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METHYLATION ARTIFACTS IN THE GAS CHROMATOGRAPHY OF SERUM EXTRACTS

COMPETITIVE SUPPRESSION WITH TRIMETHYL-d_9 ANILINIUM HYDROXIDE

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SUMMARY

In the course of the identification of drugs in body fluids by gas chromatography–mass spectrometry we have observed the appearance of N-methyl derivatives of certain compounds such as phenobarbital and diphenylhydantoin, especially in extracts of serum. With the aid of deuterium labelled phenobarbital it could, however, be shown that the N-methyl derivatives are not endogenous metabolites but artifacts generated upon injection of the serum extract into the gas chromatograph. Conversely, derivatization with trimethyl-d_9 anilinium hydroxide (TMAH-d_9) demonstrated the absence of any undeuterated methyl derivative and this reagent should be useful for the detection of endogenous N-methyl derivative which would, of course, remain undetected when using unlabelled TMAH in the course of the conventional methylation technique. There are good indications that lecithin is the methylating agent in serum responsible for the formation of these artifacts.

INTRODUCTION

N-Alkylated phenobarbitals[1,2] have recently aroused much interest from medicinal chemists due to their potential use as better anticonvulsants free from hypnotic effects. The most promising member of this newly developed class of barbiturates is N,N′-dimethoxymethylphenobarbital* (DMMP, Ia). The metabolic fate of this drug in mice has been studied extensively[3,4]. In all cases, phenobarbital (IIa) was found to be the major metabolite. N-Methyl-N′-methoxymethylphenobarbital (Ib) was detected in the blood and the brain only during the early period after introduction of DMMP to the animals or when DMMP was given in a very high dose[5]. Studies with human volunteers[6] also validated the presence of phenobarbital as the major metabolite. A minor component, namely N-methylphenobarbital (mephobarbital, Ic), was also detectable in gas chromatograms of serum

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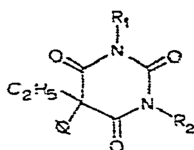
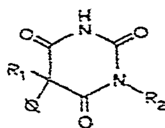
* Antalon™, eterobarb (generic).



I

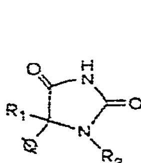


II

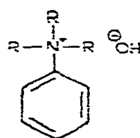
	R_1	R_2
(a)	CH_3–O–CH_2	CH_3–O–CH_2
(b)	CH_3	CH_3–O–CH_2
(c)	CH_3	H
(d)	CH_3	CH_3
(e)	CH_3	CD_3
(f)	CD_3	CD_3

	R_1	R_2
(a)	C_2H_5	H
(b)	C_2D_5	H
(c)	C_2D_5	CH_3

extracts, though the results were not very reproducible and depended very much on gas chromatographic (GC) and extraction procedures[7]. In our laboratory, preliminary gas chromatographic–mass spectrometric (GC–MS) data on serum samples from patients receiving DMMP also led to a similar observation. We further observed that, whereas urine samples from phenobarbital patients usually contained phenobarbital and no N-methylphenobarbital, analysis of the corresponding serum samples invariably indicated the presence of small amounts of N-methylphenobarbital in addition to phenobarbital. This observation held even when the level of phenobarbital in the urine sample was much higher than that in the corresponding serum sample obtained from the same individual. This phenomenon and the inconsistent results from other laboratories led us to suspect that the N-methylphenobarbital arose from some thermally induced reaction between phenobarbital and an agent in the serum extract upon injection into the gas chromatograph. Using two independent methods described in this paper, namely a coinjection experiment with phenobarbital-ethyl-d_5 (IIb) and N-trideuteromethylation of the serum extract using trimethyl-d_9 anilinium hydroxide (TMAH-d_9, IVa), we were able to show that the N-methylphenobarbital in question was indeed an artifact formed as a result of thermally induced methylation in the injection port of the gas chromatograph. Furthermore, it could be shown that lecithin (phosphatidyl choline, V) is capable of N-methylating phenobarbital in this manner. These results suggest the probable role of lecithin as the methylating agent during GC of serum extracts. Furthermore, we have demonstrated that diphenylhydantoin (IIIa) can undergo N-methylation in the same manner.



III



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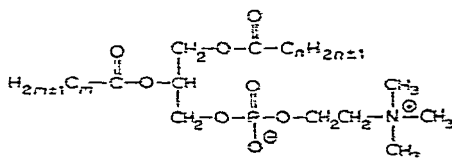


V

	R_1	R_2
(a)	Ø	H
(b)	CH_3–Ø–	H
(c)	CH_3–Ø–	CH_3

(a) R = CD_3
(b) R = CH_3

$m = 15, 17$
$n = 15, 17$

EXPERIMENTAL

Gas chromatography

A Perkin-Elmer 990 gas chromatograph was used, equipped with a 3 ft. or 6 ft. × 1/8 in. glass column of 3% OV-17 on Gas-Chrom Q (100–120 mesh). It was temperature programmed from 160° to 330° at 6°/min. The injector temperature was 220°.

Mass spectrometry

A Perkin-Elmer-Hitachi RMU-6L mass spectrometer was interfaced to the Perkin-Elmer 990 gas chromatograph via a fritted glass separator. An IBM 1800 computer was used for data acquisition and control[8]. Mass spectra were recorded continuously during an entire GC run. The ionizing voltage of the mass spectrometer was 70 eV. The temperature of the ion source was 220° and that of the manifold was held constant at 250°.

Chemicals

Phenobarbital-ethyl-d_5 had been prepared[9] by Dr. B. D. Andresen (presently at the School of Pharmacy, University of Florida, Gainesville, Fla., U.S.A.). Bovine lecithin (25 mg/ml benzene solution) was purchased from P-L Biochemicals (Milwaukee, Wisc., U.S.A.). Methyl iodide-d_3 was purchased from Merck (Quebec, Canada).

Serum samples

Serum extracts from patients receiving DMMP were provided by Dr. B. B. Gallagher (Georgetown University, School of Medicine, Washington, D.C., U.S.A.). Serum samples of phenobarbital patients were obtained from Massachusetts General Hospital.

Synthesis of trimethyl-d_9 anilinium hydroxide

Methyl-d_3 iodide (3 g) was added to 0.6 g of aniline in a small beaker. The crystalline product, trimethyl-d_9 anilinium iodide, was washed a few times with dichloromethane in a Büchner funnel over a suction flask. Some of the crystals (0.25 g) were dissolved in 2 ml of absolute ethanol in a 5-ml beaker equipped with a magnetic stirrer. Silver oxide (0.3 g) was added to precipitate the iodide. Aliquots of the mixture were withdrawn frequently and tested for iodide by acidic silver nitrate solution. Stirring continued until the assay gave a negative test. The mixture was then filtered by suction. The resulting alcoholic solution of TMAH-d_9 was stored under nitrogen.

Extraction procedure

The procedure has been described elsewhere[10]. In short, a blood sample (5 ml) was centrifuged and to the resulting serum (2.5 ml) dichloromethane (12.5 ml) was added. The serum was extracted once more with dichloromethane after adjusting the pH to about 9 by addition of solid sodium bicarbonate. The combined organic extract was dried over anhydrous sodium sulfate, concentrated on a rotary evaporator to

about 200 μl, then transferred to a culture tube (6 × 50 cm) and further concentrated to 50 μl using a stream of nitrogen.

Coinjection experiment with phenobarbital-ethyl-d_5

The extract obtained from 1 ml serum of patients receiving DMMP was mixed with 1 μl of a solution (1 μg/μl) of phenobarbital-ethyl-d_5. The mixture was then taken up with a 10-μl syringe and injected into the GC–MS system.

GC–MS analysis of phenobarbital in normal serum

Phenobarbital (1 mg) was added to 5 ml of serum obtained from a normal individual who was not receiving any drugs. The serum sample was then extracted and concentrated as described above. An aliquot of the extract (1 μl) was taken up with a 10-μl syringe and injected into the GC–MS system.

Derivatization with TMAH-d_9

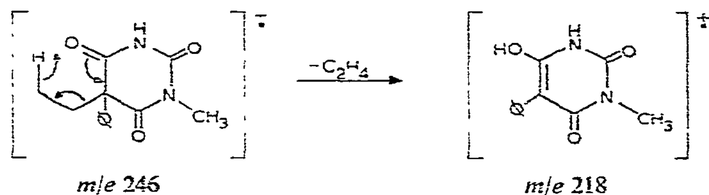
Another aliquot of the extract of serum (1 μl) obtained from a phenobarbital patient was pre-mixed with 4 μl of TMAH-d_9 reagent in a capillary tube. The resulting mixture was taken up in a 10-μl syringe and injected into the GC–MS system.

Lecithin experiment

Phenobarbital (2 mg) was dissolved in 0.5 ml of dichloromethane and 1 μl of this solution was pre-mixed with 1 μl of lecithin reagent in a capillary tube. The resulting mixture was taken up in a 10-μl syringe and injected into the GC–MS system.

RESULTS AND DISCUSSION

The mass spectra of phenobarbital and N-methylphenobarbital exhibit base peaks at *m/e* 204 and *m/e* 218, respectively. They are due to the elimination of C_2H_4 from the ethyl group in a McLafferty rearrangement of the molecular ions (Scheme I).

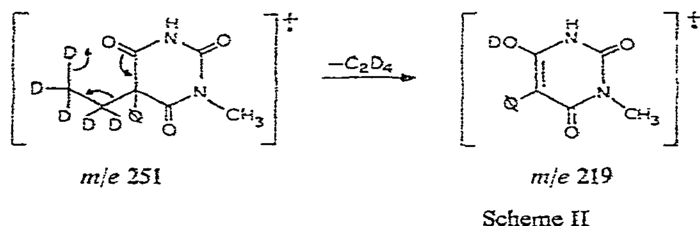


Scheme I

Thus, the record of the M — 28 ions (*i.e.*, *m/e* 204 for IIa, *m/e* 218 for Ic and *m/e* 232 for Id) provides a very sensitive means for the detection of a member of this class of compounds.

In order to determine whether the N-methylphenobarbital detected in a serum sample of a DMMP user was an artifact formed during GC, a serum extract was mixed with phenobarbital-ethyl-d_5 (IIb) and the resulting mixture analyzed by GC–MS. If there indeed existed an agent in the extract which was capable of methylating phenobarbital to N-methylphenobarbital, the sample of phenobarbital-ethyl-d_5 would

be expected to be transformed to N-methylphenobarbital-ethyl-d_5 (IIc), which should exhibit an intense peak at *m/e* 219 (Scheme II).



Scheme II

Thus, in analogy to the unlabelled compounds, a display of the M — 32 ions (*i.e.*, *m/e* 205 for IIb or *m/e* 219 for IIc) indicates the presence of these labelled materials.

The total ionization plot of such an experiment is shown in Fig. 1. Unlabelled phenobarbital was present as the major metabolite. At the retention time where N-methylphenobarbital would be expected to appear, two components were identified, namely, N-methylphenobarbital and N-methylphenobarbital-ethyl-d_5, as evidenced by the selected ion records of the ions *m/e* 218 and *m/e* 219, respectively. Another compound present in the sample, namely, phenyl-*p*-methylphenylhydantoin (IIIb) used as an internal standard for GC, was also converted to the N-methyl compound (IIIc). These results strongly indicated the presence of a methylating agent in blood serum extract.

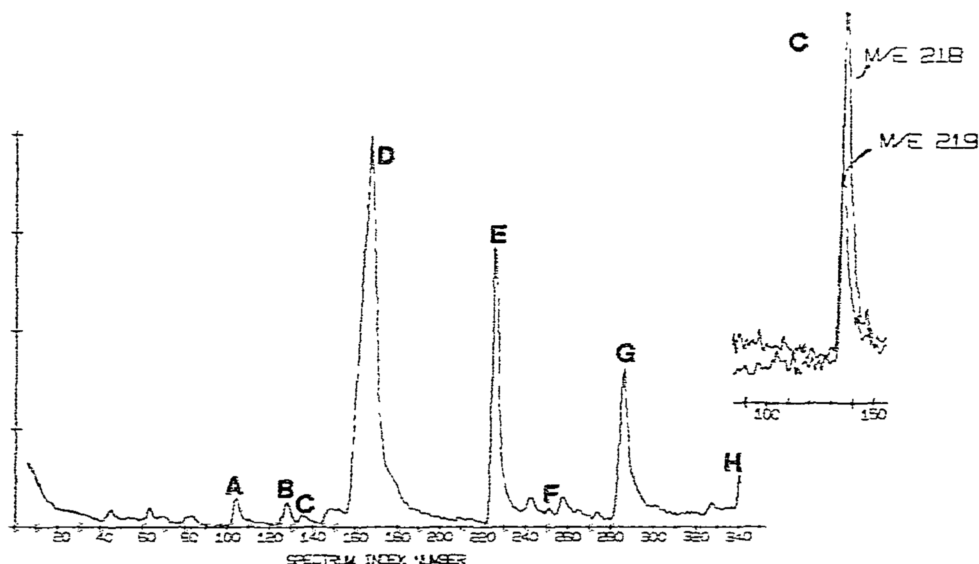


Fig. 1. Total ion plot of serum extract obtained from DMMP patient coinjected with phenobarbital-ethyl-d_5. A, B = phthalate plasticizers; C = N-methylphenobarbital (d_0 and d_5); D = phenobarbital; E = tri-2-butoxyethyl phosphate (impurity from B-D vacutainer); F = N-methylphenyl-*p*-methylphenyl hydantoin; G = phenyl-*p*-methylphenyl hydantoin; H = cholesterol. The selected ion records of ions *m/e* 218, *m/e* 219 for component C are shown in the top right insert.

In another experiment, phenobarbital was added to a serum sample which was devoid of any drug components and the resulting extract was analyzed by GC–MS. The total ionization plot of such an experiment is shown in Fig. 2a. Again, a small amount of N-methylphenobarbital was detected, as evidenced by the selected ion record of m/e 218 (Fig. 2b).

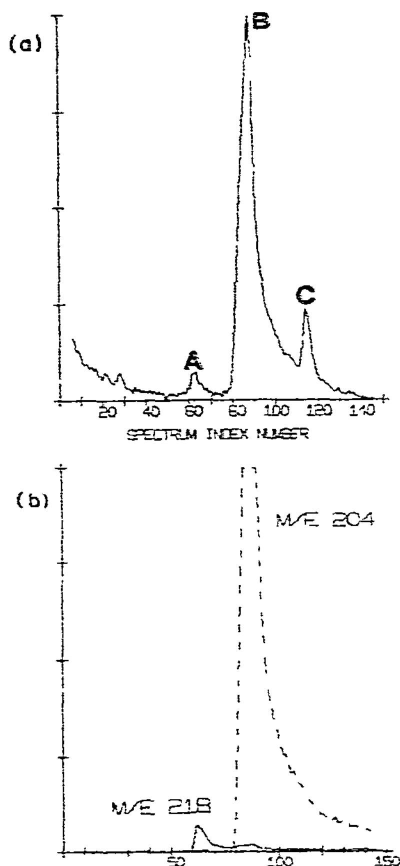


Fig. 2. (a) Total ion plot of a sample of phenobarbital in a serum extract of an individual not given any drug. A = N-methylphenobarbital, B = phenobarbital, C = dioctyl adipate. (b) Selected ion records of ions m/e 218 and m/e 204.

The two experiments described so far proved that a component in serum extract was capable of methylating phenobarbital to N-methylphenobarbital under GC conditions. They, however, did not rule out the possibility that part of the N-methylphenobarbital detected might still be due to endogenous methylation. In order to exclude this route of formation it was necessary to derivatize all available free >N–H groups with an external reagent which could competitively suppress methylation by the serum component. In doing so, the detection of any N-methyl compound could be taken as indicative of endogenous methylation. On the other hand, the absence of

any N-methyl compound could prove that *in vivo* methylation had not occurred and hence the N-methylphenobarbital detected was merely an artifact due to GC. A labelled analogue of trimethyl anilinium hydroxide (TMAH-d_9, IVa) appeared to be a suitable reagent for this purpose. Trimethyl anilinium hydroxide (TMAH, IVb) has been widely used for the analysis of barbiturates by GC[11–13] for a number of years. It is well known for its efficient derivatization of barbiturates to their more volatile N-methyl analogues having better GC properties. This allows a more accurate quantitation of the drugs. Thus, for instance, on-column derivatization of phenobarbital with TMAH yields N,N′-dimethylphenobarbital (Id) which gives a very narrow and symmetrical GC peak. With the TMAH-d_9 reagent, the >N–H groups would be converted to >N–CD_3 groups.

If N-methylphenobarbital was a metabolite resulting from *in vivo* methylation of phenobarbital, it should have been present in the serum extracts of phenobarbital or DMMP users prior to injection into the gas chromatograph. Coinjection of the blood serum extract with TMAH-d_9 reagent would have transformed N-methylphenobarbital to N-methyl-N′-methyl-d_3-phenobarbital (Ie). On the other hand, if no *in vivo* methylation had occurred and therefore no N-methylphenobarbital was present in the blood serum extract prior to GC, all phenobarbital present in the extract would have been transformed to N,N′-dimethyl-d_6-phenobarbital (If). The mass spectrum of compound Ie is shown in Fig. 3. The base peak of the mass spectrum of compound Ie is at *m/e* 235 and its molecular ion at *m/e* 263, while those of compound If are shifted 3 a.m.u. higher to *m/e* 238 and 266, respectively. When an excess of TMAH-d_9 reagent was coinjected with a blood serum extract from a patient receiving phenobarbital and the GC eluents were analyzed by mass spectrometry, only N,N′-dimethyl-d_6-phenobarbital (If) was detectable (Fig. 4b). That there was not even a trace of N-methyl-N′-methyl-d_3-phenobarbital (Ie) was concluded from the absence of *m/e* 235 in the mass spectrum (Fig. 4b). The fact that there was no peak corresponding to phenobarbital in the total ionization plot (Fig. 4a) indicated complete methylation of phenobarbital in this experiment. The same results were obtained using serum extracts from patients receiving DMMP.

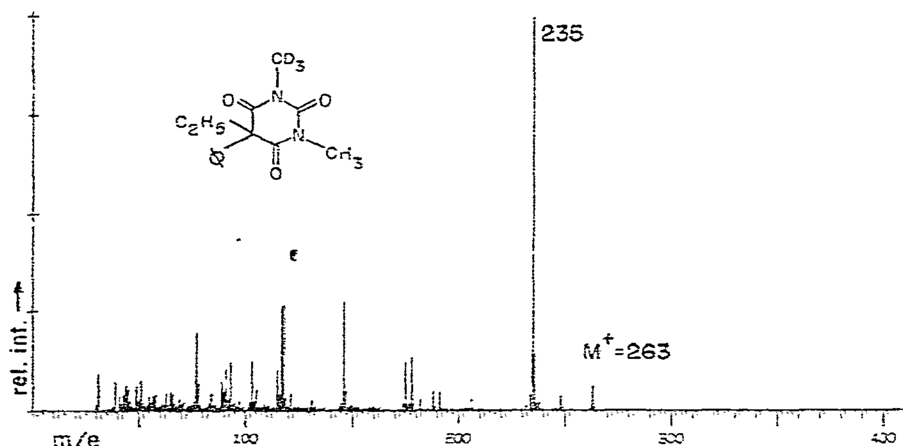


Fig. 3. Mass spectrum of N-methyl-N′-methyl-d_3-phenobarbital.

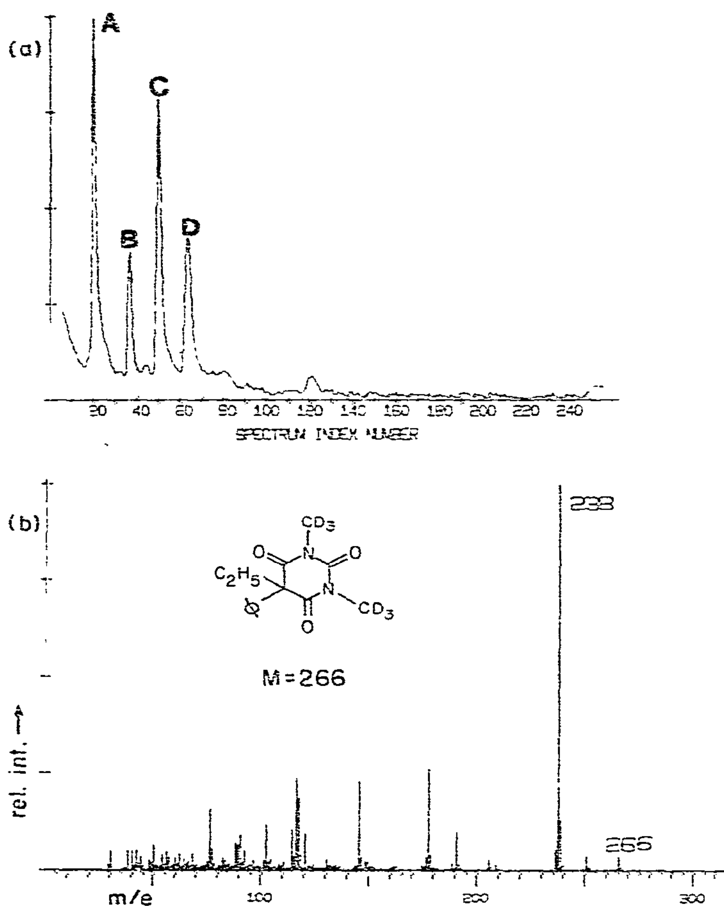
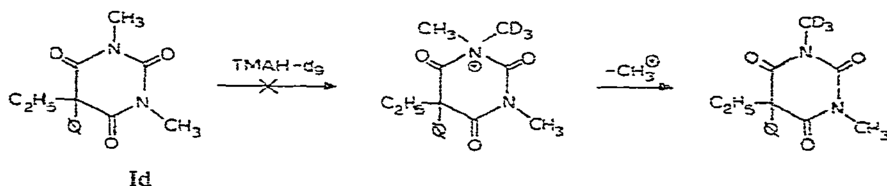


Fig. 4. (a) Total ion plot of TMAH-d_9 derivatization experiment of serum extract obtained from phenobarbital patient. A = azobenzene (impurity in TMAH-d_9 reagent), B = methyl-d_3 palmitate, C = N,N'-dimethyl-d_6-phenobarbital, D = methyl-d_3 esters of stearic, oleic and linoleic acids. (b) Mass spectrum of component C. Note that ion *m/e* 235 is absent.

Since exchange of methyl for methyl-d_3 groups in the presence of an excess of the TMAH-d_9 reagent (via the sequence of reactions shown in Scheme III) would seriously impair the utility of this approach, it had to be tested experimentally. For this purpose, N,N'-dimethylphenobarbital (Id) was coinjected with the TMAH-d_9 reagent into the gas chromatograph. Only the undeuterated species (Id), but none of



Scheme III

the tri- or hexadeutero derivatives (Ie and If, respectively) were detected, thus ruling out such replacement reactions under these experimental conditions.

The choice of using TMAH-d_9 merits further comment. In addition to testing for endogenous methylation, it can be used routinely for the quantitation of administered mephobarbital in the presence of phenobarbital for patients who have taken this drug combination. The use of the unlabelled analogue, namely TMAH, would sacrifice this bit of information since both mephobarbital and phenobarbital are converted to the identical derivative. It would also, of course, be appropriate for distinguishing other analogous drug–drug or drug–metabolite pairs. In this regard, it is noteworthy to mention that Horning *et al.*[14] have employed diazoethane to derivatize barbiturates in GC analyses. Their procedure is also capable of distinguishing between mephobarbital and phenobarbital in the presence of each other. The TMAH-d_9 derivatization procedure would seem to be more convenient if a mass spectrometer is used for detection.

It now remained to identify the endogenous methylating agent which causes the artifact. The well known ability of quaternary methyl amines to methylate primary or secondary amide groups suggested to us that the methylating agent in serum might be of similar nature. Since choline derivatives, which contain the quaternary methyl amine moieties, are present in serum (26–35 mg per 100 ml)[15], they may well be the methylating agents. In particular, lecithin (phosphatidyl choline, V) (9.34 mg per 100 ml)[16] seemed to be a very likely candidate based on chemical consideration. The fatty acid side chains of lecithin make it soluble in organic solvents[17], and therefore extractable in many of the procedures routinely employed in drug analyses. Indeed, we could demonstrate that lecithin was able to methylate phenobarbital in the manner observed to occur during analysis of blood serum extracts. GC–MS analysis of a mixture of phenobarbital and lecithin in benzene revealed the formation of N-methylphenobarbital (Ic) (Fig. 5a). The relative intensities in the selected ion records (Fig. 5b) of ions *m/e* 204 and *m/e* 218 corresponding to the relative amounts of phenobarbital and N-methyl phenobarbital, respectively, indicate that the extent of methylation by lecithin is comparable to that of the methylating agent in serum (Fig. 2b) under the experimental conditions employed.

The conflicting results of other laboratories concerning N-methylphenobarbital were probably due to the difference in extraction procedures. In some instances, a solvent such as hexane was used to remove fatty acids in the serum samples. This step would at the same time remove lecithin and hence would altogether eliminate on-column N-methylation in the injection port.

Injection port methylation can also occur with other components in serum. In the experiment illustrated in Fig. 5a, methyl palmitate was detected as a result of methylation of palmitic acid by lecithin. Thus, it is not surprising that we usually observe methyl esters of the commonly occurring fatty acids such as palmitic acid, stearic acid and oleic acid when extracts of blood samples are subjected to GC–MS analysis. The detection of methyl palmitate in this experiment therefore further substantiates the role of lecithin as the methylating agent in blood serum.

GC has been gaining popularity in drug analysis and in the search for drug metabolites, notably because of the speed and sensitivity of the analytical procedure. However, it should be realized that the results of such analyses are not always entirely unambiguous. The possibility of thermal reactions in the injection port of the gas

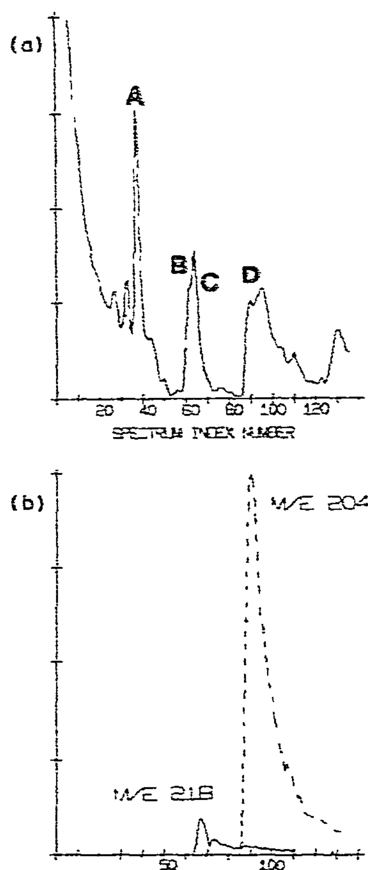


Fig. 5. (a) Total ion plot of a solution of phenobarbital mixed with a solution of lecithin. A = methyl palmitate, B = phthalate plasticizer, C = N-methylphenobarbital, D = phenobarbital. (b) Selected ion records of *m/e* 218 and *m/e* 204. Note that their relative intensities are comparable to those shown in Fig. 2b.

chromatograph should not be overlooked. The problem cited in this paper and its resolution represent an approach to the definition of this frequently unrecognized interference in GC analysis. In addition, the labelled reagent, TMAH-d_9, should prove useful in its own right for quantitation utilizing GC–MS.

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