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A METHOD FOR MEASURING GLUTETHIMIDE (DORIDEN®) IN HUMAN SERUM AFTER INTAKE OF THERAPEUTIC DOSES

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SUMMARY

A method is described that permits the determination of 0.25 μg/ml of glutethimide in 0.5 ml of serum. Because of this high sensitivity, the method is suitable for pharmacogenetic studies and for tests of bioavailability. A crucial part of the method is the extraction, purification and concentration procedure, which requires multiple steps in order to eliminate endogenous substances and possible metabolites of the drug that tend to interfere with measurements performed by gas–liquid chromatography.

INTRODUCTION

Glutethimide is marketed under the trade-name of Doriden, and is one of the most widely used hypnotics. Nevertheless, the measurement of its serum concentration by previous methods in which ultraviolet spectrophotometry[1] or thin-layer chromatography[2] are used is sensitive only to the degree that it allows the determination of glutethimide after the consumption of toxic amounts. Of the numerous gas chromatographic techniques used in clinical chemistry[2,3], only the method of GRIEVESON AND GORDON[4] is sufficiently reliable to measure serum levels of the drug after the administration of 250–500 mg doses. Even with this technique, endogenous materials and caffeine interfere with the determination and for this reason in a more recent study[5] a slightly modified method was used. The method described in this paper has numerous advantages. The assay of glutethimide down to levels of 0.25 μg/ml with a possible error of less than 0.03 μg/ml requires only 0.5 ml of serum. Technical errors commonly associated with gas chromatographic determinations are minimized by the use of an internal standard. Endogenous substances of caffeine, which could interfere with the determination, are eliminated during the purification procedure and the analysis on two columns of different characteristics confirm this. The method was developed to permit a study of genetic and environmental factors which may influence the absorption and elimination of glutethimide. In addition, owing to the high sensitivity, the bioavailability of the drug from pharmaceutical preparations can also be measured after the administration of therapeutic doses.

MATERIALS AND METHODS

Gas–liquid chromatography

A Varian Series 1700 gas chromatograph fitted with a dual flame ionization detector was used in this work, so that separate determinations could be carried out simultaneously on two different columms. The gas chromatograph was connected through a Hewlett-Packard 3378A integrator to a Varian Model 20 recorder. Stainless-steel columns of 3 mm O.D. were used. Column I was 76 cm long and was packed with 80–100 mesh Chromosorb W HP AW-DMCS coated with 4 % OV-17. Column II was 152 cm long and was packed with 80–100 mesh Chromosorb W HP AW-DMCS coated with 3.8 % SE-30. The columns were pre-conditioned by heating them at 240° for 48 h with a slow flow of nitrogen. The operating conditions were as follows: column oven 183°; detector 300°; injection port 300°; air flow-rate 150 ml/min; hydrogen flow-rate 20 ml/min; nitrogen carrier gas, 15 p.s.i. for column I and 25 p.s.i. for column II. The chromatograph was set at 1/16th of its maximum sensitivity, the integrator at 0.03 mV/min slope sensitivity and 1 mV full-scale presentation to the recorder with automatic operation.

Extraction and purification procedure

Serum samples of 0.5 ml were extracted by mixing them in a centrifuge tube for 15 sec with 2 ml of chloroform. After centrifugation at 3000 r.p.m. for 5 min the organic phase was removed with the aid of a glass syringe fitted with a 9 cm long 21 G blunt-end stainless-steel needle. The extraction procedure was repeated using 1 ml and 0.5 ml volumes of chloroform. One syringe was used for each serum sample; this syringe was not washed until the extraction had been completed. The pooled organic phase was washed with 1 ml of 0.1 *N* NaOH, followed by 1 ml of 0.1 *N* HCl. After each wash the tubes were centrifuged to enhance separation. The organic phase was evaporated at about 40° with the aid of a fine nitrogen stream. The dry residue was re-dissolved in 4 ml of diethyl ether and further purified by washing with 1 ml of 0.1 *N* NaOH and 1 ml of 0.1 *N* HCl. The organic phase was transferred to a dry test-tube. To achieve complete recovery of glutethimide, the NaOH and HCl were washed through, in this order, with 2 ml and then with 1 ml of diethyl ether. The organic solvent fractions were pooled and evaporated as before. The purified extract transferred from the test-tube into a Reacti-vial of 0.3 ml total capacity and 0.1 ml cone capacity with three 0.1 ml volumes of chloroform, drying under vacuum after each transfer.

The glutethimide used as standard at 10–40 μg/ml concentration was dissolved in chloroform containing 60 μg/ml of pentobarbital for column I and in methanol containing 100 μg/ml of pentobarbital for column II. Each day the performance of the chromatograph was determined by injecting several 1.0 μl doses of the standard solution. As shown in Fig. 1, the area under the glutethimide peak divided by the area under the pentobarbital peak was plotted against glutethimide concentration. Following this, 1.0 μl of a blank extract from serum of the volunteer was applied to the columns. The slope of the standard curve was re-determined 30 min later. Usually there was a slight shift of this curve, but thereafter it remained stable for several hours in spite of repeated serum extract injections.

The purified serum extract containing the glutethimide was dissolved in

10–40 μl of the appropriate organic solvent containing the internal standard and 1.0 μl was injected into the column. As the integrator was set at automatic operation the peak time and the area under the curve were recorded on the print-out. The unknown glutethimide concentration was estimated from the standard curve by interpolation. The peak retention times on column I were 1.5–1.9 min for pentobarbital and 3.1–3.4 min for glutethimide, and on column II 2–2.2 min and 4.1–4.4 min, respectively.

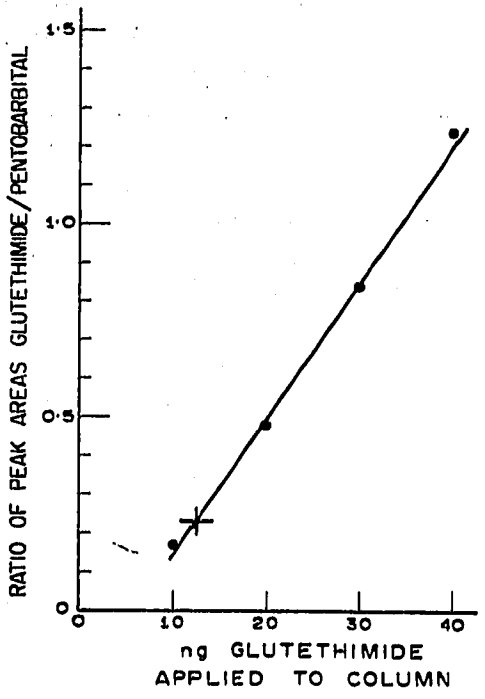

Fig. 1. Example of a standard curve used to estimate serum concentrations of glutethimide. The dots are experimental points obtained by measuring drug (glutethimide) and internal standard (pentobarbital) added to chloroform using column I; the cross indicates the reading obtained with a starting material of 0.5 ml of serum containing 0.125 μg of glutethimide if the described extraction method is used. No correction procedure was used (*e.g.*, by changing concentration of internal standard) to make the curve intersect with the origin of the co-ordinates.

The recovery of the glutethimide from serum was tested by drying a known volume of standard solution in methanol on to test-tubes and dissolving the residue in serum. The blood samples from normal volunteers taking glutethimide were collected at different time intervals by venepuncture. After clotting and centrifugation of the blood samples, the serum was frozen and stored at —20° until the assay was carried out. All chemicals used in this study were of analytical quality.

RESULTS AND DISCUSSION

The variation in the ratios of the peak areas of the standard was found to be less than 1%. After the injection of the first serum extract, the standard curve shifted to the right and the retention times were shortened slightly, but remained stable thereafter.

It was found during the preliminary recovery studies that serum samples containing 5 μg/ml or less of glutethimide were sometimes estimated to contain more

than double the added amount of drug. If saline with glutethimide was extracted, the amount recovered was the same or slightly less than expected. After this observation, serum samples of several individuals were extracted and subjected to gas chromatographic analysis. Endogenous substances in various concentrations with peak retention times of 2.2–2.4, 3.6–4.1 and 5–5.3 min on the OV-17 column were detected in all serum samples tested. The corresponding retention times on the SE-30 column were 1.4–1.7, 3.4–3.9 and 4.6–5.1 min. It was observed with serum from given individuals that the amounts of these substances were high if the lipid content was high. As the retention times were close to those of pentobarbital and glutethimide, it was necessary to eliminate these substances so as to minimize the interference with the determination. Washing the chloroform extract with water was not successful. Lowering the column temperature resulted in loss of glutethimide on the column and several-fold reduction in sensitivity.

Fig. 2A represents the record obtained from the base–acid-washed chloroform extract of a serum without, and Fig. 2B of the same serum with, 1.20 μg/ml of glute-

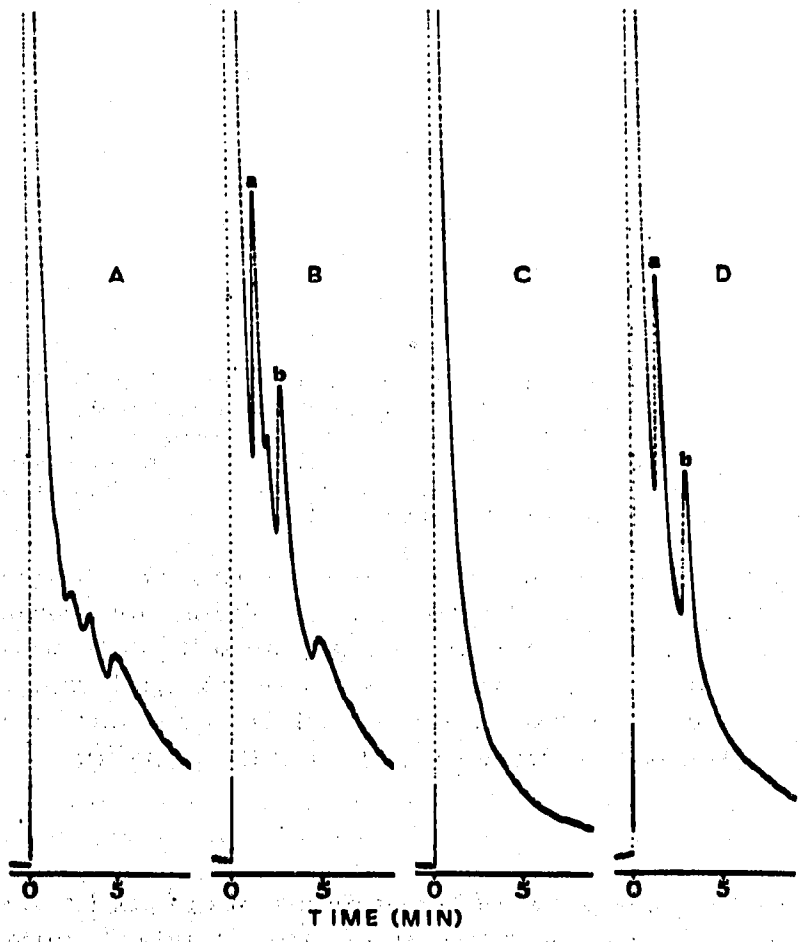

Fig. 2. Chromatograms from column I of serum extracts after various stages of purification. A = washed chloroform extract of serum containing no glutethimide; B = washed chloroform extract of serum containing pentobarbital internal standard (a) and 1.20 μg/ml of glutethimide (b); C = same as A but chloroform extract re-dissolved in diethyl ether and washed; D = same as B but chloroform extract re-dissolved in diethyl ether and washed.

thimide and pentobarbital internal standard added. The interference is not always as serious as that illustrated, but nevertheless, it makes the estimation difficult, especially if the serum samples were taken after meals (high lipid content). Serum samples extracted with diethyl ether instead of chloroform and washed as described were free of the interfering substances, but the recovery of glutethimide was less than 60%. These observations led to the consecutive use of chloroform and diethyl ether in the finally adopted extraction procedure, which leads to the recovery of at least 95% of the drug. Fig. 2C represents the pattern obtained without and Fig. 2D with 1.20 μg of glutethimide per millilitre of serum and pentobarbital as internal standard using the described method. This method can be used to estimate a 0.25 μg/ml concentration of glutethimide in serum and the accuracy is 95–101% even if the amounts added are very small (Table I).

TABLE I

RECOVERY OF GLUTETHIMIDE FROM SERUM

0.5 ml of serum used for extraction; concentrations expressed per millilitre of serum.

Subject	*Amount of glutethimide added (ug/ml)*	*Apparent concentration (μg/ml)*		*Recovery (%) (average of two columns)*
		Column I	*Column II*	
1	1.00	0.99	0.95	97
	0.50	0.52	0.48	100
	0.25	0.25	0.24	98
2	0.45	0.45	0.46	101
	0.25	0.25	0.24	98
3	0.50	0.48	0.51	99
	0.25	0.24	0.26	100
4	1.00	1.02	1.01	101
	0.50	0.48	0.49	97
	0.25	0.26	0.24	100
5	1.00	0.98	0.98	98
	0.50	0.52	0.49	101

Fig. 3 illustrates the serum concentration of glutethimide at various time intervals for four healthy males of comparable body weight (average 150 lb) and age (average 32 years), taking the commercially available product in doses of 750 mg for A, 250 mg for B and C and 125 mg for D. The drug was taken on an empty stomach 2 h before breakfast for A, C and D and 15 min before breakfast for B. The plasma concentration curves show a relatively rapid absorption rate, reaching a maximum in 1–2 h if taken on an empty stomach and a somewhat delayed peak if taken shortly before meals. The sharp fall of serum concentration after reaching the maximum indicates that the drug, owing to its high lipid solubility, is probably taken up in the adipose tissue. Equilibrium is reached only after 4–6 h and the gradual decrease of serum concentration after this time is influenced mainly by the release and metabolism of the drug. The same phenomenon was observed by CURRY *et al.*[5]. The clinical observations were closely related to that of the serum concentration, that is, D

noticed no effect while the other two subjects taking 250 mg felt sedated for 2–3 h after drug intake. Subject A was heavily sedated during the experiment.

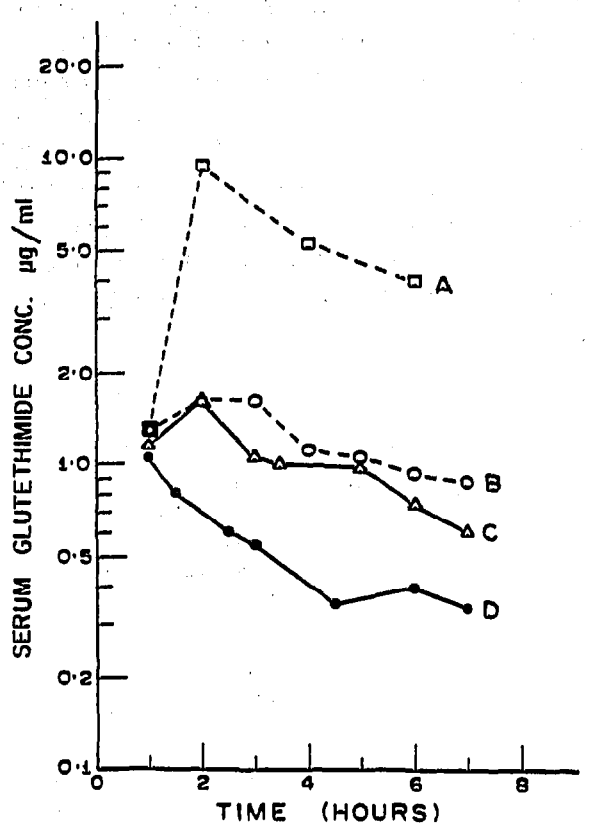

Fig. 3. Serum concentrations of glutethimide after oral administration of the drug. Subject A received 750 mg, B and C 250 mg and D 125 mg.

The method used measures the unchanged glutethimide in serum. Endogenous substances and metabolites do not interfere with the determination. SUNSHINE *et al.*[2] found it necessary to wash the chloroform extract of urine of glutethimide-intoxicated patients with NaOH solution in order to eliminate glutethimide metabolites before the gas chromatographic analysis. Although the doses administered here are low in comparison with the toxic doses and the concentration of metabolites in the serum is usually lower than that of the parent drug, the double wash procedure gives added reliability to the assay. Serum samples containing pentobarbital, secobarbital, amobarbital and phenobarbital in addition to glutethimide were analysed by the described method and it was found that the barbiturates are removed from the chloroform extract into the alkaline aqueous layer. As glutethimide, after therapeutic doses, is not excreted in unchanged form in the urine[6] and the metabolites were not available in pure form, no attempt was made to measure the urinary concentration of the unchanged drug or its metabolities.

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