GAS CHROMATOGRAPHIC ANALYSIS OF BARBITURATES BY FLASH HEATING METHYLATION WITH TRIMETHYLPHENYLAMMONIUM ACETATE

M.MRÁZ and V.ŠEDIVEC

Institute of Hygiene and Epidemiology, 100 42 Prague 10

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Flash heating methylation of barbituric acids with trimethylphenylammonium hydroxide or tetramethylammonium hydroxide does not take place quantitatively; some barbiturates are partly cleaved in the presence of the alkaline reagents, while others are methylated incompletely. New methylation reagents are proposed, trimethylphenylammonium acetate or tetramethylammonium acetate, the use of which does not cause side reactions; the only product of methylation is 1,3-dimethyl derivative of the corresponding barbituric acid.

Due to their polar character gas chromatographic determination of 5,5-disubstituted barbituric acids is accompanied by certain difficulties. A strong tailing is observed or even irreversible adsorption may take place. The disturbing effect is especially strongly felt when small quantities are determined, which may occur, for example, in the analysis of blood[1]. A substantial improvement of chromatographic properties may be achieved if disubstituted barbituric acids are converted to 1,3-dimethyl derivatives[2,3]. For this purpose the technique named "flash heating methylation" is most commonly used[4]: a solution containing in addition to barbiturate a suitable amount of tetramethylammonium hydroxide[5] or trimethylphenylammonium hydroxide[6] or some other quaternary base[7-10] is injected into the evaporation cell of the gas chromatograph. By the thermal decomposition of the quaternary salt 1,3-dimethyl derivative of the corresponding barbituric acid and trimethylamine or dimethylaniline, respectively, are formed. Even in this simple and rapid method of methylation certain complications arise caused by the cleavage of the barbiturate molecule with the alkaline reagent[5], or by the formation of lower methylation products[6].

We investigated the methylation course of various disubstituted barbituric acids and tried to find such conditions as would not cause the above-mentioned difficulties. On the basis of our findings we have proposed new methylation reagent, trimethylphenylammonium acetate or tetramethylammonium acetate, the use of which causes neither cleavage nor formation of lower methylation products. The sole reaction product is the expected 1,3-dimethyl derivative.

EXPERIMENTAL

Chemicals and Apparatus

Trimethylphenylammonium iodide: One mol of dimethylaniline was dissolved in ethyl acetate, 1,2 mol of methyl iodide were added and the mixture allowed to stand at room temperature for several hours (best overnight). The rough crystalline product was filtered off and recrystallized from absolute ethanol.

0·5M *solution of trimethylphenylammonium acetate* (TMPA-Ac): 1·315 g of trimethylphenylammonium iodide was dissolved in 10 ml dry methanol in a 100 ml flask. Solid silver acetate (1·0 g) was added, the flask stoppered with a glass stopper and the content was stirred with a magnetic stirrer for 2 hours. Several drops of supernatant were then withdrawn, acidified with nitric acid and 2—3 drops of silver nitrate solution were added in order to check the presence of iodides. If the test was positive the stirring was continued. When the reaction was complete the solution was filtered. The reagent should be stored in a well stoppered brown glass flask; it is stable for several months.

0·5M *trimethylphenylammonium hydroxide solution* (TMPA-H): 1·315 g of trimethylphenylammonium iodide and 0·875 g of powdered silver oxide were worked up in the above described manner. 0·5M tetramethylammonium hydroxide and tetramethylammonium acetate were prepared analogously from the corresponding iodide. The stock solutions of barbituric acid derivatives (allobarbital, aprobarbital, amobarbital, pentobarbital, hexobarbital, cyclobarbital, phenobarbital and prominal) were prepared by dissolving 100 mg of substance in 10 ml of methanol.

Chromatograph Chrom 4 with flame ionization detector. The columns were of stainless steel (250 cm × 3 mm and 120 cm × 3 mm), packed with 5% GE XE 60 or 10% NPGS on Chromosorb W AW-DMCS. 100/120 mesh. Nitrogen flow 30 ml/min, hydrogen 20 ml/min, air 500 ml/min. Record sensitivity 1/20 and 1/50, chart shift 10 mm/min.

Procedure

A suitable volume of a methanolic or ethereal solution of barbiturate was measured into a 10 ml conical test-tube, the solvent was evaporated and the residue dissolved in 50 µl of methylation reagent. A 3 µl sample was withdrawn from the solution and injected into the injection port (250°C) of the gas chromatograph; dimethylated derivatives of allobarbital, aprobarbital, amobarbital and pentobarbital were chromatographed at 170°C, the derivatives of cyclobarbital, hexobarbital phenobarbital and prominal (which have longer retention times) at 190°C.

RESULTS AND DISCUSSION

Formation of By-products During the Methylation of Barbiturates with Trimethylphenylammonium Hydroxide

The course of the "flash heating methylation" of 5,5-phenylethylbarbituric acid with trimethylphenylammonium hydroxide or tetramethylammonium hydroxide has been investigated in detail by other authors. It was shown that in addition to 1,3-dimethyl derivative another substance with a shorter retention time is also formed (initially labelled as "early phenobarbital"[11] and later identified as N-methyl-

-α-phenylbutyramide[12]); this is due to the cleavage of the barbiturate molecule in contact with the alkaline methylation reagent. The degree of cleavage is dependent on the reagent concentration. Under certain conditions (1·8M) the degradation product is formed in such concentration that the measurement of the parameters of its peak may serve for quantitative determination of phenylethylbarbituric acid[12]. Other authors did not observe the degradation[13,14] but have found that some barbiturates afforded not only the corresponding 1,3-dimethyl derivative after methylation with tetramethylammonium hydroxide, but also small amounts of by-products with longer retention times (monomethyl derivatives or even the original unchanged barbiturates).

In view of the mentioned discrepancies we investigated the course of methylation of eight different barbiturates. In our first experiments we used a relatively concentrated solution (0·5M) of trimethylphenylammonium hydroxide and we showed that all barbiturates afforded not only the peak of the corresponding 1,3-dimethyl derivative but also two additional peaks with shorter retention times, belonging to degradation products; cyclobarbital was an exception, because of only a single degradation product. The quantity of degradation products may differ considerably for various barbiturates. In some instances the respective peaks were small, but in others they indicated that the degradation products are formed in considerable amounts.

TABLE I

Retention Times of the Products Formed during the Methylation of Barbiturates with 0·5M Trimethylphenylammonium Hydroxide

DMD 1,3-dimethyl derivative; R ratio of the heights of peaks of the dimethyl derivative and the main degradation product (No 2).

Barbiturates	Alkyls (aryls) in position			Retention time DMD, min	Relative retention times of by-products			R
	5,5-		N—R					
	R_1	R_2	R_3		1	2	3	
Allobarbital[a]	allyl	allyl		7·87	0·641	0·895		1·03
Aprobarbital[a]	isopropyl	allyl		7·54	0·567	0·713	2·37	9·12
Amobarbital[a]	ethyl	isoamyl		10·87	0·552	0·674	3·31	3·39
Pentobarbital[a]	ethyl	1-methylbutyl		12·07	0·484	0·582	2·97	27·5
Hexobarbital[b]	methyl	1-cyclohexen-1-yl	methyl	17·33	0·255	0·318		0·102
Cyclobarbital[b]	ethyl	1-cyclohexen-1-yl		16·47	0·382			0·875
Phenobarbital[b]	ethyl	phenyl		19·68	0·269	0·412		0·076
Prominal[b]	ethyl	phenyl	methyl	19·68	0·270	0·412		0·079

Temperature of the column (5% GE XE 60 on Chromosorb W 100/120 mesh): [a] 170°; [b] 190°C.

The peak of the degradation product with the longer retention time was always substantially higher than the peak of the degradation product with the shorter retention time.

In order to obtain at least an approximate criterion of the stability or instability of individual barbiturates against the alkaline reagent we calculated the ratio of peak heights of the 1,3-dimethyl derivative and of the main degradation product. This ratio assumed high values when the methylation led predominantly to 1,3-dimethyl derivative; its value decreased with an increasing amount of the degradation product. From the data presented in Table I it is evident that among the barbiturates investigated pentobarbital is most stable and phenobarbital and prominal are least stable.

The cleavage of barbiturates with alkaline methylation reagents is not caused by the high temperature of the injection port alone, but it also takes place at room temperature[15,16]. If barbiturates are left in contact with the reagent for a certain time before injection, the peaks of the degradation products are considerably higher than in the case of samples analysed immediately after mixing.

In further experiments we used several reagents the concentration of which was gradually decreased from the initial 0·5M to 0·005M. We found that the amount of the degradation products decreased with a decreasing concentration of the reagent, so that the ratio of heights, R, assumed higher values. For two especially labile barbiturates (phenobarbital and hexobarbital) this dependence is shown in Fig. 1.

From the facts mentioned it might seem that reagents of a concentration as low as possible would be advantageous for the methylation of barbiturates. However, we observed that under such conditions small peaks appear on the chromatograms

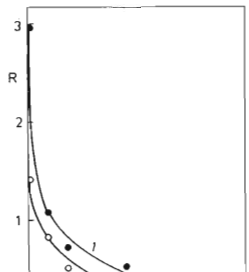

FIG. 1

Effect of Concentration of Alkaline Methylation Reagent on the Cleavage of Barbiturates

1 Phenobarbital; 2 hexobarbital; R ratio of the peak heights of dimethyl derivative and the main product of degradation.

of the majority of barbiturates, which have longer retention times than the corresponding 1,3-dimethyl derivatives, but shorter than the initial barbiturates. These peaks probably belong to lower methylated products, *i.e.* to monomethyl derivatives. In the case of phenobarbital this assumption was confirmed by comparison of the retention time of the peak under observation with the retention time of the prominal (N-methyl derivative of phenobarbital). The fact is worthy of mention that in some barbiturates (aprobarbital, amobarbital, pentobarbital) the formation of small amounts of monomethyl derivatives was observed even when a concentrated reagent solution (0·5M) had been used. Quite similar results were also obtained when instead of trimethylphenylammonium hydroxide tetramethylammonium hydroxide was used.

The experiments supplied a satisfactory explanation of the discrepancies in earlier data on the methylation course of barbiturates and have shown that both the amount and the character of the by-products are dependent not only on the concentration of the methylation reagent used, but also on the type of the barbiturate methylated. For purposes of quantitative analysis methylation reagents should be used which have a constant, well defined, concentration. Only under such conditions is the yield of the corresponding 1,3-dimethyl derivative acceptably reproducible. The optimum concentration of the reagent at which the by-products are formed in small amounts only is different, however, for each barbiturate and it should therefore be selected from case to case. Of course, this requirement cannot be fulfilled in the analysis of mixtures of two or more barbiturates, or in cases when the identity of the barbiturates sought is unknown beforehand.

Methylation of Barbiturates with Trimethylphenylammonium Acetate

As mentioned before the main source of difficulties in the methylation of barbiturates is the strongly alkaline reaction of the methylation reagent. Therefore we wondered whether methylation would also take place when the alkaline reagent was neutralized beforehand by a low-boiling acid. We assumed that after the injection of the sample the volatile acid might be displaced by the unvolatile barbituric acid during the first phase of heating. Then the quaternary salt of barbituric acid formed would decompose in the subsequent phase of heating in the usual manner to the corresponding 1,3-dimethyl derivative and dimethylaniline.

In our first experiments we employed the reagent (0·05M) prepared by mixing equivalent amounts of methanolic solutions of trimethylphenylammonium hydroxide and acetic acid. For each analysis 25 μg of barbiturate were dissolved in 50 μl of reagent and a part of the solution (3 μl) was injected into the injection port (250°C) of the gas chromatograph. The results were unusually favourable: none of the barbiturates investigated gave rise to the formation of reaction by-products. A single peak appeared on the chromatogram, corresponding to the respective 1,3-dimethyl derivative. The record line before and after this peak was quite smooth. For control

we also recorded chromatograms at a lower or a higher temperature so that the presence of the degradation or lower methylated products would not escape our attention.

Equally favourable results were also obtained when reagents of higher concentration (0·1M and 0·5M) were used for the methylation. Even in such cases the corresponding 1,3-dimethyl derivative was the only reaction product obtained. We also demonstrated that a long contact of the reagent (24 hours) with the barbiturate does not affect the course or the result of the methylation. In consequence of the neutral reaction of the reagent undesirable changes of the sample cannot take place during storage. The temperature of the injection port, from 200 to 300°C, does not affect the methylation course either. The only requirement is that the reagent should be in sufficient excess (at least a five-fold amount of the equivalent); a large excess (up to 500-fold amount) does not interfere.

In order to check to what extent the methylation could be affected by the excess of the acetic acid we also prepared reagents with an identical concentration of trimethylphenylammonium ion, but containing a double or even a five-fold amount of acetic acid. When these acid reagents were used the presence of the degradation product on the chromatograms was not observed, but with all barbiturates small peaks appeared after the peaks of 1,3-dimethyl derivatives, which indicated that monomethyl derivative was also formed (Fig. 2C). A list of the reaction products formed during the methylation of various barbiturates with alkaline, neutral and acid methylation reagent is given in Table II. From the results it follows that an alkaline or acid methylation reagent is unsuitable for the methylation of barbiturates;

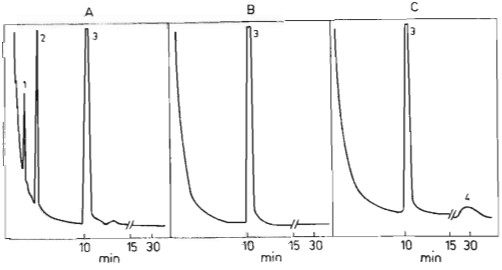

FIG. 2

Methylation of Phenobarbital with Alkaline (A), Neutral (B) and Acid (C) Reagent
1, *2* Degradation products, *3* dimethyl derivative, *4* monomethyl derivative.

only a neutral reagent methylates the barbiturates to corresponding dimethyl derivatives without a simultaneous formation of undesirable by-products.

TABLE II

Reaction Products of Methylation

S cleavage of barbiturate; M formation of monomethyl derivative; × distinct cleavage or formation of monomethyl derivative; (×) the degradation product or the monomethyl derivative is formed in low amounts only; 0 no degradation product or monomethyl derivative is formed.

Barbiturate	Reagent 0·05M					
	alkaline		neutral		acid	
	S	M	S	M	S	M
Allobarbital	×	(×)	0	0	0	×
Aprobarbital	(×)	×	0	0	0	×
Amobarbital	(×)	×	0	0	0	×
Pentobartital	(×)	×	0	0	0	×
Hexobarbital	×	0	—	—	0	—
Cyclobarbital	×	0	0	0	0	0
Phenobarbital	×	0	0	0	0	×
Prominal	×	—	0	—	0	—

TABLE III

Methylation of Barbiturates with Trimethylphenylammonium Hydroxide and Acetate

Conditions of methylation	Hydroxide	Acetate
Increasing concentration of the methylation reagent (0·005—0·5M)	degree of cleavage of barbiturate increases	without effect
Prolonged effect of the methylation reagent at room temperature	the majority of barbiturates undergoes gradual degradation	without effect
Efficiency of methylation	some barbiturates give monomethyl derivative	quantitative methylation
Temperature of the injection port 200—300°C	degradation of barbiturates increases with temperature	without effect

The neutral reagent can be prepared either by mixing equivalent amounts of methanolic solutions of the base and acetic acid, or more simply and rapidly, by the reaction of silver acetate with the methanolic solution of the quaternary halogenide. For current use the reagent of 0·1M concentration was found best. It can be prepared by dilution of the 0·5M methanolic stock solution, taking care that the final reagent should contain approximately equal volumes of methanol and acetone. The presence of acetone improves the dissolution of the barbiturate residues.

Equally successful was also the methylation of barbiturates with tetramethylammonium acetate which was prepared by reaction of the corresponding halogenide with silver acetate.

CONCLUSION

Methylation of barbiturates with alkaline methylation reagents (trimethylphenylammonium hydroxide) is accompanied by side reactions during which either degradation products or lower methylation products are formed. The character and the amount of these by-products are dependent on the configuration of the methylated compound, on the reagent concentration, on the temperature of the injection port and on the time of the contact with the barbiturate (Table III). When operation conditions are strictly followed reproducible yields of 1,3-dimethyl derivative may be achieved, but a simultaneous formation of by-products complicates the chromatographic records and decreases the sensitivity of the detection or of the determination.

When barbiturates are methylated with trimethylphenylammonium acetate or tetramethylammonium acetate the formation of by-products does not take place; corresponding 1,3-dimethyl derivatives are the sole reaction products even with high concentrations of the reagent and high temperatures in the injection port (Table III). The changes in the reagent concentration which may take place, for example, by partial evaporation of the solvent during the dissolution of the residues do not affect the reproducibility of the results. In consequence of the unambiguous course of the methylation the chromatographic records are simpler and the detection sensitivity and determination accuracy are higher. For these reasons trimethylphenylammonium acetate and tetramethylammonium acetate are very suitable methylation reagents for the detection and the determination of small amounts of barbiturates.

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