Journal of Chromatography, 237 (1982) 155–158
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,574

Note

Studies of barbiturate degradation following methylation with dimethyl sulfate

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(Received November 24th, 1981)

Barbiturates are frequently converted to their dimethyl derivatives for either more ready and improved gas chromatographic analyses[1] or as part of a gas chromatographic confirmation procedure[2–9]. Methylation with dimethyl sulfate has been found to be easily done and to yield quite clean chromatograms, making it superior to other methylating reagents and techniques[2].

In our laboratory, methylation is done by adding 1 ml of carbonate buffer and 0.1 ml of dimethyl sulfate to barbiturate extraction residues. The mixture is heated in a 75–85°C water bath in an open tube, which is occasionally vortexed until the dimethyl sulfate layer has disappeared, signalling completion of the methylation. The tube is then removed from the water bath and allowed to cool in a cold water bath. The solution is then extracted with carbon tetrachloride and an aliquot of the extract is injected into a gas chromatograph to confirm the presence of barbiturates found in our free barbiturate screening procedure[8–9].

This methylation procedure is performed on 20–50 samples per day. Occasionally we have found that quality-control samples that screened positive as free barbiturates for short- and intermediate-acting barbiturates, gave negative or very weak results as methylated derivatives; the internal standard was present but not the barbiturate(s) that was originally found and supposed to be there. When this had happened, it has been found that the tubes had been left in the water bath for a period of time after methylation was completed. This result has prompted us to intiate this study of the methylation procedure. We have sought to ascertain the extent of the adverse affects of over-heating upon barbiturate solutions left sitting in the hot water bath after methylation has been completed.

EXPERIMENTAL

Reagents

Dimethyl sulfate and carbon tetrachloride were used as received. The barbiturate solution consisted of 1 *N* sodium carbonate solution containing 15 μg/ml of each barbiturate: amobarbital, butabarbital, ibomal, pentobarbital, phenobarbital, and secobarbital.

0021-9673/82/0000–0000/$02.75

Chromatography

Gas chromatographic determinations were performed on a Hewlett-Packard Model 1500 gas chromatograph equipped with a flame ionization detector and a 1.83 m × 2 mm I.D. glass column packed with 3% OV-1 on Chromosorb W run at 170°C.

Methylation

Open-tube methylation. A 1-ml volume of barbiturate solution and 0.1 ml of dimethyl sulfate were added to a conical centrifuge tube. The tube was then placed in a 75°C water bath, and vortexed every few seconds until the dimethyl sulfate layer disappeared (completion of methylation). At that point the time was noted. The tube was left in the water bath for the desired amount of time (0, 10, 25, 45, or 60 min), after which it was removed, placed in an ice–water bath for 3 min, and then extracted with 0.1 ml of carbon tetrachloride. An aliquot of the organic layer was then chromatographed and the barbiturate peak heights measured.

Closed-tube methylation. The reagents were added to a centrifuge tube as above, then the tube was sealed with a screw cap. The tube was heated and vortexed until the dimethyl sulfate layer disappeared, then the tube was allowed to stand in the water bath for an additional 60 min. The tube was removed from the water bath and cooled; then the solution was extracted and chromatographed as above.

RESULTS AND DISCUSSION

The results (Table I) indicate that for open-tube methylation carried out under the conditions described (using dimethyl sulfate and aqueous alkaline barbiturate solutions, heated at 75°C) that short- and intermediate-acting barbiturates which have a shorter retention time than ibomal (the internal standard) such as amobarbital, butabarbital, pentobarbital, and secobarbital are rapidly lost as time passes following the completion of methylation (disappearance of the dimethyl sulfate layer). The losses for these barbiturates become significant after as little as 10 min of continued heating (24–31% for several of the barbiturates), reach nearly 30% for all the barbiturates after 25 min, and reach at least 45% for all the barbiturates after 45

TABLE I

CONCENTRATION CHANGES *VERSUS* TIME LEFT IN WATER BATH

Based on ibomal internal standard.

Barbiturate	*Minutes left in water bath*					
	Without cap					*Capped: 60*
	0	*10*	*25*	*45*	*60*	
Butabarbital	0%	−18.2%	−29.4%	−47.2%	−52.4%	− 8.2%
Amibarbital	0%	−29.8%	−51.0%	−67.3%	−77.9%	−21.6%
Pentobarbital	0%	−24.3%	−46.2%	−66.9%	−72.8%	−17.2%
Secobarbital	0%	−30.7%	−53.6%	−62.9%	−79.3%	−21.4%
Ibomal	0%	0%	0%	0%	0%	0%
Phenobarbital	0%	+ 7.2%	+10.3%	+20.1%	+20.6%	+ 0.5%

min. The losses are clearly great enough that quantitations will be seriously lowered and some positive specimens may give negative (too small) results.

Phenobarbital, a long-acting barbiturate with a longer retention time than ibomal, was found to increase slightly in concentration under the described methylation conditions (Table I). This can only reflect a slight loss in ibomal, rather than an actual increase in phenobarbital concentration. The fact that ibomal slightly decreased during the study means that the short- and intermediate-acting barbiturates actually decreased more than is indicated in Table I. The results in Table II illustrate the losses based on phenobarbital and more closely reflect the actual barbiturate losses during overheating. (Note that since even phenobarbital may be lost relative to a barbiturate with an even longer retention time that the loss values in Table II represent a lower limit for the percent loss).

TABLE II

CONCENTRATION CHANGES *VERSUS* TIME LEFT IN WATER BATH

Based on phenobarbital.

Barbiturate	*Minutes left in waterbath*					
	Without cap					*Capped: 60*
	0	*10*	*25*	*45*	*60*	
Butabarbital	0%	−23.5%	−36.1%	−55.5%	−60.5%	− 8.4%
Amobarbital	0%	−35.2%	−55.6%	−73.1%	−81.5%	−22.2%
Pentobarbital	0%	−29.9%	−50.6%	−72.4%	−77.0%	−17.3%
Secobarbital	0%	−34.7%	−56.9%	−69.4%	−83.3%	−22.2%
Ibomal	0%	− 7.7%	− 9.6%	−17.3%	−19.2%	− 1.9%
Phenobarbital	0%	0%	0%	0%	0%	0%

The results for methylation carried out with dimethyl sulfate at 75°C using closed tubes show that all barbiturates experience a slight loss with respect to phenobarbital as time passes following continued heating after the completion of methylation. This loss, however, is only about 1/4 or less than that for open-tube methylation.

The reason(s) behind the loss of the barbiturates during overheating is not completely understood. We suspect that it may be due largely to the fact that methylated barbiturates are more volatile than free acid barbiturates and may boil away as they stand in the hot water bath after methylation is completed. This factor is supported by the fact that the barbiturate losses increase with time and the fact that closed-tube methylation, which limited the amount of barbiturate that could be lost via boil-away, yielded much less of a barbiturate loss than open-tube methylation. Another possible factor for the barbiturate loss is demethylation and/or barbiturate breakdown due to the heat. Demethylation seems unlikely since the overheated solutions did not contain peaks on the chromatogram for free barbiturates. Breakdown seems more likely and is supported by the fact that closed-tube methylation at 85°C carried out in strong acid and in strong base both lead to complete destruction of all the barbiturates after only 20 min, while methylation of a neutral solution under these conditions lead to only partial destruction.

The results of this study indicate that while methylation with dimethyl sulfate may be faster and lead to cleaner chromatograms than other methylation agents, that steps must be taken to prevent barbiturate loss that may yield false negative or falsely lowered results. Several steps can be taken to minimize possible barbiturate degradation: (1) remove the tubes from the heated water bath as soon as methylation is completed; (2) use closed-tube rather than open-tube methylation; (3) methylate a calibration standard to be used for quantitation with each batch of samples methylated.

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