pharm. Sci.*, 68 (1979) 386.
111 M. G. Horning, E. A. Boucher, M. Stafford and E. C. Horning, *Clin. Chim. Acta*, 37 (1972) 381.
112 D. J. Harvey, L. Glazener, C. Stratton, D. B. Johnson, R. M. Hill, E. C. Horning and M. G. Horning, *Res. Commun. Chem. Path. Pharm.*, 4 (1972) 247.
113 D. J. Harvey, D. B. Johnson and M. G. Horning, *Anal. Lett.*, 5 (1972) 745.
114 H. Ehrsson, *Anal. Chem.*, 46 (1974) 922.
115 M. Garle and I. Petters, *J. Chromatogr.*, 140 (1977) 165.
116 F. N. IJdenberg, *Pharm. Weekbl.*, 110 (1975) 21.
117 R. H. Greeley, *Clin. Chem.*, 20 (1974) 192.
118 H. Roseboom and A. Hulshoff, *J. Chromatogr.*, 173 (1979) 65.
119 J. F. Menez, F. Berthou, D. Picart, L. Bardou and H. H. Floch, *J. Chromatogr.*, 129 (1976) 155.
120 V. S. Venturella, V. M. Gualario and R. E. Lang, *J. Pharm. Sci.*, 62 (1973) 662.
121 R. M. Thompson, *J. Pharm. Sci.*, 65 (1976) 288.
122 R. M. Thompson, N. Gerber, R. A. Seibert and D. M. Desiderio, *Drug. Metab. Dispos.*, 1 (1973) 489.
123 T. Walle, *J. Chromatogr.*, 114 (1975) 345.
124 O. Gyllenhaal, H. Brötell and B. Sandgren, *J. Chromatogr.*, 122 (1976) 471.
125 S. Sun and A. H. C. Chun, *J. Pharm. Sci.*, 66 (1977) 477.
126 S. Dilli and D. Pillai, *J. Chromatogr.*, 190 (1980) 113.
127 E. W. Robb and J. J. Westbrook, *Anal. Chem.*, 35 (1963) 1644.
128 J. J. Kamm and E. J. van Loon, *Clin. Chem.*, 12 (1966) 789.
129 H. V. Street, *J. Chromatogr.*, 41 (1969) 358.
130 J. L. Holtzmann and D. S. Alberts, *Anal. Biochem.*, 43 (1971) 48.
131 H. V. Street, *Clin. Chim. Acta*, 34 (1971) 357.
132 E. Brochmann-Hanssen and T. O. Oke, *J. Pharm. Sci.*, 58 (1969) 370.
133 G. W. Stevenson, *Anal. Chem.*, 38 (1966) 1948.
134 K. D. Parker, J. A. Wright, A. F. Halpern and C. H. Hine, *J. Forens. Sci. Soc.*, 8 (1968) 125.
135 J. MacGee, *Clin. Chem.*, 17 (1971) 587.
136 C. E. Pippenger and H. Kutt, *Clin. Chem.*, 19 (1973) 666.
137 R. J. Perchalski and B. J. Wilder, *J. Pharm. Sci.*, 63 (1974) 806.
138 F. Vajda, F. M. Williams, B. M. Davidson, M. A. Falconer and A. Breckenridge, *Clin. Pharmacol. Ther.*, 15 (1974) 597.
139 E. B. Solow, J. M. Metaxas and T. R. Summers, *J. Chromatogr. Sci.*, 12 (1974) 256.
140 F. Dorrity and M. Linnoila, *Clin. Chem.*, 22 (1976) 860.
141 A. Sengupta and M. A. Peat, *J. Chromatogr.*, 137 (1977) 206.
142 H. J. Kupferberg, *Clin. Chim. Acta*, 29 (1970) 283.
143 N. Kallberg, S. Agurell, B. Jalling and L. O. Boreus, *Europ. J. Clin. Pharmacol.* 3 (1971) 185.
144 M. Ehrnebo, S. Agurell and L. O. Boreus, *Europ. J. Clin. Pharmacol.*, 4 (1972) 191.
145 H. M. H. G. Cremers and P. E. Verheesen, *Clin. Chim. Acta*, 48 (1973) 413.
146 R. J. Perchalski, K. N. Scott, B. J. Wilder and R. H. Hammer, *J. Pharm. Sci.*, 62 (1973) 1735.
147 J. Roger, G. Rodgers and A. Soo, *Clin. Chem.*, 19 (1973) 590.
148 J. H. Goudie and D. Burnett, *Clin. Chim. Acta*, 43 (1973) 423.
149 R. J. Perchalski and B. J. Wilder, *Clin. Chem.*, 19 (1973) 788.
150 A. L. Sherwin, A. A. Eisen and C. D. Sokolowski, *Arch. Neurol.*, 29 (1973) 73.
151 R. Osiewicz, V. Aggarwal, R. M. Young and I. Sunshine, *J. Chromatogr.*, 88 (1974) 157.
152 M. Ehrnebo, *J. Pharm. Sci.*, 63 (1974) 1114.
153 J. Pecci and T. J. Giovanniello, *J. Chromatogr.*, 109 (1975) 163.

154 D. Schmidt and H. J. Kupferberg, *Epilepsia*, 16 (1975) 735.
155 N. Kallberg, S. Agurell, O. Ericsson, E. Bucht, B. Jalling and L. O. Boreus, *Europ. J. Clin. Pharmacol.*, 9 (1975) 161.
156 H. L. Davis, K. J. Falk and D. G. Bailey, *J. Chromatogr.*, 107 (1975) 61.
157 D. P. Lehane, P. Menyharth, G. Lum and A. L. Levy, *Ann. Clin. Lab. Sci.*, 6 (1976) 404.
158 L. K. Wong, C. E. Costello and K. Biemann, *J. Chromatogr.*, 116 (1976) 321.
159 C. V. Abraham, *Microchem. J.*, 21 (1976) 272.
160 A. N. Latham and G. Varlow, *Brit. J. Clin. Pharmacol.*, 3 (1976) 145.
161 P. Menyharth, A. L. Levy and D. P. Lehane, *Chromatogr. Newslett.*, 4 (1976) 15.
162 C. V. Abraham and H. D. Joslin, *Clin. Chem.*, 22 (1976) 769.
163 J. de Graeve and J. Vanroy, *J. Chromatogr.*, 129 (1976) 171.
164 R. E. Hill and A. N. Latham, *J. Chromatogr.*, 131 (1977) 341.
165 R. C. Kelly, J. C. Valentour and I. Sunshine, *J. Chromatogr.*, 138 (1977) 413.
166 C. J. Least, G. F. Johnson and H. M. Solomon, *Clin. Chem.*, 23 (1977) 593.
167 J. J. McAuliffe, A. L. Sherwin, I. E. Leppik, S. A. Fayle and C. E. Pippenger, *Neurology*, 27 (1977) 409.
168 W. J. Serfontein and L. S. De Villiers, *J. Chromatogr.*, 130 (1977) 342.
169 C. V. Abraham and D. Gresham, *J. Chromatogr.*, 136 (1977) 332.
170 R. H. Smith, J. A. MacDonald, D. S. Thompson and W. E. Flacke, *Clin. Chem.*, 23 (1977) 1306.
171 R. Varma, *J. Chromatogr.*, 155 (1978) 182.
172 H. Malkus, P. I. Jatlow and A. Castro, *Clin. Chim. Acta*, 82 (1978) 113.
173 Z. K. Shihabi, *Clin. Chem.*, 24 (1978) 1630.
174 K. Kurata, M. Takeuchi and K. Yoshida, *J. Pharm. Sci.*, 68 (1979) 1187.
175 F. Vincent, C. Feuerstein, M. Gavend and J. Faure, *Clin. Chim. Acta*, 93 (1979) 391.
176 H. Aspelund, *Acta Aboensis. Math. Phys.*, 20 (1955) 16; *C.A.*, 50 (1956) 11351f.
177 J. C. van Meter and H. W. Gillen, *Clin. Chem.*, 19 (1973) 359.
178 A. Wu, *Clin. Chem.*, 20 (1974) 630.
179 M. T. Bush and E. S. Bush in D. M. Woodbury, J. K. Penry and R. P. Schmidt (Editors), *Antiepileptic Drugs*, Raven Press, New York, 1971, p. 293.
180 P. S. Callery and J. Leslie, *Clin. Chem.*, 22 (1976) 926.
181 P. S. Callery and J. Leslie, *J. Pharm. Sci.*, 66 (1977) 578.
182 M. Mraz and V. Sedivec, *Collect. Czech. Chem. Commun.*, 42 (1977) 1338.
183 M. Mraz and V. Sedivec, *Collect. Czech. Chem. Commun.*, 42 (1977) 1347.
184 K. M. Williams and B. Halpern, *J. Chromatogr.*, 97 (1974) 267.
185 R. N. Gupta and P. M. Keane, *Clin. Chem.*, 21 (1975) 1346.
186 T. Nishina, K. Okoshi and M. Kitamura, *Clin. Chim. Acta*, 73 (1976) 463.
187 T. C. Butler and W. J. Waddell, *Neurology*, 8 (1958) Suppl. 1, 106.
188 A. Kumps and Y. Mardens, *Clin. Chim. Acta*, 62 (1975) 371.
189 P. Friel and A. S. Troupin, *Clin. Chem.*, 21 (1975) 751.
190 M. Kowblansky, B. M. Scheinthal, G. D. Cravello and L. Chafetz, *J. Chromatogr.*, 76 (1973) 467.
191 W. D. Hooper, D. K. Dubetz, M. J. Eadie and J. H. Tyrer, *J. Chromatogr.*, 110 (1975) 206.
192 A. Hulshoff, A. G. J. van der Houwen, D. M. Barends and H. B. Kostenbauder, *Anal. Chim. Acta*, 105 (1979) 139.
193 T. J. Giovanniello and J. Pecci, *Clin. Chim. Acta*, 67 (1976) 7.
194 T. J. Giovanniello and J. Pecci, *Clin. Chem.*, 23 (1977) 2154.
195 R. Bonnichsen, A. C. Maehly, Y. Marde, R. Ryhage and B. Schubert, *Zacchia*, 6 (1970) 371; *C.A.*, 74 (1971) 74418s.
196 J. N. T. Gilbert, B. J. Millard and J. W. Powell, *J. Pharm. Pharmacol.*, 22 (1970) 897.
197 R. F. Skinner, E. G. Gallagher and D. B. Predmore, *Anal. Chem.*, 45 (1973) 574.
198 G. H. Draffan, R. A. Clare and F. M. Williams, *J. Chromatogr.*, 75 (1973) 45.
199 D. J. Harvey, J. Nowlin, P. Hickert, C. Butler, O. Gansow and M. G. Horning, *Biomed. Mass Spectrom.*, 1 (1974) 340.
200 E. C. Horning and M. G. Horning, *Clin. Chem.*, 17 (1971) 802.
201 D. J. Harvey, L. Glazener, C. Stratton, J. Nowlin, R. M. Hill and M. G. Horning, *Res. Comm. Chem. Path. Pharm.*, 3 (1972) 557.
202 B. S. Finke and D. M. Taylor, *J. Chromatogr. Sci.*, 10 (1972) 312.
203 R. Bonnichsen, C.-G. Fri, B. Hedfjall and R. Ryhage, *Z. Rechtsmedizin*, 70 (1972) 150.
204 W. Ehrenthal, K. Pfleger and M. Moller, *Varian MAT Publication*, No. 22.
205 J. B. Knight, *Finnigan Application Tips*, No. 33 Finnigan, Sunnyvale, CA, 1971, p. 1.
206 C. van Peteghem, A. Heyndrickx and A. Moulin, *Acta Pharmacol. Toxicol., Suppl.* 41 (1977) 212.

207 M. G. Horning, J. Nowlin, K. Lertratanangkoon, R. N. Stillwell, W. G. Stillwell and R. M. Hill, *Clin. Chem.*, 19 (1973) 845.
208 M. G. Horning, W. G. Stillwell, J. Nowlin, K. Lertratanangkoon, D. Carroll, D. Dzidic, R. N. Stillwell, E. C. Horning and R. M. Hill, *J. Chromatogr.*, 91 (1974) 413.
209 B. K. Tang, T. Inaba and M. Kalow, *Drug Metab. Dispos.*, 3 (1975) 479.
210 T. Inaba, B. K. Tang, L. Endrenyi and W. Kalow, *Clin. Pharmacol. Ther.*, 20 (1976) 439.
211 J. N. T. Gilbert, B. J. Millard, J. W. Powell, W. B. Whalley and B. J. Wilkins, *J. Pharm. Pharmac.*, 26 (1973) 119.
212 J. N. T. Gilbert, B. J. Millard and J. W. Powell, *Brit. J. Pharmacol.*, 47 (1973) 665.
213 J. N. T. Gilbert, B. J. Millard, J. W. Powell and W. B. Whalley, *J. Pharm. Pharmac.*, 26 (1974) 123.
214 J. N. T. Gilbert and J. W. Powell, *Biomed. Mass Spectrom.*, 1 (1974) 142.
215 M. G. Horning, P. Gregory, J. Nowlin, M. Stafford, K. Lertratanangkoon, C. Butler, W. G. Stillwell and R. M. Hill, *Clin. Chem.*, 20 (1974) 282.
216 M. G. Horning, K. Lertratanangkoon, J. Nowlin, R. N. Stillwell, T. E. Zion, P. Kellaway and R. M. Hill, *J. Chromatogr. Sci.*, 12 (1974) 630.
217 B. K. Tang, T. Inaba and W. Kalow, *Biomed. Mass Spectrom.*, 4 (1977) 73.
218 W. Baldeo, J. N. T. Gilbert, J. W. Powell, L. V. Jones and M. J. Whitehouse, *J. Pharm. Pharmac.*, 29 (1977) 254.
219 D. E. Games, A. H. Jackson, K. T. Taylor and N. J. Haskins, *Advan. Mass Spectrom. Biochem. Med.*, 1 (1976) 383.
220 H. S. Fraser, F. M. Williams, D. L. Davies, G. H. Draffan and D. S. Davies, *Xenobiotica*, 6 (1976)
221 M. G. Horning, W. G. Stillwell, J. Nowlin, K. Lertratanangkoon, R. N. Stillwell and R. M. Hill, *Mod. Probl. Paediat.*, 15 (1975) 73.
222 B. Krauer, G. H. Draffan, F. M. Williams, R. A. Clare, C. T. Dollery and D. F. Hawkins, *Clin. Pharmacol. Ther.*, 14 (1973) 442.
223 M. G. Horning, J. Nowlim, M. Butler, K. Lertratanangkoon, K. Sommer and R. M. Hill, *Clin. Chem.*, 21 (1975) 1282.
224 M. G. Horning, J. Nowlin, M. Stafford, K. Lertratanangkoon, K. R. Sommer, R. M. Hill and R. N. Stillwell, *J. Chromatogr.*, 112 (1975) 605.
225 L. Endrenyi, T. Inaba and W. Kalow, *Clin. Pharmacol. Ther.*, 20 (1976) 701.
226 M. G. Horning, L. Brown, J. Nowlin, K. Lertratanangkoon, P. Kellaway and T. E. Zion, *Clin. Chem.*, 23 (1977) 157.
227 M. L. Bastos, G. E. Kananen, R. M. Young, J. R. Monforte and I. Sunshine, *Clin. Chem.*, 16 (1970) 931.
228 S. K. Niyogi and F. Rieders, *Acta Pharmacol. Toxicol*, 29 (1971) 113.
229 J. G. H. Cook, *Proc. Assoc. Clin. Biochem.*, 2 (1963) 199.
230 S. Dilli and A. Weekley, *J. Chromatogr.*, 195 (1980) 349.
231 L. M. St. Onge, E. Dolar, M. A. Anglim and C. J. Least, *Clin. Chem.*, 25 (1979) 1373.
232 A. Arbin, *J. Chromatogr.*, 144 (1977) 85.
233 W. Godolphin and J. Thoma, *Clin. Chem.*, 24 (1978) 483.
234 K. W. Leal, A. J. Wilensky and R. L. Rapport, *J. Anal. Toxicol.*, 2 (1978) 214.
235 D. R. Deans, *Chromatographia*, 1 (1968) 18.
236 F. L. Vandemark and R. F. Adams, *Clin. Chem.*, 22 (1976) 1062.
237 C. E. Pippenger, J. K. Penry and H. Kugg, *Anti-Epileptic Drugs: Quantitative Analysis and Interpretation*, Raven Press, New York, 1978, p. 77.
238 G. M. Schier and I. E. T. Gan, *Clin. Chem.*, 25 (1979) 1191.
239 D. C. Fenimore, C. M. Davis, J. H. Whitford and C. A. Harrington, *Anal. Chem.*, 14 (1976) 2289.
240 P. I. Jaakonmaki, K. L. Knox, E. C. Horning and M. G. Horning, *Eur. J. Pharmacol.*, 1 (1967) 63.
241 S. J. Mulé, M. L. Bastos, D. Jukofsky and E. Saffer, *J. Chromatogr.*, 63 (1971) 289.
242 N. Weissman, M. L. Lowe, J. M. Beattie and J. A. Demetriou, *Clin. Chem.*, 17 (1971) 875.
243 M. L. Bastos, D. Jukofsky, E. Saffer, M. Chedekel and S. J. Mulé, *J. Chromatogr.*, 71 (1972) 549.
244 M. P. Kullberg, W. L. Miller, F. J. McGowan and B. P. Doctor, *Biochem. Med.*, 7 (1973) 323.
245 A. W. Missen and J. F. Lewin, *Clin. Chim. Acta*, 53 (1974) 389.
246 P. A. F. Pranitis, J. R. Milzoff and A. Stolman, *J. Forens. Sci.*, 19 (1974) 917.
247 M. Schwartz and W. Read, in *Rapid Drug Screening by Gas Chromatography, III*, Bendix Corp., 1972, p. 15.
248 L. B. Hetland, D. A. Knowlton and D. Couri, *Clin. Chim. Acta*, 36 (1972) 473.
249 R. K. Lantz and R. B. Eisenberg, *Clin. Chem.*, 24 (1978) 821.
250 P. Holtzbein, G. Bohn and G. Rucker, *Z. Anal. Chem.*, 292 (1978) 216.