Gas-Chromatographic Simultaneous Analysis for Glutethimide and an Active Hydroxylated Metabolite in Tissues, Plasma, and Urine

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We report a gas-chromatographic method for the simultaneous quantitative analysis of both glutethimide and 4-hydroxy-2-ethyl-2-phenylglutarimide (4-HG), an active metabolite of glutethimide, in tissues, plasma, and urine. The method, applied to postmortem tissues from fatal glutethimide overdose and to plasma samples obtained from a patient in coma from acute glutethimide intoxication, proved to be accurate, sensitive, and specific. Results of the analysis confirm that 4-HG accumulates in plasma and tissues during glutethimide intoxication. Because 4-HG has been shown previously to be at least as potent as glutethimide itself, its accumulation may play an important role in the acute toxicity of glutethimide.

Glutethimide, 2-ethyl-2-phenylglutarimide, is a sedative-hypnotic similar in structure and biological activity to the barbiturates. Although early reports claimed that glutethimide was relatively free from the adverse effects often associated with acute overdoses of the barbiturate drugs (1), many cases of serious intoxication with glutethimide were subsequently reported (2–12). The management of glutethimide poisoning is now clearly established as a serious clinical problem (13–17).

Ambre and Fischer have recently reported that 4-HG,1 a metabolite of glutethimide (Figure 1), accumulated to concentrations much higher than the concentrations of unchanged drug in the plasma of humans intoxicated by glutethimide overdose (18, 19). The metabolite was subsequently isolated and purified from the urine of dogs given large doses of glutethimide. This biosynthetically prepared 4-HG possessed sedative-hypnotic activity in mice that was equal to or greater than that of glutethimide itself (19). Thus, it is quite likely that 4-HG could play an important role in the etiology of glutethimide intoxication.

This laboratory has continued the investigation of glutethimide poisoning with the aim of elucidating the role of the active metabolite, 4-HG. These studies required an assay for both glutethimide and 4-HG in plasma, urine, and tissues. Although many assays for glutethimide have been published (20–31), they were incapable of the accurate, simultaneous determination of low concentrations of both glutethimide and the hydroxylated metabolite, 4-HG. Therefore, we developed a new gas-chromatographic assay for glutethimide and an active metabolite, 4-HG, in plasma, urine, and tissues. The assay was used to examine postmortem tissues from overdose fatalities and plasma samples from an individual hospitalized with acute, nonfatal glutethimide intoxication.

Materials and Methods

Apparatus

Gas chromatograph: A Model 800 with flame-ionization detectors (Packard Instrument Co., Downers Grove, Ill. 60515) was used. The columns were 1.8 m by 4 mm (i.d.) coiled glass packed with either “8% SE-30” or “3% OV-225” on 80/100 mesh “Supelcoport” (Supelco, Bellefonte, Pa. 16823). The SE-30 columns were conditioned with “Silyl-8” (Pierce Chemical Co., Rockford, Ill. 61105). Column temperatures were 215 °C for SE-30 and 225 °C for OV-225, and the injection port and detector were maintained at 245 °C. Nitrogen carrier gas flow was 60 ml/min. Electrometer sensitivity was 1 × 10⁻¹⁰ to 1 × 10⁻⁹ A/mV.

Fig. 1. Structures of glutethimide and 4-HG, an active metabolite that accumulates in the plasma of humans intoxicated by glutethimide overdose.
Mass spectra: Mass spectra of the chromatographic peaks attributed to glutethimide and 4-HG were obtained on a 1015 S/L combined GC-MS instrument (Finnegan Corp., Sunnyvale, Calif. 94086). The gas chromatographic component was a Model 1400 (Varian Associates, Palo Alto, Calif. 94303). A 1.5 m by 2 mm coiled glass column packed with “3% OV-1” (Supelco) was used with helium carrier gas flowing at 20 ml/min and an oven temperature of 180 °C. Operating conditions for the mass spectrometer were as follows: Electron energy, 70 eV; emission ion current, 200 A; power supply, 2.5 kV; sensitivity, $10^{-7}$ A/V; and scan speed, 1 s.

Reagents

All commercially available reagents were AR grade or the equivalent.

- Diethyl ether, anhydrous
- Ethanol, anhydrous
- Hexanes, mixture of isomers washed once with 0.1 mole/liter HCl
- Pyridine, freshly distilled
- Methanol, anhydrous
- Acetic anhydride
- HCl, 0.1 mol/liter

Standards

The internal standard consisted of 0.5 or 1.0 mg of $p$-hydroxybenzophenone per milliliter of methanol. Drug standard solutions contained 0.1, 0.5, 1.0, 2.5, and 5.0 mg of glutethimide or 4-HG per milliliter of methanol. The 4-HG was isolated from dog urine as described previously by Ambre and Fischer (19). Final purity of the 4-HG was greater than 99%, as determined by gas chromatography and thin-layer chromatography (19).

Assay procedure

The extraction procedure is an adaptation of the method described by Goldbaum and Domanski (22). Tissue homogenates were prepared by grinding tissue samples with either 3 or 7 volumes of distilled water in a Potter-Elvehjem homogenizer with a glass pestle. Some tissues (such as kidney) containing large amounts of fibrous material usually required thorough chopping in a Waring blender before use of the glass-glass homogenizer.

Up to 2.0 ml of a tissue homogenate, plasma, or urine was added to a 45-ml glass-stoppered centrifuge tube together with 10 μl of internal standard solution. Next, 1.0 ml of HCl and 15 ml of diethyl ether were added, and the samples were shaken for 10 min. The ether layer was separated by centrifugation and 10 ml was transferred to another set of 45-ml centrifuge tubes. The aqueous phase was extracted again with an additional 15 ml of diethyl ether, and the ether extracts were combined and washed once with 5 ml of HCl. The ether layer was transferred to 45-ml tubes and evaporated under a stream of nitrogen at 50 °C.

The residue was dissolved in 2 ml of absolute etha-

nol and 5 ml of acid-washed hexane. A two-phase system was then formed by adding 0.5 ml of HCl. Glutethimide and the hydroxylated metabolite partitioned into the aqueous-ethanol phase, while most of the interfering substances were dissolved in the hexane, which was then aspirated and discarded. The aqueous-ethanol phase was shaken with an additional 5 ml of the acid-washed hexane. After the hexane was removed, the samples were dried under nitrogen at 50 °C. Acetate derivatives of 4-HG and of the internal standard were formed by adding 10 μl of pyridine and 100 μl of acetic anhydride and allowing the samples to remain at room temperature overnight. Finally the samples were dried under nitrogen at 50 °C, and reconstituted in 50 μl of methanol, 1 to 2 μl of which was injected into the gas chromatograph. Columns packed with 8% SE-30 were used for the analysis of all samples except urine, which required the use of columns containing OV-225.

For rapid clinical toxicology evaluations of plasma and urine samples, this method can be shortened by adding the pyridine and acetic anhydride to the residue obtained by evaporating the acid-washed ether extracts. The mixture is heated for 5 min in a boiling water bath; then 1.0- to 2.0-μl samples are injected directly on the GLC columns. In this way an entire analysis can be performed in less than 2 h.

Quantitation

Human tissues, obtained at autopsy from persons with a history of little or no drug ingestion immediately before death, were used as control blanks. Standard curves were obtained by pipetting 10-μl aliquots of the standard solutions into samples of blank human tissue homogenates, plasma, or urine together with 10 μl of the internal standard. Appropriate standards were carried through the entire analytical procedure in parallel with the samples to be analyzed. The ratio of the GLC peak heights (expressed as the height of the drug or metabolite peak divided by the height of the internal standard peak) were calculated for both glutethimide and 4-HG and plotted vs. the amount of glutethimide or 4-HG added to the sample. The resulting plots were straight lines passing through the origin. Both the glutethimide and 4-HG concentrations in the experimental samples were measured by determining the peak height ratios as described above and calculating the equivalent concentrations from the standard curves.

Results

Standard Curves and Blanks

The gas chromatograms of blank liver, kidney, plasma, and urine samples carried through the entire extraction procedure were remarkably free of interfering peaks. Extracts of human brain tissue homogenates were the most difficult to obtain free of extraneous contaminants, and the GLC tracings resulting
from the analysis of human brain on SE-30 columns usually contained a peak that eluted just before the internal standard peak. However, this peak did not compromise the quantitative analysis. None of the blank samples had peaks with retention times similar to those for glutethimide or 4-HG.

Recovery of glutethimide and 4-HG was determined by comparing absolute peak heights of extracted standards with the peak heights obtained with unextracted primary standards. After correction for aliquot factors, the actual recovery of both glutethimide and of 4-HG from tissue homogenates, plasma, or urine was greater than 90%. Nonetheless, extracted standards were included in all assays.

Typically, standard curves were linear over at least a 50-fold range of concentrations for both glutethimide and 4-HG (Figure 2).

Sensitivity

As little as 5 ng of glutethimide and 10 ng of 4-HG (as the acetate derivative) could be detected by direct injection onto SE-30. The practical limits of sensitivity for the assay as described were about 0.2 μg of glutethimide and 0.5 μg of 4-HG per milliliter of plasma or 0.4 μg of glutethimide and 10 μg of 4-HG per gram of tissue. Sensitivity could be increased by concentrating the final sample to 20–30 μl.

Precision

The coefficient of variation obtained by extraction and analysis of six replicate standards, prepared by adding 10 μg each of glutethimide and 4-HG to brain tissue homogenates, was less than 6%. Reproducibility did not vary significantly from day to day.

Specificity of the Assay

Analysis of postmortem tissue samples: Samples of tissues from cases of fatal glutethimide intoxication were analyzed as described above. Typical gas chromatograms extracts of brain tissue are shown in Figure 3. The identity of the drug-related peaks shown in Figure 3 was confirmed by mass spectrometry. Figure 4A shows the mass spectrum of the substance in the peak attributed to glutethimide in the brain extract from Case A; Figure 4B shows the mass spectrum of authentic glutethimide. The mass spectrum of the substance in the peak attributed to the 4-HG-acetate from Case B (Figure 3) and the mass spectrum of the acetate derivative of the standard 4-HG isolated and purified from dog urine are shown in Figure 5. It is apparent that the mass spectra of the glutethimide and 4-HG extracted from post-mortem brain tissue are identical to their respective standards. These data confirm that the GLC peaks attributed to glutethimide and 4-HG were correctly identified and were free of contamination by extraneous material having similar retention times. Similar results were obtained when the drug and its me-

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**Fig. 2.** Standard curves for the quantitative analysis of glutethimide and 4-HG added to human brain homogenates. Data shown are means and ranges of duplicate assays.

**Fig. 3.** Typical gas chromatograms on SE-30 columns of brain tissue extracts from two cases of fatal glutethimide intoxication. Sample size, 0.25 g in each case; electrometer sensitivity, 1 × 10⁻¹⁰ A/mV. The identified peaks are: G, glutethimide; IS, the internal standard (p-hydroxybenzophenone) chromatographed as the acetate derivative; and the acetate derivative of 4-HG. The unlabeled peaks are unrelated to glutethimide, but are generally not seen in extracts of liver, kidney, whole blood, plasma, or urine.
Table 1. Retention Times for Glutethimide, Glutaconimide, and the Acetate Derivatives of 4-HG and the Internal Standard on 8% SE-30 and 3% OV-225 GLC Columns

<table>
<thead>
<tr>
<th>Column</th>
<th>Glutethimide</th>
<th>Glutaconimide</th>
<th>Internal standard</th>
<th>4-HG</th>
</tr>
</thead>
<tbody>
<tr>
<td>8% SE-30</td>
<td>5.0</td>
<td>5.0</td>
<td>8.0</td>
<td>9.2</td>
</tr>
<tr>
<td>3% OV-225</td>
<td>7.0</td>
<td>7.6</td>
<td>9.4</td>
<td>14.4</td>
</tr>
</tbody>
</table>

* The GLC conditions are given in the "Methods" section.

Table 2. Concentrations of Glutethimide and 4-HG in Human Postmortem Tissue Samples

<table>
<thead>
<tr>
<th>Case</th>
<th>Tissue</th>
<th>Glutethimide, mg/g wet wt tissue</th>
<th>4-HG, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Brain</td>
<td>72 (71-72)</td>
<td>5 (3-6)</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>190 (166-214)</td>
<td>8 (8-9)</td>
</tr>
<tr>
<td>B</td>
<td>Brain</td>
<td>6 (5-7)</td>
<td>44 (43-46)</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>10 (9-11)</td>
<td>37 (34-39)</td>
</tr>
</tbody>
</table>

* The assay is described in detail in the "Methods" section.

Data from postmortem tissue analyses of two cases of fatal glutethimide poisoning are summarized in Table 2. Glutethimide concentrations in the brain and liver of Case A were very high, but concentrations of the hydroxylated metabolite were low. Tissues from Case B contained rather small quantities of glutethimide, but concentrations of the me-
tabolite were four to seven times those of the unchanged drug.

Analysis of urine samples: A β-glucuronidase hydrolysate of urine from a glutethimide-intoxicated patient was analyzed because this sample was likely to contain the highest concentrations of glutethimide metabolites that might interfere with the analysis of 4-HG. Mass spectra again showed that even in urine samples, the 4-HG acetate peak on SE-30 columns was free of interfering substances. However, the concentration of glutethimide in urine could not be reliably assayed on SE-30 columns. Fischer and Amberg have recently shown that extracts of urine obtained from patients intoxicated with glutethimide contained interfering quantities of 2-ethyl-2-phenylglutaconimide, a metabolite that cannot be separated from glutethimide on SE-30 (32). Peaks attributed to glutethimide and its glutaconimide metabolite were adequately resolved on OV-225 columns (32), and mass spectra of the peak owing to the acetate derivative of 4-HG demonstrated that the peak was also free of interfering substances when the analysis was performed with use of this column. Thus, OV-225 should be used in the analysis of urine samples containing glutethimide and its metabolites. The SE-30 columns were satisfactory for all other samples we have analyzed, since the glutaconimide metabolite was not found in concentrations high enough to interfere with the quantitation of glutethimide in any samples other than urine.

Concentrations of Glutethimide and 4-HG in the Plasma of a Case of Acute Glutethimide Intoxication

Ambre and Fischer have previously reported that the plasma of patients hospitalized for acute glutethimide intoxication contained high concentrations of 4-HG (18, 19). A recent case of glutethimide poisoning referred to the University of Iowa Hospitals concerned a patient who had ingested about 6 g of glutethimide 7 h before admission. Blood samples were drawn at 7.5, 14, and 21 h after drug ingestion. The patient was comatose but responded to painful stimuli at these times and recovered consciousness completely about 30 h after ingesting the drug. The plasma samples were assayed for both glutethimide and 4-HG (Table 3). Glutethimide concentrations were high upon admission but decreased with time, while the concentrations of 4-HG appeared to peak about 14 h after drug ingestion. Unfortunately, no blood samples were taken at the time of recovery, so that we cannot correlate plasma concentration of the metabolite with recovery of the patient.

**Discussion**

**Methodology**

The gas-chromatographic assay for glutethimide and an active metabolite, 4-HG, described in this report proved to be satisfactory for the quantitative analysis of both these materials in plasma and tissues and is, therefore, a significant improvement over previously published analyses designed to detect glutethimide alone (20-31). The colorimetric procedures are incapable of accurately distinguishing between glutethimide and metabolites (20). Many of the previously reported GLC methods might have the necessary specificity but were not used with an appropriate extraction procedure. Thus, when samples containing glutethimide and its metabolites were extracted with a very nonpolar solvent such as petroleum ether, glutethimide but no 4-HG was extracted (20). If ether or chloroform were used as the solvent, glutethimide and 4-HG were extracted (20, 22), but this method suffered from serious shortcomings. First, the unwashed ether extracts of tissues contained so much extraneous material that the GLC tracings were rendered useless by large interfering peaks. Secondly, the quantitation of the 4-HG peak, even in relatively clean samples, was sometimes compromised by peak tailing and an apparent breakdown or isomerization of the metabolite on the column (19). Finally, some procedures removed interfering materials by washing chloroform or ether extracts of the samples with dilute base (20, 30), a step that must be avoided because it effectively removes 4-HG from the extracts. We circumvented these problems by adapting the extraction procedure described by Goldbaum and Domanski (22) to a GLC analysis.

Since speed and the qualitative identification of the intoxicating agent may be more important than a high degree of accuracy, the abbreviated version of the analysis (described under “Methods”) probably should be considered when rapid clinical toxicology evaluations of plasma or urine samples are required. However, in our experience, when the shorter extraction method was used, GLC tracings of blank urine and plasma samples sometimes contained a variable number of unidentified peaks, and extracts of tissue homogenates contained very large amounts of lipid. The great advantage of the modified Goldbaum extraction procedure described here is its ability to ef-

**Table 3. Concentrations of Glutethimide and 4-HG in the Plasma of a Case of Nonfatal, Acute Glutethimide Intoxication**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time after drug ingestion, h</th>
<th>Concentration, μg/ml plasmaabc</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.5</td>
<td>12.1 (11.1-13.1)</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>5.7 (5.6-5.8)</td>
</tr>
<tr>
<td>3</td>
<td>19</td>
<td>3.8 (3.6-3.9)</td>
</tr>
</tbody>
</table>

* This patient's clinical course is summarized in the "Results" section. The patient was in a light coma throughout the period during which plasma samples were taken and recovered about 30 hours after drug ingestion.

* Details of the analysis are given in the "Methods" section.

* Data shown are means and the ranges of duplicate assays.

* 4-Hydroxy-2-ethyl-2-phenylglutarimide.
fect a nearly quantitative extraction of neutral, water-insoluble substances such as glutethimide and 4-HG from difficult samples such as tissue homogenates. When coupled with gas chromatography on columns of either SE-30 or OV-225, this procedure was accurate, specific, and sensitive. Urine from individuals who have ingested glutethimide must be analyzed on OV-225 columns regardless of which of the two extraction procedures was used to prepare the samples. This is because urine contains a glutaconimid metabolite that interferes with the quantification of glutethimide if the analysis were to be performed on SE-30 or similar nonpolar GLC columns (32).

In our experience, the glutaconimid metabolite was not found in tissues or plasma in concentrations high enough to interfere with quantitation of glutethimide on SE-30 columns. However, we periodically screen samples for the presence of this potentially interfering metabolite by GLC analysis on OV-225 columns. An OV-225 column is not used in the routine analysis for glutethimide and 4-HG in most samples primarily because the retention time of the 4-HG acetate peak is considerably greater on OV-225 than on SE-30 columns.

The accurate quantitative measurement of 4-HG concentrations obviously requires authentic 4-HG for standards. Thus far, however, 4-HG is not readily available because the metabolite has been obtained only by isolating it from dog urine by a time-consuming procedure (19). Because most analysts will not have immediate access to authentic 4-HG, the following method might be used to calculate the 4-HG concentrations in unknown samples.

The relative responses of a flame ionization detector to different substances can be determined by measuring the areas under the peaks in the chromatogram. Using this technique, the detector response to glutethimide on the Packard gas chromatograph was within 5% of the detector response to an equal amount of 4-HG (chromatographed as the acetate derivative). Similar detector responses were also obtained when a Varian Model 2100 was used. This similarity in detector response makes it valid to use glutethimide standards to quantify 4-HG. Thus, if instead of peak height ratios, the peak area ratio (peak area of glutethimide divided by peak area of the internal standard) is plotted vs. the amount glutethimide, the resulting standard curve can be used directly to calculate the amounts of both glutethimide and 4-HG in the unknown samples.

Cases of Acute Glutethimide Intoxication

Data from the analysis of plasma from the single case of nonfatal, acute glutethimide intoxication reported here were in complete accord with the earlier and more comprehensive studies of Ambre and Fischer (18). High concentrations of 4-HG were seen 19 h after drug ingestion. At that time the patient was still in a semi-comatose state although plasma concentrations of glutethimide had fallen to concentrations below those attained after the administration of normal sedative doses of the drug (18, 26).

Analysis of postmortem tissues from two cases of fatal glutethimide poisoning yielded markedly different results. In Case A, glutethimide concentrations in brain tissue were probably sufficiently high to be the immediate cause of death. However, the concentration of glutethimide in the brain of Case B was quite low, whereas 4-HG concentrations were high. There is insufficient data to allow us to state unequivocally that 4-HG was the cause of death in Case B. Delayed death in glutethimide intoxication might be due to factors other than direct depression of the central nervous system, and such pathology could be indirectly related to concentrations of glutethimide or the active metabolite in brain or blood. Nonetheless, because, in mice, 4-HG has been shown to be about twice as potent as glutethimide (19), the data presented here suggest that 4-HG may play an important role in the etiology and outcome of at least some cases of acute glutethimide intoxication. Thus, the importance of assaying for both glutethimide and 4-HG in studying cases of glutethimide intoxication is obvious.

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References