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DIRECT DERIVATIZATION AND GAS CHROMATOGRAPHIC DETERMINATION OF BARBITURATES IN AUTOPSY LIVER TISSUES

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SUMMARY

A new gas chromatographic procedure is presented for the determination of barbiturates in autopsy tissues (liver and blood). The barbiturates are separated from the interference of co-extracted, free fatty acids in an acid-catalyzed methylation of the fatty acids and quantified as the N,N-dimethyl derivatives following reaction with dimethyl sulphate. Derivatization and recovery are shown to be simple, efficient procedures leading to substantially higher analytical results.

INTRODUCTION

The extraction of small amounts of drugs from biological materials is usually accompanied by troublesome lipids unless, as in the case of urine or saliva, the sample is essentially lipid-free. The amount and nature of the lipids present is determined by the type of material constituting the sample. Thus, the extraction of liver tissues by common organic solvents leads to the isolation[1] of large amounts of free fatty acids in addition to much smaller amounts of lipids of varying polarity, such as triglycerides, phospholipids, cholesteryl esters and cholesterol. As a consequence, the recovery from liver tissues and blood of *acidic* drugs or other toxic substances, as distinct from *basic* compounds, is always complicated by the co-extracted fatty acids since their behaviour will be very similar in any separation process based on chromatography or partitioning between two immiscible solvents under controlled pH. When considering the estimation of an acidic compound present in tissues, the solvent chosen ought to result in the complete removal of the compound regardless of the amount of co-extracted material. Thereafter, the procedure is required to cope with the removal of lipids (or, indeed, other co-extractives) in a manner which does not lead to the simultaneous loss of the acidic compound. Although obvious, these two requirements are, in practice, difficult to meet fully and existing methodologies have either ignored them or accepted the analytical results as, at best, semi-quantitative (for a review, see ref. 2).

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In this paper, we present a new procedure for the estimation of barbiturates isolated from autopsy liver tissues after extraction with methanol. Interference from free fatty acids, the major component of the co-extractives, is eliminated by an acid-catalyzed esterification (using methanol–hydrogen chloride) and the unaffected free barbituric acids are recovered with the aid of dilute alkali. The acids are finally alkylated with dimethyl sulphate prior to gas chromatography (GC) on a non-polar (SE-30) column.

EXPERIMENTAL

Synthesis of barbituric acid derivatives

The N,N-dimethyl derivatives of amobarbital, pentobarbital and phenobarbital were prepared[3] by reacting the free acid (1 g) with dimethyl sulphate in a mildly alkaline medium. Crude derivatives were isolated by extraction of the solvent-free residues with hexane (2 × 40 ml) and conveniently purified by passing the extract (dried with anhydrous sodium sulphate) through a short column of alumina (10 × 2.5 cm) with further development of the column using 1% methanol (v/v) in hexane. Fractions (11 × 10 ml) showing a single compound when examined by GC were combined, the solvent removed and the product dried under vacuum, over phosphorus(V) oxide. Identity and purity were confirmed by microanalysis, infrared and mass spectroscopy.

Reagents and standard solutions

All solvents and reagents were chemically pure, commercially available materials. Apart from the solvents which were re-distilled from an all-glass apparatus, the reagents were used without further treatment.

Potassium carbonate. Saturated aqueous solution.

Sodium hydroxide. 0.1 *M* in water.

Methanol–hydrogen chloride. Prepared by slowly saturating anhydrous methanol (700 ml, 10 min) with dry hydrogen chloride (Matheson, Coleman & Bell, East Rutherford, NJ, U.S.A.) and diluting to 1 l. The acid concentration was determined by titrating aliquots (10 ml) with standardized alkali (1.00 *M*) using phenolphthalein indicator. It was usually 1.5–2.0 *M* and suitable for use without further dilution.

Solutions of barbituric acids. Prepared by dissolving an accurately weighed amount of amobarbital, pentobarbital or phenobarbital (about 0.030 g) in methanol and diluting to 100 ml. A ten-fold dilution of the solution gave a convenient working concentration.

Solutions of N,N-dimethyl derivatives of barbituric acids. Prepared by dissolving the appropriate and accurately weighed, pure dimethyl derivative (about 0.025 g) in hexane and diluting to 100 ml. A working solution was obtained by making a ten-fold dilution of each stock solution.

Solution of internal standard. 0.1% (w/v) *n*-octadecane in CS_2.

Gas chromatography

A Becker Model 417 gas chromatograph was used. It was fitted with a coiled borosilicate column (5 ft. × 1/4 in. O.D.) packed with 10% (w/w) SE-30 on Chromosorb W AW DMCS (80–100 mesh). General operating conditions were:

carrier gas nitrogen flow, 45 ml/min; injection port temperature, 210°C; column, 180°C; flame-ionization detector, 210°C.

A solution (1–2 μl) of the barbiturate derivative (from extract or standard solution) contained in a known volume of the internal standard solution was injected on the column. Concentrations of the barbituric acid were calculated from a calibration plot of peak height ratio (derivative/*n*-octadecane) *versus* concentrations of the corresponding dimethyl derivative. The dilution of the solution to be chromatographed was adjusted to correspond to the concentration range 0.030–0.140 mg derivative per ml of the calibration plot (see captions to Table IV). However, a calibration plot was prepared for each batch of five or six determinations.

Conversion studies

(i) Amobarbital (30 μg) was added to a test-tube (10 × 1.8 cm O.D.) and the solvent carefully removed under vacuum at 35°C). Aqueous potassium carbonate (0.3 ml), dimethyl sulphate (0.07 g) and methanol (0.2 ml) were added. The tube was then lightly stoppered and placed in boiling water until the reaction had ceased (*ca.* 10 min). The contents of the tube were diluted with water (2 ml), extracted with hexane (2 ml) and the extract carefully reduced to dryness. This residue was dissolved in 2.0 ml solution containing the internal standard, then analyzed by GC, as above.

(ii) A variation of (i) differed only in that benzene was used as the extracting solvent.

Efficiency of the conversion reaction was determined separately for pentobarbital (32.4 μg) and phenobarbital (33.5 μg) using benzene only in the extraction step. Conditions for GC remained the same.

Recovery studies

Analyses were carried out with an homogeneous matrix obtained from a freeze-dried, drug-free tissue (5 g). This was exhaustively extracted by refluxing in boiling methanol (160 ml) for 1.5 h. The cooled mixture was filtered under vacuum, then the extract was divided into five equal portions and thereafter handled as a set of five replicates. To each was added a known amount of amobarbital (30 μg) and the solvent removed under vacuum (at 35°C). The dry residue was then heated in a water-bath at 85–90°C with the methanol–hydrogen chloride reagent (50 ml) in a tightly stoppered test-tube. When cool, the contents of the tube were reduced to approximately 20 ml, diluted with water (50 ml) and extracted with chloroform (2 × 20 ml). The combined organic phase was reduced in volume (*ca.* 20 ml) and extracted with sodium hydroxide solution (2 × 10 ml). The aqueous phase was immediately acidified with hydrochloric acid (10 *M*, 0.5 ml) then re-extracted with chloroform (2 × 10 ml). Solvent was completely removed from the extract and the residue treated as in (ii), above.

Tissue analyses

Although similar procedurally, some details are repeated because of differences in the final scale adopted for the actual analyses.

Fresh tissues (Method B). A sample (5 g) of the comminuted tissue was boiled in methanol (50 ml, 30 min) under reflux. The cold methanolic extract* was decanted

* A single extraction in methanol is referred to later as Method A.

through a small, coarse filter-paper and the residue subjected to two similar extractions. The combined extracts were reduced in volume to *ca.* 20 ml in a rotary evaporator, 50 ml 0.2 *M* hydrochloric acid were added and the solution extracted with chloroform (3 × 20 ml). The combined extracts were again reduced to dryness under vacuum (at 35°C) and the *dry* residue reacted with methanol–hydrogen chloride reagent (30 ml) in a tightly stoppered test-tube at 85°C (in a water-bath) for 30 min. The cooled contents of the tube were reduced to about 10 ml, diluted with water (20 ml) and extracted with chloroform (2 × 10 ml). The extracts were combined, extracted with sodium hydroxide solution (2 × 10 ml) and the total alkaline extract immediately acidified with hydrochloric acid (10 *M*) and re-extracted with chloroform (2 × 10 ml) to recover the barbituric acid. This extract was reduced to dryness and, with the aid of a minimal amount of chloroform, quantitatively transferred to a methylation tube (10 × 1.8 cm O.D.). Solvent was carefully removed and methylation completed (≈ 5 min) by heating the stoppered tube at 85°C after adding dimethyl sulphate (0.07 g), potassium carbonate solution (0.3 ml) and methanol (0.3 ml). When cold, the product was diluted with water (2 ml) and extracted with benzene (2 × 2 ml). The organic phase was taken to dryness and the residue dissolved in an accurately measured volume of the internal standard solution. Aliquots (1–2 μl) were injected onto the column and the concentration of barbituric acid derivative determined from the calibration plot. The concentration of the barbituric acid in the tissue was obtained from:

$$\text{Barbituric acid (mg/kg sample)} = (\text{concentration of derivative in extract, } \mu g/\mu l) \times V \times \frac{1000}{W} \times F$$

where W = mass (g) of tissue taken, V = final volume (ml) of internal standard solution and F = (molecular weight of the barbituric acid)/(molecular weight of corresponding N,N-dimethyl derivative).

Freeze-dried tissues. The same procedure was employed for freeze-dried tissues, however, a smaller sample (1 g) was used and the dry methanol-soluble residue methylated directly, thereby eliminating the acid–chloroform extraction step.

Blood. The same procedure was employed as in *Fresh tissues* except that the acidified blood (2 ml) was extracted directly with chloroform (3 × 10 ml) and the combined extracts reduced to a dry residue for reaction with methanol–hydrogen chloride (20 ml).

RESULTS AND DISCUSSION

This procedure for the analysis of barbiturates was developed primarily because of two problems in the application of existing spectrophotometric methods to the toxicological analysis of liver tissues and, to a lesser extent, blood. In the first of these, the inevitable precipitation of fatty acids (isolated by centrifugation and confirmed by GC) at low pH precludes measurement of the absorption* of radiation at 240 nm and, despite effective methylation of the fatty acids (as outlined in the Experimental section) and the removal of the barbiturate from the resulting substrate,

* Unsaturated fatty acids have insignificant absorption at 240 nm.

the yellow-brown colour and high absorbance values of the extracts (and aqueous residues) persist. The second problem is related to the, as yet, unidentified chromogens originating from other matrix components[4], and is also partially dependent on the analytical procedure employed.

The selective esterification of free fatty acids in the presence of the barbiturates is the basis for the separation of these two classes of compounds. The mechanism probably involves protonation of the pyrimidine nucleus of the barbiturate, thereby effectively blocking any alkylation, with the simultaneous, unhindered esterification of carboxylic acids of lipid origin by the oxonium ion derived from the alcohol (here, $CH_3\overset{+}{O}H_2$). At the same time, any other carboxylic acids present in the original extract would also be expected to undergo esterification, and remain (or be lost) with the fatty acid esters when separated from the barbiturate in the prevailing acidic environment at the completion of the esterification reaction. Identification of these carboxylic acids would be dependent only upon an effective separation from the fatty acid esters of similar GC retention times. Again, the acid-catalyzed esterification ought to be equally effective in separating fatty acids from other acidic drugs possessing a cyclic amide structure provided that the compounds are not acid-sensitive.

Efficiency of the barbiturate derivatization

Based on the use of the pure derivative, the combined efficiency of the conversion of amobarbital into the N,N-dimethyl derivative and its extraction by hexane and benzene is compared in Table I. The data show significantly better results when extraction of the derivative is carried out with benzene, although the reproducibility in both cases is comparable. The efficiency attainable with benzene

TABLE I

COMPARISON OF CONVERSION EFFICIENCIES FOR AMOBARBITAL USING HEXANE AND BENZENE SOLVENTS

Values obtained for 30 μg amobarbital.

Conversion efficiency (%)	
Hexane extraction	*Benzene extraction*
81.6	95.7
77.6	87.7
76.2	95.2
77.6	94.1
74.9	88.2
79.8	94.1
79.8	90.9
84.3	
79.8	
86.1	
81.6	
Mean 79.9	92.3
n 11	7
S.D. 3.36	3.33

is confirmed by the data for amobarbital, pentobarbital and phenobarbital shown in Table II where, in each case, the two-step process of conversion and extraction has an efficiency exceeding 95%, with standard deviations ranging between 4.0 and 6.0%.

TABLE II

CONVERSION EFFICIENCIES FOR AMOBARBITAL, PENTOBARBITAL AND PHENOBARBITAL

Values obtained for ≈30 μg of free acid with benzene as solvent in the extraction of the derivatized drug.

Conversion efficiencies (%)			
*Amobarbital**		*Pentobarbital*	*Phenobarbital*
	100.0	99.5	91.6
	93.5	100.6	87.4
	95.0	90.5	91.6
	90.0	100.6	94.8
	92.0	102.2	95.9
	95.0	93.4	98.6
	97.5	102.2	100.1
	91.8	96.7	104.9
	92.8	94.5	104.9
	94.0		
	100.5		
	98.5		
	103.6		
Mean	95.7	97.8	96.6
n	13	9	9
S.D.	4.02	4.21	6.05

* Overall amobarbital results from Tables I and II ($n = 20$): mean 94.5; S.D. 4.1; R.S.D. 4.3%. Results are for samples processed in five batches.

Recovery of barbiturate from a tissue matrix

Table III shows the recovery of amobarbital added to methanol-soluble tissue components which, with the described procedure, averages greater than 90% with a standard deviation of *ca.* 2.7%. The lowest recovery (see Set 3) was obtained when the solutions were left to stand overnight before completing the analysis, and can reasonably be attributed[5,6] to adsorption of the barbiturate onto the surfaces of the glass containers.

Analysis of fresh tissues

Results for the analysis of amobarbital in liver tissues and one blood specimen are shown in Table IV. The simpler procedure involving only a single extraction in methanol (Method A) yielded results which, when compared with those obtained following the triple extraction of Method B, can only be regarded as semi-quantitative. Nevertheless, in most cases, even Method A yielded higher values than the official results for the tissue analyses. Apart from the more effective recovery of soluble material by the triple extraction procedure, explanation[4] for the improved barbiturate recovery lies in the progressive dehydration of the tissue by each

TABLE III

RECOVERY OF AMOBARBITAL FROM TISSUE EXTRACTS AFTER METHYLATION AND ALKYLATION REACTIONS

Values for the recovery of 30 μg of free acid after extraction, derivatization and isolation of the dimethyl derivative. Overall mean 90.6; S.D. 2.7; R.S.D. 3.0%.

Recovery (%)			
	Set 1	*Set 2*	*Set 3*
	93.50	91.9	89.1
	90.8	93.2	88.6
	88.1	94.7	90.2
	90.8	90.2	86.5
	93.5	91.9	85.5
Mean	91.3	92.4	88.0
n	5	5	5
S.D.	2.26	1.68	1.93

TABLE IV

DATA FOR ANALYSES OF "FRESH" LIVER TISSUES

Age of samples at time of analysis (official results, mg/kg); 1, 18 months (12.7); 2, 5 years (32.0); 3 18 months (18.0); 4 3 months (20.0); 5, 3 months (83.0); 6, 3 months (20.0).

Method	*Sample no.*	*Final volume* (ml)*	*Sample mass (g)*	*Amobarbital concentration*	
				*mg/kg***	*Mean*
A	1	2.0	4.9	21.0	
			4.5	18.9	20.2
			4.7	20.7	
A	2	4.0	4.9	61.0	61.5
			5.1	61.9	
A	3	2.0	5.0	20.8	
			4.3	24.5	22.5
			4.6	22.3	
A	4	2.0	4.8	41.8	
			6.0	49.4	42.7
			3.6	36.8	
A	5	10.0	4.6	71.4	
			4.7	89.2	80.6
			4.0	81.2	
—	6***	2.0		25.8	
				24.9	24.6
				23.1	
B	1	4.0	5.8	20.3	
			5.0	20.9	22.3
			8.2	25.6	
B	2	4.0	5.1	80.0	
			4.6	78.7	80.1
			5.5	81.5	
B	4	4.0	5.9	60.8	57.8
			5.1	54.7	
B	5	10.0	8.4	110.9	
			4.3	119.5	120.2
			5.0	130.2	

* Adjusted to correspond to calibration plot.

** Values are means from duplicate determinations.

*** Blood specimen; results in mg/l.

successive volume of methanol employed for the extraction. Indeed, comparison of the result for a freeze-dried tissue (84.7 mg/kg for sample 2 of Table IV) suggests that after three extractions the recovery of the barbiturate from fresh tissue is still incomplete, and that solvent extraction of fresh tissue is less efficient and slower than for a freeze-dried form of the same tissue containing less than 10% moisture. The variation observed for sample 5 (and, to a lesser extent, sample 4) with both methods implies that, because of the small sample required for analysis, great care is needed when preparing the entire specimen prior to sampling. Although obvious precautions such as thorough mixing of the thawed tissues were exercised, close attention to the actual comminution of the specimen (especially fibrous tissues in it) may be essential. It is difficult, furthermore, to assess the effect upon the data of Table IV of chemical changes which accompany lengthy storage of tissues and lead to the release of additional amounts of the drug. In fact, there is little information available for comparison, and the conflict which exists may even implicate the analytical methods employed. For example, the release of protein-bound barbiturate[7,8] and loss of water from decomposing tissue[7] were considered to account for increasing concentrations found in tissues held at room temperatures for several months. However, in another study[9], under similar conditions, a marked fall in the concentration occurred, yet, when stored at 4°C, little loss of the drug was observed. Additional support for the data of Table IV as an improvement in methodology (rather than as evidence for the release of protein-bound barbiturate) is provided by the analytical results for fresh tissues (see Table V) obtained using three alternative extraction[10] procedures. Significantly, all values fall below that of the freeze-dried tissue for reasons which probably involve inadequacies in methodology (including, incomplete extraction of samples and losses due to adsorption on precipitates). With the exception of ketones, for reasons given below, correct use of polar, water-miscible solvents, such as

TABLE V

RESULTS OF AMOBARBITAL ANALYSES FOLLOWING EXTRACTION OF FRESH TISSUES BY DIFFERENT METHODS

For details of the extraction see ref. 10. After treatment, residue from each sample (5 g) was rinsed two or three times with the appropriate solvent (water or chloroform). Regardless of the method of extraction, estimation was completed as in *Tissue analyses*. Value obtained for the corresponding freeze-dried tissue, 86.0 mg/kg. Official result, 32 mg/kg.

Method of extraction	*Sample mass (g)*	*Amobarbital concentration*	
		*mg/kg**	*Mean*
Stas–Otto Extraction**	6.5	61.3	62.2
	5.5	63.1	
Tungstate protein precipitation***	5.3	52.9	51.9
	5.1	50.9	
Direct chloroform extraction‡	6.8	47.1	47.4
	5.6	47.7	

* Final volume 2.0 ml. Concentration range for calibration plot 0.061–0.244 mg dimethyl derivative per ml.

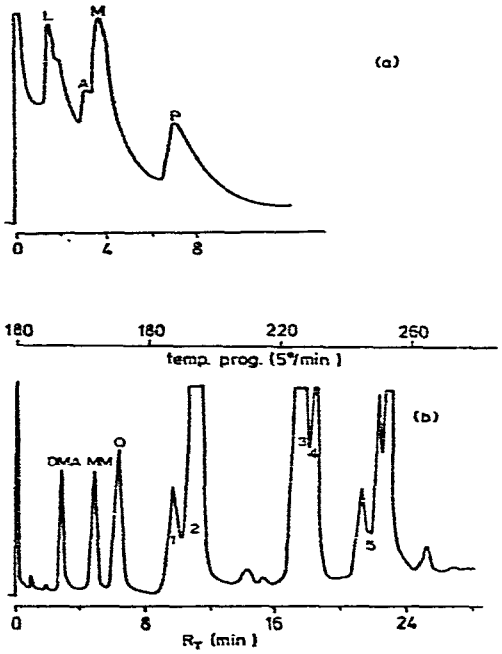
** Two extractions with ethanol (50 ml; 2 h then 1 h).

*** Using 30 ml of specified reagent solutions.

‡ Extraction in refluxing solvent (50 ml, 1.5 h).

methanol and ethanol, greatly improves the likelihood of complete drug recovery from fresh tissues and, more effectively than other water-miscible solvents (or water-immiscible solvents which function by partitioning rather than extraction), facilitates the denaturing and insolubilizing of proteins. Of course, emphasis on the removal of proteins[10,11] seems an irrelevance in comparison with the more difficult problems posed by lipids and other co-extractives of a more polar and reactive nature or, still related to the recovery problem, the potential losses introduced by the use of metal salts as protein precipitants[11–13]. Thus, in our view, extraction in boiling (or re-cycling) solvent is successful for removing free or loosely-bound drug. Presumably, enzymatic degradation of protein[11,14] can liberate, in addition, any occluded or more strongly protein-bound drug, whereas acid hydrolysis or specific enzymes are needed to free the drug covalently-bound to the protein.

Typical chromatograms of tissue extracts are reproduced in Figs. 1 and 2. In Fig. 1a, the chromatogram of an alkaline extract of methanol-soluble compounds



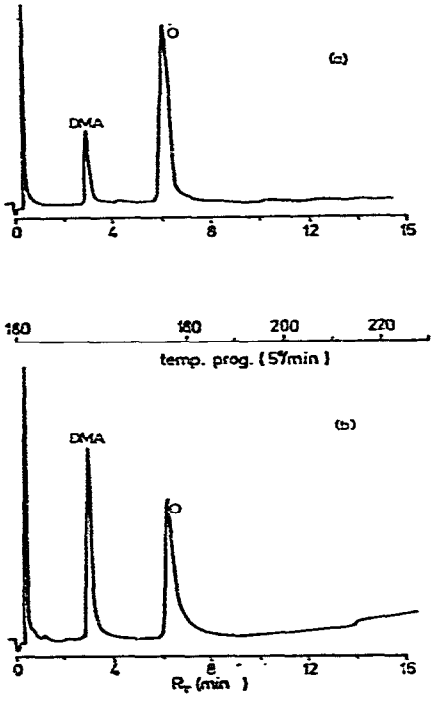


Fig. 1. Chromatograms of tissue extracts. (a), Without derivatization and under isothermal conditions at 190°C. Amobarbital (A) appears as a small peak ahead of the main source of interference, myristic acid (M). Broad peaks due to lauric (L) and palmitic (P) acids are also shown on an elevated baseline. (b), After alkylation with dimethyl sulphate. In the isothermal portion, dimethyl amobarbital (DMA) is clearly separated from methyl myristate (MM) and the internal standard, octadecane (O). Other fatty acid esters elute as the temperature rises: 1 = methyl palmitoleate; 2 = methyl palmitate; 3 = methyl oleate; 4 = methyl stearate; and 5 = higher fatty acid esters.

Fig. 2. Chromatograms of typical extracts obtained after methylation and alkylation steps in the new procedure. The complete absence of fatty acid esters is shown in the isothermal run (a) and confirmed in (b) under temperature programmed conditions (after elution of octadecane) for the same sample. See Fig. 1 for abbreviations.

shows the presence of amobarbital as a small shoulder on the larger myristic acid peak. Poor resolution of the peaks due to lauric and myristic acids, together with an elevated baseline, precludes useful identification and estimation of barbiturates. As shown in the isothermal region of Fig. 1b, conversion of fatty acids and the barbituric acid to the corresponding methyl derivatives facilitates a complete separation of the N,N-dimethylamobarbital from the laurate (under the solvent peak) and myristate esters. For these experimental conditions, the most suitable internal standard was *n*-octadecane. However, this applies only in the absence of the metabolite 3'-hydroxyamobarbital whose retention time on this column is close to that of the internal standard. The efficacy of the new procedure in removing interfering lipids is demonstrated by typical chromatograms of lipid-free extracts (see Fig. 2). There is no evidence in either case of traces of fatty acid esters in the isothermal (Fig. 2a) or temperature-programmed runs (Fig. 2b) so that greater flexibility in the choice of an internal standard is possible and the presence of other acidic drugs, particularly those with longer retention times, is more easily observed.

Finally, as referred to earlier, despite the removal of UV-absorbing endogenous[15] carboxylic acids with the long-chain fatty acids, there is no overall improvement in the composition of the remaining extract which simplifies the subsequent measurement of barbiturates by spectrophotometry. Advantage in the methylation procedure is gained only when the analysis is completed by GC. As will be discussed elsewhere, the absorption of UV-radiation (as well as the formation of intense colour) is related to complex and on-going reactions involving compounds present in all tissues. Perhaps of greater interest is the fact that this reaction (the Maillard reaction) acts as a source of highly reactive intermediates, especially carbonyl compounds[4], which may be responsible for the loss of, or failure to detect, certain drugs other than barbiturates because of the scavenging action of the numerous intermediates.

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