CHROM 4029

GAS–LIQUID CHROMATOGRAPHY OF SUBMICROGRAM AMOUNTS OF DRUGS

IV. IDENTIFICATION OF BARBITURATES, HYDANTOINS, AMIDES, IMIDES, CARBAMATES, PHENYLBUTAZONE, CARBOXYLIC ACIDS AND HYDRAZINE DERIVATIVES BY DIRECT DERIVATIVE FORMATION WITHIN THE GAS CHROMATOGRAPH

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SUMMARY

By the single injection of a mixture of N,O-bistrimethylsilylacetamide and drug into the gas chromatograph, trimethylsilyl derivatives of carboxylic acids, barbiturates, amides, imides, hydantoins, carbamates, phenylbutazone, hydrazine derivatives, alcohols, phenols and certain amines can be formed within the apparatus. The retention time (t_r) of the derivative may be less than, equal to, or greater than the t_r of the parent drug; peak shapes are more symmetrical; sensitivity is increased. These factors, together with information obtained by a similar procedure of derivative formation (where applicable) using aldehydes, ketones and acid anhydrides, provide additional parameters for use in drug identification both in simple solution and in extracts of biological material.

INTRODUCTION

In the application of gas–liquid chromatography (GLC) to the qualitative analysis of drugs in biological material, it is necessary to have at least one parameter other than the retention time (t_r) of the drug in order to make the identification of the drug more certain. The parameters employed in this respect may involve other procedures making use of absorption characteristics of the drug in the ultraviolet or infrared regions of the spectrum, mass spectrometry, individual 'spot tests' and colour reactions, R_F values obtained by thin-layer or paper chromatography, etc. With the possible exception, in certain cases, of mass spectrometry and infrared spectrometry, these other procedures are not generally sufficiently sensitive for work in the submicrogram region. Determination of melting point or boiling point is also excluded when dealing with submicrogram quantities of compounds. On the macro scale, one of the most useful parameters for identification purposes is the formation of derivatives of the unknown compound, the physical characteristics of which are then compared with those formed from appropriate standards. The formation of

derivatives of drugs is particularly suitable in GLC analysis for three main reasons. Firstly, the derivative has, in most cases, a t_r which differs sufficiently from the t_r of the parent drug to be regarded as an additional parameter in characterising the drug. Secondly, since derivatives are formed by reaction with functional polar groups of the drug, the adsorption of the derivative by the column support material is less than that of the drug itself. This produces more symmetrical peak shapes and greater sensitivity, thus making the process suitable for work with submicrogram quantities of drugs. Thirdly, the derivatives can be formed *within* the gas chromatograph; and it is with this aspect of the subject that this paper deals.

Two good review articles relating to peak identification have been produced recently. That by BEROZA AND COAD[1] deals with the broad topic of reaction gas chromatography whilst that by PERRY[2] relates more specifically to peak identification in gas chromatography. In the work described below, derivatives of compounds such as barbiturates, hydantoins, amides, imides, carboxylic acids, etc., are formed within the gas chromatograph by simple, single injection of a mixture of reagent and drug.

EXPERIMENTAL

Gas chromatography details including preparation of metal column, treatment of diatomaceous earth, application of liquid phase (SE-30 or SE-52), packing of column, purification of reagents, etc., have been described in previous articles by STREET[3,4] and MCMARTIN AND STREET[5,6]. The apparatus used for the work described in this paper was an F & M Model 810 gas chromatograph equipped with flame ionisation detectors. The carrier gas was nitrogen, flow rate 23 ml/min.

Drug nomenclature

Where possible, the names of the drugs studied conform to those given in the Merck Index, 7th edition, 1960. Mefenamic acid is N-(2,3-xylyl) anthranilic acid; ethinamate is ethynylcyclohexylcarbamate; phenacetin is acetophenetidine; paracetamol is 4-hydroxyacetanilide. In the 8th edition of the Merck Index, 1968, Primidone is listed under Primaclone.

Reagents used for formation of derivatives

Table I gives a list of the reagents used for derivative formation within the gas chromatograph. Benzaldehyde, methyl ethyl ketone and the acid anhydrides were used without dilution whereas N,O-bistrimethylsilylacetamide (BSA) was used either undiluted, or as a 10 or 20% solution in acetone, *n*-hexane, or acetonitrile. Acetone (or other ketones) cannot be used as a solvent when searching for primary amines by GLC. Trimethylsilyldiethylamine and N-trimethylsilylimidazole (described by HORNING, MOSS AND HORNING[7]) were also tried as trimethylsilyl donors but were found to be not as effective as BSA.

Procedure

The treatment of urine, blood and liver samples is carried out as described previously by STREET[3,4] and by STREET AND MCMARTIN[8]. These procedures give rise to the following five fractions:

TABLE I

REAGENTS USED AND TYPES OF GROUPS AND DRUGS SUITABLE FOR DERIVATIVE FORMATION WITHIN THE GAS CHROMATOGRAPH

Reagents	*Reacting group present in drug molecules*	*Examples of drugs*
N,O-bistrimethylsilylacetamide (BSA)	Carboxylic acids	Aspirin, salicylic acid, benzoic acid, mefenamic acid
N.B. BSA also reacts with primary and secondary amines, but the reagents listed below are more suitable for reaction with these groups because there appears to be some inconsistency in results, particularly with secondary amines, *e.g.* the trimethylsilyl derivative of nortriptyline is unstable at the temperature required to 'run' the parent drug.	5,5- and 1,5,5-substituted barbituric acid derivatives	Barbiturates
	Amides or substituted amides	Lidocaine, phenacetin, paracetamol (also possesses a phenolic—OH group), primidone (a substituted cyclic diamide)
	Imides	Glutethimide, Bemegride
	Hydantoins	Mesantoin, diphenylhydantoin
	Carbamates	Ethinamate, physostigmine
	Hydrazine derivatives	Iproniazid, Isocarboxazid
	Enolic form of 3,5-dioxopyrazolidines	Phenylbutazone
	Alcohols and phenols	See below
Aldehydes, *e.g.* benzaldehyde Ketones, *e.g.* methylethyl ketone. (Higher mol. wt. ketones are more suitable for high mol. wt. drugs).	Primary amines[a]	Amphetamine, bisnortriptyline
Acid anhydrides, $(R \cdot CO)_2O$. R varied from CH_3 to C_6H_{13}, but acetic and butyric anhydrides most useful	Primary amines	Chlorphentermine
	Secondary amines	N-Methylamphetamine, nortriptyline
	Alcohols	Quinine, codeine, morphine
	Phenols	Morphine, paracetamol, certain constituents of cannabis

[a] Compounds containing the group —C(OH)—C(HN—R)— (*e.g.* ephedrine) also react with aldehydes and ketones.

(1) strongly acidic drugs, *e.g.* salicylic acid, mefenamic acid; (2) weakly acidic drugs, *e.g.* barbiturates, glutethimide; (3) 'neutral' drugs, *e.g.* ethinamate, phenacetin; (4) basic drugs, *e.g.* amphetamine, phenothiazines; (5) 'amphoteric' drugs, *e.g.* morphine.

From 1 to 5 μl of reagent are drawn up into a 10 μl SGE* syringe. This is followed in the same syringe by 1 or 2 μl of pure solution of drug, or a solution of the residue of the biological extract. This mixture is then injected directly into the gas chromatograph.

* Scientific Glass Engineering Pty. Ltd., London and Melbourne.

In some preliminary experiments where relatively large amounts of starting material were available, *e.g.* when injecting extracts of cannabis, a stream splitter was used to collect the various fractions as they emerged from the column. This was done by holding a glass melting-point tube in the exit pipe of the splitter. The tube was passed through a silicone rubber septum which acted as a seal. These collected fractions were then subjected to further tests, *e.g.* using thin-layer chromatography and the elevated temperature reversed-phase paper chromatography described by STREET[9–12].

For injection into the gas chromatograph, the choice of solvent used to dissolve the residue containing the drug is important. Acetone is a good general solvent suitable for use with BSA, but must *Not* be used with extracts containing primary amines, owing to the formation of Schiff bases. Chloroform is a suitable alternative to acetone, but should be freshly distilled.

RESULTS

The type of reactive groups and drugs which are amenable to derivative formation under the conditions described above are listed in Table I. Detailed information relating to some of the types of drugs which form trimethylsilyl (TMS) derivatives under the above conditions is given in Table II. The results obtained by injecting a mixture of BSA and nine barbiturates are shown in Fig. 1a. A chromatogram of the nine (unreacted) barbiturates is shown for comparison purposes in Fig. 1b.

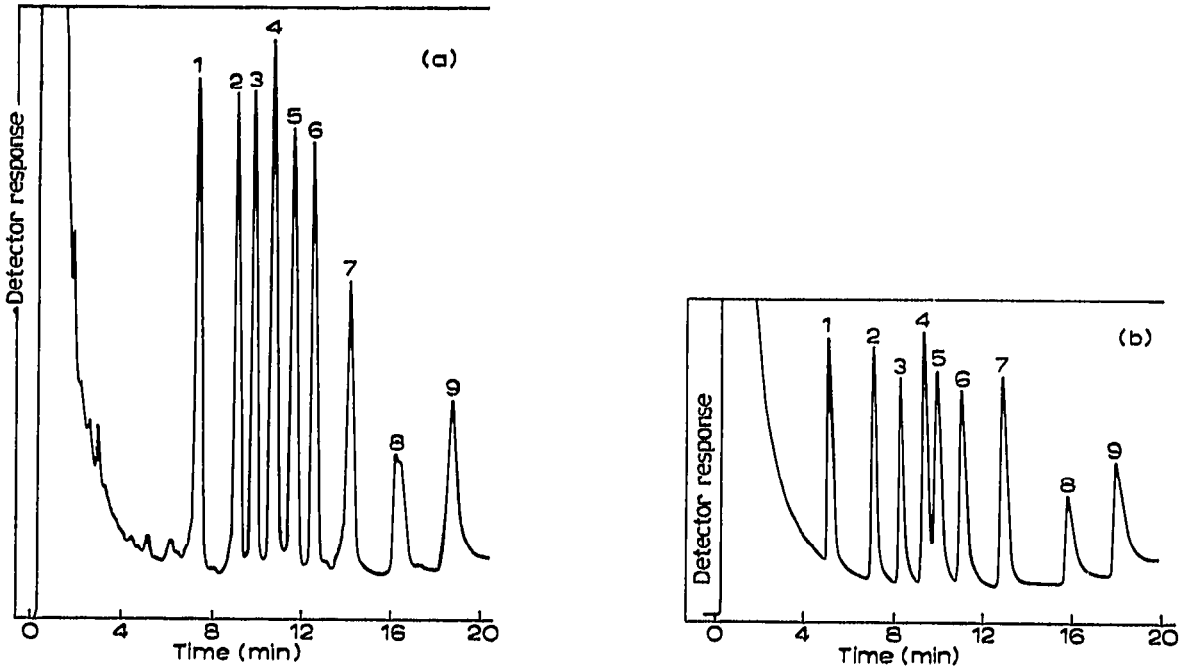


Fig. 1. (a) Gas–liquid chromatogram of TMS derivatives of barbiturates obtained by injecting 3 μl of 20% BSA (in *n*-hexane) together with 1 μl of a chloroform solution containing 0.5 μg of each of the following barbiturates: 1 = barbital; 2 = diallylbarbituric acid; 3 = butethal; 4 = amobarbital; 5 = pentobarbital; 6 = secobarbital; 7 = hexobarbital; 8 = cyclobarbital; 9 = heptabarbital. Column temperature: 160° at start, programmed at 4°/min; attenuation × 100. (b) As in (a) but *without* BSA reagent. Note the change in retention times.

TABLE II

DETAILS OF THE CONDITIONS FOR TMS DERIVATIVE FORMATION, WITHIN THE GAS CHROMATOGRAPH, OF A NUMBER OF DRUGS POSSESSING THE FUNCTIONAL GROUPS REFERRED TO IN THE TEXT

Drug	*Mol. wt.*	*Amount of drug injected (μg)*	*Volume of solvent injected (μl)*	*Volume of BSA reagent injected*	*GLC column temperature (°C)*	*GLC injector port temperature (°C)*	*GLC attenuation factor*	*t_r of drug (min)*	*t_r of derivative (min)*
Salicylic acid	138	0.8	0.8	2 μl	137	250	80	7.20[a]	15.32
Paracetamol	151	0.8	0.8	2 μl	183	253	160	8.80[a]	4.84[b]
Ephedrine	165	0.8	0.8	0.4 μl 10% in acetone	137	270	100	10.44	10.20
Ethinamate	167	0.8	0.8	1 μl	162	272	100	5.60	3.48
Iproniazid	179	0.8	0.8	1 μl 20% in *n*-hexane	172	263	100	7.48[a]	7.60[c]
Phenacetin	179	0.4	0.4	1 μl 20% in *n*-hexane	185	305	100	6.48	4.00
Acetylsalicylic acid	180	0.8	0.8	2 μl	137	250	80	6.84	16.68
Glutethimide	217	0.8	0.8	1 μl	183	255	80	13.60	13.80
Primidone	218	0.8	0.8	4 μl 10% in acetone	218	260	320	17.60	3.44[d]
Marplan	231	0.8	0.8	2 μl 10% in acetone	210	263	100	8.00	6.00
Lidocaine	234	0.8	0.8	1 μl 10% in acetone	187	278	100	13.64	8.60
Mefenamic acid	241	0.25	1.0	1 μl	227	267	100	9.40[a]	9.20
Diphenylhydantoin	252	0.8	0.8	2 μl	240	267	160	11.80	6.80
Physostigmine	275	0.8	0.8	1 μl 10% in acetone	195	258	100	8.48	8.52
Morphine	285	0.1	1.0	1 μl	253	275	40	8.00	8.80
Phenylbutazone	308	0.8	0.8	2 μl	252	275	80	6.80	10.92
Quinine	324	0.1	1.0	1 μl 10% in acetone	252	275	10	18.80[a]	12.28

[a] In these cases, it was necessary to use more than 1 μg of drug to obtain a peak for the *unchanged drug* at the attenuation stated.

[b] Insufficient BSA produces a second peak (t_r = 8.40 min) presumably due to formation of a mono-TMS derivative (probably the TMS *ether*) of paracetamol.

[c] A smaller peak (t_r = 8.68 min) was also present in this case. This may be due to the formation of a di-TMS derivative but the relative heights of the two peaks did not change significantly as the proportion of BSA to drug was varied. The second peak may, therefore, be due to an impurity in the original drug sample; this is being investigated further.

[d] With amounts of BSA less than those stated, a second peak (t_r = 7.40 min) is present. This is probably due to the formation of a mono-TMS derivative. See Fig. 3, and Discussion.

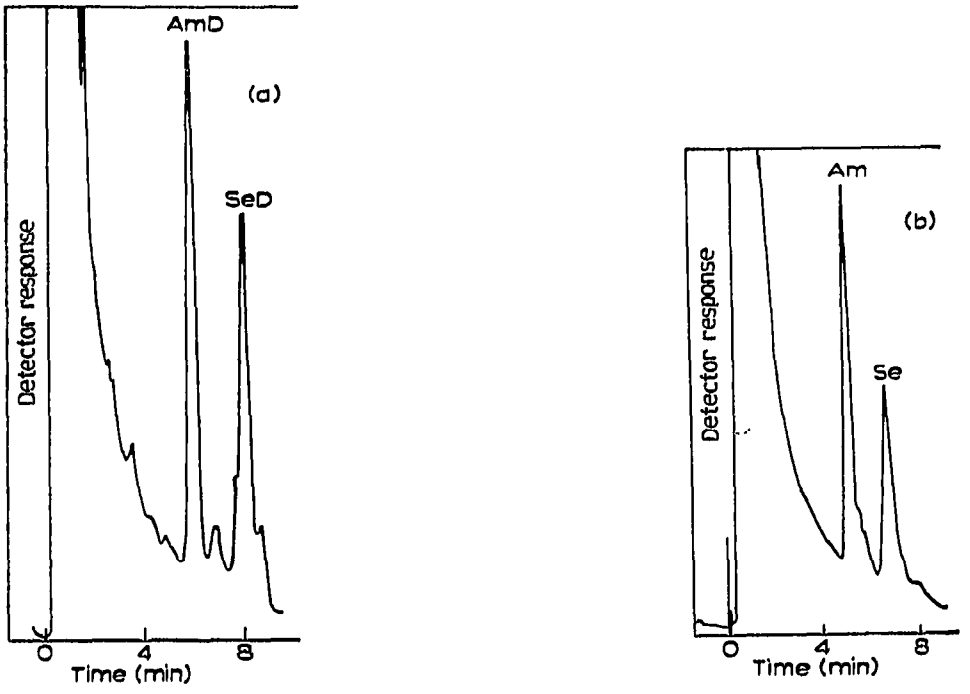


Fig. 2. (a) Gas–liquid chromatogram of 5 μl of an extract (dissolved in acetone) of post-mortem liver injected together with 3 μl of BSA. Death was due to ingestion of an overdose of amobarbital and secobarbital. AmD and SeD = TMS derivatives of amobarbital and secobarbital, respectively. Column temperature: 197°. Attenuation × 160. (b) As in (a) but *without* BSA; and attenuation × 100. Am = amobarbital; Se = secobarbital.

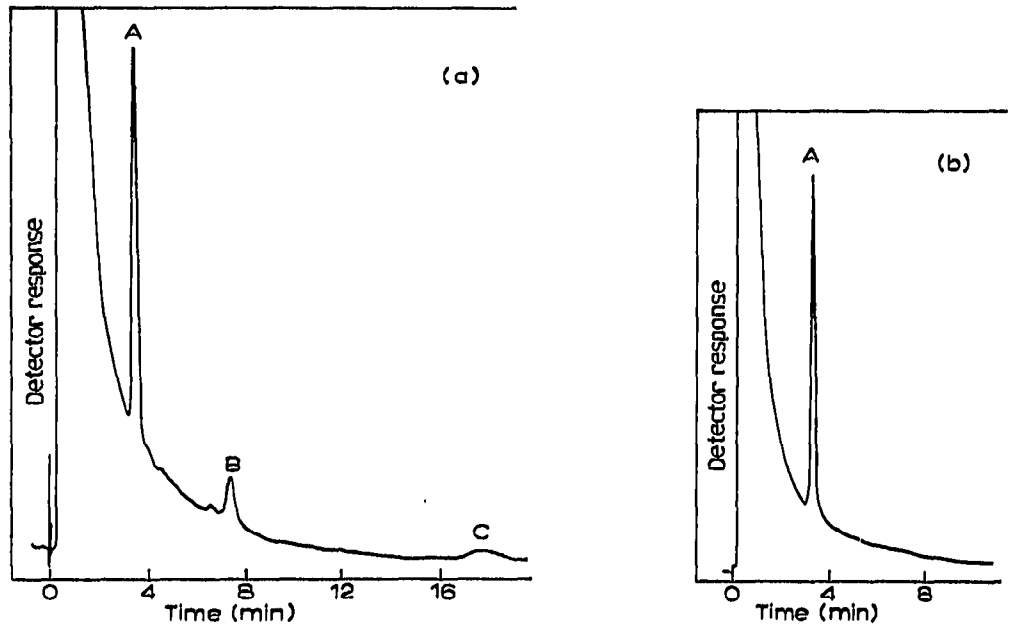


Fig. 3. (a) Gas–liquid chromatogram obtained by injecting 0.6 μl of 10% BSA (in acetone) together with 0.8 μl (0.8 μg) of Primidone (in acetone). Column temperature: 218°; attenuation × 80. A is probably the di-TMS derivative of Primidone; B is probably a mono-TMS derivative of Primidone; C is unchanged Primidone. (b) As in (a) but with 4 μl of 10% BSA (in acetone) and attenuation × 320.

Results of application of the procedure to an actual case of death by ingestion of an overdose of Tuinal are illustrated in Fig. 2, which shows the TMS derivatives of amobarbital and secobarbital from an extract of liver.

The effect on derivative formation of varying the amount of BSA is illustrated in the case of Primidone in Fig. 3.

DISCUSSION

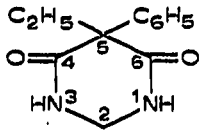
The technique of 'on-column' esterification was introduced by ANDERS AND MANNERING[13,14] to form derivatives of amines, alcohols and phenols. Amphetamine was identified by BECKETT AND ROWLAND[15] by the alteration in retention time after reaction with acetone. This was a modification of a procedure originally described by BROCHMANN-HANSSEN AND SVENDSEN[16]. Formation of derivatives of amines and alkaloids, within the gas chromatograph, has also been discussed by STREET[4]. In 1966, STEVENSON[17] showed that barbituric acids could be methylated on the column by injecting a mixture consisting of the drug and tetramethylammonium hydroxide (TMAH). However, this procedure gave rise to two peaks for each barbiturate and the relative heights of these peaks were related to injector port temperature and amount of TMAH. The two peaks were due to methylation of either one or both of the nitrogen atoms of the 5,5-disubstituted barbituric acids. ROWLAND AND RIEGELMAN[18] found that in the silylation of submicrogram amounts of carboxylic acids, the response of the flame ionisation detector (F.I.D.) to the usual solvents was so great as to obscure the peak due to the compound being estimated. They suggested the use of carbon disulphide as a solvent (because the response of the F.I.D. to this compound is very low), and found that by this means they could determine less than 1 μg of acetylsalicylic acid per millilitre of plasma.

The use of BSA as a highly reactive silyl donor has been described by KLEBE, FINKBEINER AND WHITE[19] although the compound was first reported by BIRKOFER, RITTER AND GIESSLER[20]. The fact that it has now been found that BSA can be injected directly into the gas chromatograph means that less time is wasted in forming derivatives, and also that there is no chance of the hydrolysis which sometimes occurs with certain derivatives in damp atmospheres necessitating special precautions being taken to avoid introducing moisture.

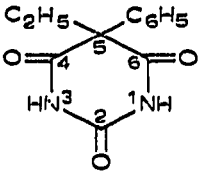
In all the above procedures, except those described by the present writer, the derivatives are either prepared before injection into the gas chromatograph or are formed on the column by first injecting the drug solution followed by a second injection of reagent. In the procedures described in this paper and in the paper by STREET[4], there is only one injection, the drug and the reagent being taken up into the same syringe. These procedures give highly reproducible retention times for the derivatives and because of the increase in sensitivity, due to modification of polar groups, have been found to be applicable to work in the sub-microgram region. In several cases, *e.g.* with paracetamol, no peak is seen when less than 2 μg of drug is injected but when injected with BSA, less than 1 μg of derivative is readily detected. This means that a tentative identification of certain drugs which may have been 'missed' during GLC analysis can now be made. KLEBE *et al.*[19] found that, in the reaction of BSA with *p*-tolylmethylurea, only one of the two nitrogen-bound protons in the urea was displaced. They suggested that this was because the di-silyl derivative would

be on an energy level almost as high as BSA itself. For this reason, it is probable that the 5,5-disubstituted barbituric acids referred to in the present work are only mono-silylated on one of the nitrogen atoms. Similar thermodynamic considerations also probably account for the fact that hexobarbital, which already has a methyl group on one of its nitrogen atoms, is more difficult to silylate than those barbiturates which have protons attached to their nitrogen atoms.

Because Primidone (I) is oxidised in the body to phenobarbital (II), it is often considered by biochemists that the drug is chemically similar to the barbituric acid derivatives.



(I) Primidone



(II) Phenobarbital

However, if one looks at the structural formulae of these two compounds, one sees that, in phenobarbital, each of the two nitrogen atoms is attached to two =C=O groups whereas in Primidone, only one =C=O group is attached to each of the two nitrogen atoms. Furthermore, the electron-attracting effects due to the oxygen atoms are in the same general direction and are not opposed by an oxygen on C_2. This would tend to weaken the two =N—H bonds in Primidone. Hence it would be expected that the nitrogen-bound protons of Primidone could be replaced more easily than those of phenobarbital. Indeed, it has been found that Primidone can be N-acetylated by injection of acetic anhydride whereas phenobarbital cannot be acetylated in this way. It may, therefore, be more satisfactory to regard Primidone as a mono-substituted cyclic di-amide than as a derivative of pyrimidine. But even as a mono-substituted amide it should not react with acetic anhydride, just as acetophenetedin does not N-acetylate under the above conditions. Hence, in order to account for the acetylation of Primidone, it would seem to be necessary to take into consideration the effect and position of the oxygen atoms and possibly the phenyl group, and the fact that there is no strongly electro-negative element attached to C_2 of the ring. However, the facts that Primidone can be acetylated by injection and phenobarbital cannot, and that both these drugs do form TMS derivatives by injection, provide useful additional parameters in the analysis of these compounds.

It will be noted that the retention time (t_r) of the TMS derivative is greater than that of the parent drug in some cases (*e.g.* all the barbiturates investigated except phenobarbital), whilst it is smaller in other cases (*e.g.* Primidone, Marplan). This probably depends on the relative dipole moments of drug and TMS derivative. For example, the dipole moment of barbital would be expected to be less than that of its mono-TMS derivative and hence the t_r of this latter compound would be greater than the t_r of barbital. In the case of Primidone, which forms a mono- and/or a di-TMS derivative, the dipole moments would be in the descending order Primidone, mono-TMS, di-TMS. This would be in keeping with the position and shapes of the peaks shown in Fig. 3. Factors other than dipole moments also affect relative t_r values, *e.g.* hydrogen bonding. However, when columns are used which show only

slight tendency to donate or accept protons (*i.e.* have low adsorption, see ref. 4), the t_r values are only minimally affected. In a few instances, there is very little difference between t_r of drug and t_r of TMS derivative, *e.g.* physostigmine. The only evidence (from the chromatogram) that a derivative has been formed is the production of a more nearly symmetrical peak and a big increase in peak height, both of which may be attributed to reduction of polarity and, therefore, reduction of adsorption by the column.

The TMS derivative of phenobarbital has a shorter t_r than unchanged phenobarbital, thus providing an exception to the general pattern observed with those barbiturates referred to in Fig. 1a and b where the t_r of the TMS derivative is greater than the t_r of the parent drug. Again this is probably an effect due to polarity differences, although it is interesting to note that rutonal, which is a 5-methyl-5-phenylbarbituric acid derivative (as compared with phenobarbital, which is a 5-ethyl-5-phenyl derivative) has a shorter t_r than its TMS derivative. This difference between the behaviour of phenobarbital and the other barbiturates investigated is of value in discriminating between cyclo- and phenobarbital which, in the unmodified states, have very similar t_r values on SE-30 and SE-52.

Using BSA and the other reagents listed in Table I, it is now possible to identify a wide range of drugs using only a gas chromatograph. When combined with other parameters, the procedure provides one step nearer to a fully comprehensive scheme of toxicological analysis. Further work in this sphere could be profitably directed towards 'on-column' derivative formation of drug metabolites, especially glucuronides.

ACKNOWLEDGEMENT

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