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POSSIBLE INTERFERENCE BY A METABOLITE IN GAS CHROMATOGRAPHIC ASSAYS FOR GLUTETHIMIDE WHICH EMPLOY CERTAIN NON-SELECTIVE LIQUID PHASES

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SUMMARY

The specificity of gas chromatographic assays for glutethimide was assessed using combined gas chromatography–mass spectrometry. Analysis of urine from patients intoxicated with glutethimide indicated that a drug metabolite was eluted with glutethimide from columns containing SE-30, OV-1 and OV-17. This would lead to an overestimation of the unchanged drug in the urine when these non-selective columns were used. The metabolite, identified as α-phenyl α-ethyl glutaconimide by gas chromatography–mass spectrometry, could be separated from glutethimide using OV-225 or Carbowax 20M liquid phases. The concentration of the metabolite in the urine increased relative to glutethimide with time after the intoxicating dose. Plasma from the same patients contained very little of the glutaconimide metabolite relative to glutethimide. It is recommended that biological samples containing glutethimide be analyzed using gas chromatographic columns which are capable of separating the drug from its potentially interfering metabolites. Use of OV-225 or Carbowax 20M liquid phases would eliminate the possible overestimation of glutethimide in biological samples.

INTRODUCTION

Gas chromatographic (GC) methods have replaced spectrophotometric methods for the quantitative determination of glutethimide in biological samples. This is because of the inherent specificity of GC coupled with the relative ease of sample preparation. Numerous GC methods for glutethimide analysis have been reported employing a variety of extraction procedures and GC conditions[1–13]. Until now the specificity of these assays for glutethimide has not been determined using the powerful analytical tool of mass spectrometry (MS).

The purpose of this study was to assess, using combined GC–MS, the ability of various commonly-used GC column packings to separate glutethimide from its

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metabolites. It was found that some of the GC columns used in the analysis of glutethimide could not separate the drug from one of its metabolites while other more selective columns could perform this function. The results reported here will allow the selection of a specific assay for glutethimide from the large numbers of GC assays now available.

EXPERIMENTAL

Methods

Urine and plasma samples were obtained from patient VL and five other patients who were admitted to the hospital with the diagnosis of glutethimide intoxication. The patients had ingested unknown quantities of the drug and were in coma at the time of admission. All patients subsequently recovered without incident. Urine and plasma samples collected from the patients were frozen until analysis. Samples of authentic glutethimide and α-phenyl glutarimide were obtained from Ciba-Geigy (Summit, N.J., U.S.A.). Authentic α-phenyl α-ethyl glutaconimide was not available for use as a standard.

Sample preparation. A 5-ml aliquot of urine was extracted with two successive 10-ml portions of diethyl ether (reagent grade). The combined ether extracts were washed with 5 ml of 0.1 *N* NaOH followed by a wash with 5 ml of 0.1 *N* HCl. The washed ether extract was evaporated to dryness at 30° by swirling under reduced pressure (Evapomix–Buchler Instruments). The residue was dissolved in 0.1 ml of methanol and 1 μl injected into the gas chromatograph. Addition of known amounts of glutethimide to the blank urine samples in separate experiments indicated that recovery of glutethimide using this extraction and washing procedure was 75%. The same procedure was used to analyze 1.5 ml of plasma from the patients.

Gas chromatography. Analyses were performed using Varian Models 1400 and 2100 gas chromatographs equipped with hydrogen flame detectors. Nitrogen carrier gas flows and oven temperatures are given in Table I. Injection port and detector temperatures were 245°.

The instruments contained 6 ft. × 0.25 in. glass columns. The column packings, purchased from Supelco (Bellefonte, Pa., U.S.A.), were 3% SE-30, 3% OV-1,

TABLE I

GAS CHROMATOGRAPHY OF A WASHED ETHER EXTRACT OF URINE OBTAINED FROM A PATIENT (VL) INTOXICATED WITH GLUTETHIMIDE

Column	*Oven temp. (°C) (isothermal)*	*Carrier gas flow (ml/min)*	*Retention time (t_R) of authentic glutethimide (min)*	*Number of peaks observed in in urine extracts*	*$t_{Rglut.}$ of GC peaks in in urine extracts**	*$t_{Rglut.}$ of authentic α-phenyl glutarimide**
3% SE-30	200	20	4.1	1	1.00	0.90
3% OV-1	200	20	3.9	1	1.00	0.81
3% OV-17	220	20	3.8	1	1.00	1.00
2% OV-225	200	20	6.0	2	1.00, 1.09	1.34
2% Carbowax 20M	220	40	8.5	2	1.00, 1.30	1.50

* Retention time relative to authentic glutethimide.

3% OV-17, 2 and 3% OV-225 on 80–100 mesh Supelcoport and 2% Carbowax 20M on 100–120 mesh Supelcoport.

Gas chromatography–mass spectrometry. A Finnigan Model 1015 SL quadrupole instrument was used. Glass columns (5 ft. × 0.25 in.) containing 3% OV-1 or 2% OV-225 were employed in the GC–MS analyses. Operating temperatures were: oven, 200°; injection port, 245°; separator, 210°. The conditions at which the mass spectra were taken were: electron energy, 70 eV; emission current, 200 μA; high voltage, 2.5 kV; sensitivity, 10^{-7}; scan time, 1 sec.

RESULTS

Urine from patients hospitalized with glutethimide intoxication was used as a source of the drug and its metabolites. An aliquot of a washed ether extract of urine collected between 25 and 43 h after hospital admission from patient VL was injected into a combined gas chromatograph–mass spectrometer containing a 3% OV-1 column. The total ion current chromatogram shown in Fig. 1 shows a single peak,

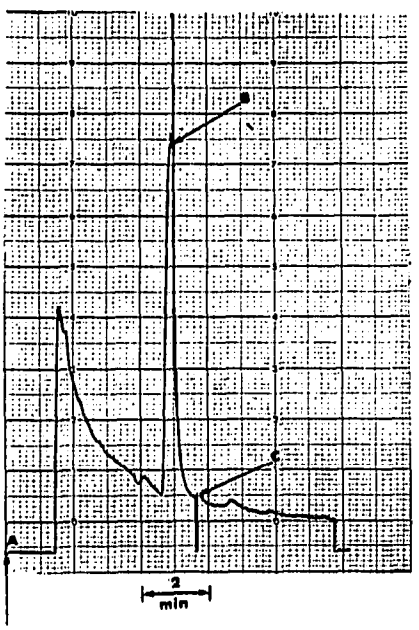


Fig. 1. Total ion current chromatogram on OV-1 of a 12–25-h urine extract from patient VL. Point A indicates injection of the sample. The ionizer was not operating for 1.5 min after injection thereby eliminating most of the solvent peak. Mass spectra, taken at points B (sample 483, Fig. 2) and C (background) in the chromatogram produced the deflections shown at those points.

having a retention time identical to that obtained by injection of authentic glutethimide. The mass spectrum taken at the apex of the chromatographic peak is shown in Fig. 2 (sample 483). The spectrum shows ion peaks typical of authentic glutethimide, with the molecular ion (M^+) at 217 and $M-C_2H_4$ (molecular ion minus C_2H_4) at 189. Additional ion peaks are seen in the spectrum, however, when compared to

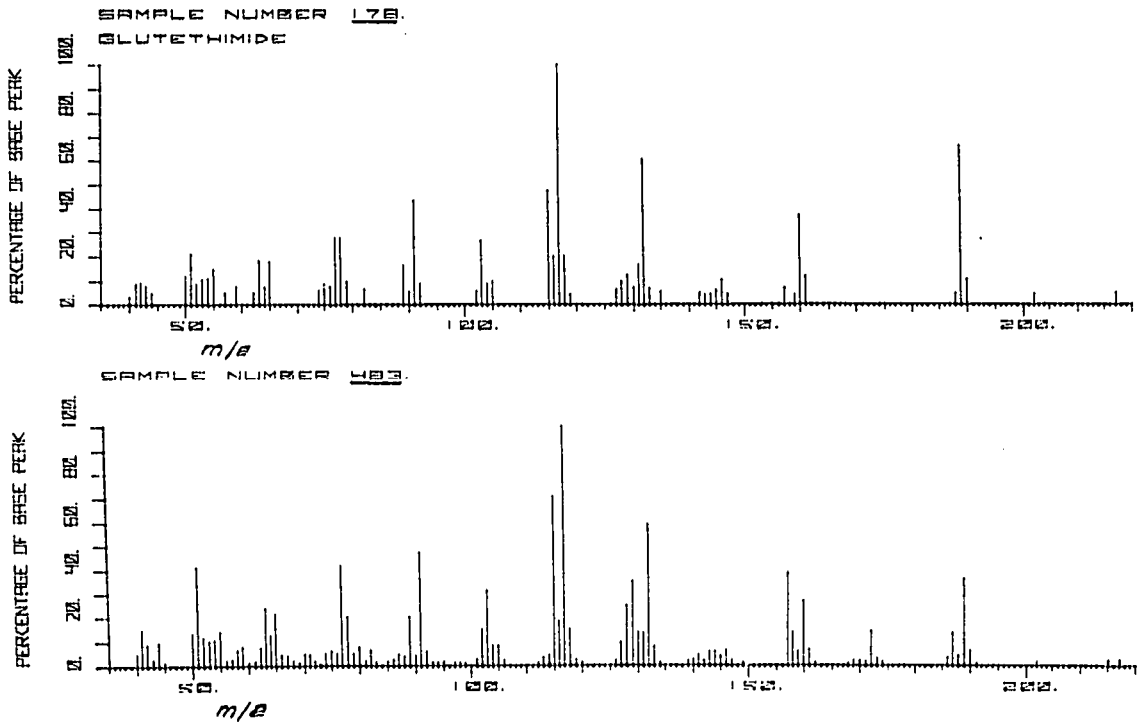
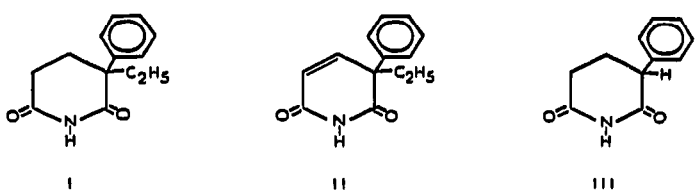


Fig. 2. Mass spectra of standard glutethimide (sample 178) and of the substances in the single peak eluting from OV-1 after injection of a washed urine extract from patient VL (sample 483).

that of authentic glutethimide. Most notable are peaks at *m/e* 215, 187, 172 and 158 which do not appear in the spectrum of glutethimide. These peaks did not appear in extracts of blank urine and the fact that the 215, 187 and 158 peaks are 2 mass units lower than corresponding peaks in the glutethimide spectrum suggested that a substance was contaminating the GC peak and that it may be the previously reported[14] dehydro metabolite of the drug, α-phenyl α-ethyl glutaconimide. This metabolite contains two hydrogen atoms less than glutethimide and thus its mass spectrum would contain some ions similar to glutethimide but having two mass units less. The chemical structures of glutethimide and two of its previously reported metabolites are shown in Fig. 3.

The GC–MS evidence for non-homogeniety of the glutethimide peak in urine extracts chromatographed on OV-1 prompted an investigation of the ability of



I II III

Fig. 3. Chemical structures of glutethimide (I) and two of its metabolites: α-phenyl α-ethyl glutaconimide (II) and α-phenyl glutarimide (III).

various GC columns to separate glutethimide from the contaminating material. These studies were performed using a conventional gas chromatograph equipped with a hydrogen flame detector. The retention times relative to glutethimide were determined for the substances in a washed ether extract of urine obtained from patient VL. In addition, the relative retention time of authentic α-phenyl glutarimide, another known metabolite of glutethimide[14], was determined on five different columns.

The results in Table I show that only one GC peak was observed when the urine extract was chromatographed on SE-30, OV-1 and OV-17 columns. Lowering the column temperature to increase retention time did not produce additional peaks attributable to glutethimide-related substances (data not shown). On Carbowax 20M and OV-225 columns, however, two peaks were observed in the gas chromatogram of the urine extracts. Comparison of the retention times suggested that glutethimide was present in the sample in addition to a substance having a longer retention time. Carbowax 20M gave better separation of these substances than OV-225. GC–MS evidence, similar to that shown in Fig. 2, indicated that the substance eluting after glutethimide on OV-225 or Carbowax 20M was not separated from the drug on SE-30 or OV-17 columns. All columns except OV-17 were capable of separating glutethimide from authentic α-phenyl glutarimide. The short relative retention time of α-phenyl glutarimide on SE-30 and OV-1 columns indicated that this metabolite was not the substance contaminating the glutethimide peak on those columns.

The time course for the appearance in the urine of the substance eluting after glutethimide also suggested that it may be a metabolite of the drug. Urine collected from patient VL at various times after admission to the hospital was extracted and chromatographed on an OV-225 column. The results are shown in Fig. 4. In urine collected from admission to 12 h, the peak due to the apparent metabolite contained 1/5 of the area of the glutethimide peak. The concentration of glutethimide in this urine sample was 0.010 mg/ml. Urine collected between 12 and 25 h after admission contained relatively more of the metabolite; the ratio of the peak areas was approximately 2:1. The 25–43-h urine sample contained predominately the metabolite, the peak areas being in a ratio of 1:7.

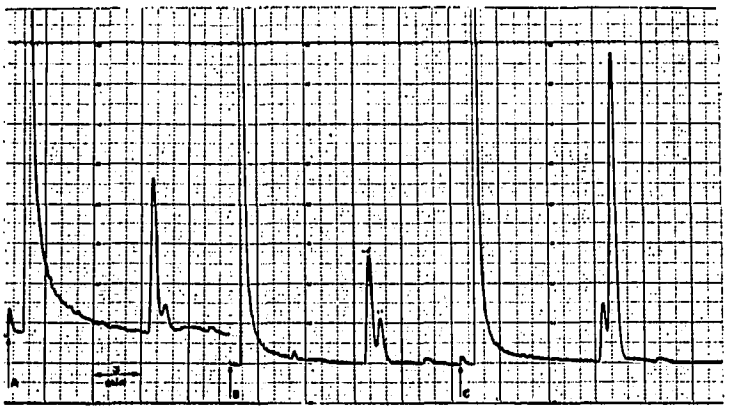


Fig. 4. Gas chromatograms of extracts from urine collected at various times after patient VL was admitted to the hospital. A 3% OV-225 column and a conventional flame ionization detector were used. Arrows indicate sample injection: A, 0–12 h sample; B, 12–25 h sample; and C, 25–43 h sample. Retention times: glutethimide, 9.0 min; metabolite, 9.5 min.

The data shown in Fig. 4 for patient VL are typical of the six patients we have observed with glutethimide intoxication. In all cases, the urinary metabolite peak increased relative to glutethimide with time after admission. The metabolite excretion followed the same pattern after pharmacologic doses (1 g) to humans and after intoxicating doses (400 mg/kg) given to dogs (data not shown).

Fig. 5 shows the GC–MS analysis of the peak eluting after glutethimide when the urine extracts of patient VL were chromatographed on OV-225. The spectrum in Fig. 5 is consistent with the structure α-phenyl α-ethyl glutaconimide. The chemical

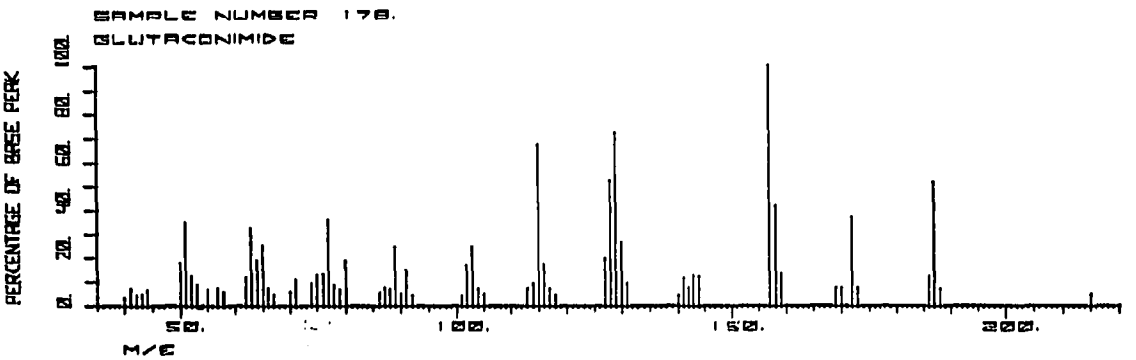


Fig. 5. Mass spectrum of α-phenyl α-ethyl glutaconimide which was separated from glutethimide on OV-225.

structure of this metabolite, shown in Fig. 3, is simply that of glutethimide with a double bond between carbons 4 and 5 of the imide ring. The spectrum exhibits a molecular ion at *m/e* 215 which has a relative abundance similar to the molecular ion of glutethimide at 217. A McLafferty rearrangement (see ref. 15) could account for the intense peak at *m/e* 187 just as the same reaction results in the *m/e* 189 in the glutethimide spectra[16]. The large peaks at *m/e* 172 (M—NHCO) and *m/e* 157 in the metabolite spectra are probably due to ions which have undergone rearrangement and ring contraction similar to those reported for glutethimide[16].

Extracts of plasma have also been analyzed for the presence of the metabolite α-phenyl α-ethyl glutaconimide by GC–MS, and by GC on OV-225. Plasma was obtained from glutethimide intoxicated patients and from normal volunteers given pharmacologic doses (1 g) of the drug. The extracts were found to contain the metabolite in amounts no more than 1/10 of the amount of glutethimide in any sample. When the metabolite did appear, it was in samples taken at times later than 24 h after the dose or hospital admission. In our limited experience, the glutaconimide metabolite does not accumulate to levels higher than glutethimide in the plasma.

DISCUSSION

The practical GC assay which is specific for glutethimide depends on two separation procedures. The first is an extraction step to selectively remove the drug from an aqueous biological sample. The second is injection of the extract on to a GC column which is capable of separating the drug from all other substances in the extracted

sample. Sunshine *et al.*[5] have reported on the effectiveness of various extraction procedures to remove possible interfering substances from glutethimide. It was found that many of the metabolites of glutethimide, including α-phenyl glutarimide, could be removed by washing chloroform extracts of urine (or plasma) with NaOH. Kadar and Kalow[13] also found that washing chloroform extracts of plasma with HCl and NaOH removed interfering substances. In a previous report from this laboratory it was shown that unwashed ether extracts of plasma taken from humans intoxicated with the drug contained glutethimide and several of its metabolites[17]. In the present study, all ether extracts of plasma or urine were washed with acid and base to remove glutethimide metabolites and other interfering substances. It has been shown here, however, that one glutethimide metabolite, α-phenyl α-ethyl glutaconimide, cannot be removed by the washing procedure. This metabolite when injected with glutethimide on to relatively non-polar columns was not separated from the drug. Thus, extraction procedures alone cannot be used to separate glutethimide from all of its known metabolites.

The columns used in this study were chosen because they are commonly used for drug analysis or because their separation characteristics, specified by McReynolds constants[18] are widely different. This study has provided data to show that SE-30, OV-1 and OV-17 do not separate glutethimide from α-phenyl α-ethyl glutaconimide. A frequently used column for glutethimide analysis is SE-30 (refs. 1, 3–5, 8–10). This column has separation characteristics identical to OV-1 (ref. 18) and like OV-1 it did not separate glutethimide from its glutaconimide metabolite.

The present study has shown that columns exhibiting polarity greater than OV-17 are needed to separate glutethimide from its potentially interfering metabolite. Carbowax 20M, which has been used for glutethimide analysis[2,7], gave the best separation. This column has the disadvantage, however, that the retention times for the drug and the glutaconimide metabolite are somewhat long for a rapid analysis. The separation of these substances on OV-225 appears to be adequate for quantitative analysis[19] and retention times are of a shorter duration on that column.

There are certain types of samples in which the metabolite α-phenyl α-ethyl glutaconimide can be found in concentrations high enough to interfere with the analysis of glutethimide when a non-selective GC column is used. Analysis of glutethimide in urine would be in error when SE-30, OV-1, OV-17 or similar columns were used. The drug would be overestimated to an extent that would depend on the time after the dose during which the urine sample was collected. At later collection times, most of the apparent glutethimide analyzed on non-selective columns would actually be the glutaconimide metabolite. This laboratory has not had the opportunity to analyze hemodialyzates obtained from intoxicated patients but these may contain the interfering metabolite as well. If this were the case, the amount of drug removed by hemodialysis would be overestimated using non-selective GC columns.

The interfering metabolite, which is biologically less active than glutethimide[20], apparently does not accumulate in plasma. Therefore, values for plasma glutethimide obtained using non-selective GC columns may not always be in error. It is possible, however, that the metabolite could appear in the plasma of certain patients or laboratory animals which have a quantitatively different metabolic pathway for the drug. It seems reasonable, therefore, to analyze all biological samples (including plasma and tissue samples), using GC columns capable of separating glutethimide from its glutaconimide metabolite. Using OV-225 or Carbowax 20M instead of non-

selective liquid phases and following the published procedures, utilizing acid and base wash of the organic solvent used for extraction, would insure that metabolites would not interfere with glutethimide analysis from any type of biological sample.

Mass fragmentography offers an alternative to those laboratories equipped with GC–MS instrumentation. By monitoring the *m/e* 189 ion either alone or in combination with other appropriate ions in the spectrum of glutethimide, which do not appear in the glutaconimide spectrum, it is possible to obtain specific detection of the unchanged drug. This procedure is routinely used in our laboratory as a check on the specificity of glutethimide analysis when samples are being analyzed for which adequate blanks are not obtainable.

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