Determination of Glutethimide (Doriden) and Its Metabolites in Biologic Specimens

Irving Sunshine, Robert Maes, and Rosalie Faracci

A gas chromatographic method for the quantitative determination of glutethimide was developed. This method was applied to samples obtained from patients who were comatose because of acute glutethimide intoxication. The results obtained from this procedure were compared with those obtained by using an ultraviolet method. The patients' clinical conditions were assessed and correlated with the blood concentrations of glutethimide. The samples were processed to isolate glutethimide, as well as its metabolites. The metabolites were separated using thin-layer chromatography and were checked for purity by both thin-layer and gas chromatography.

Several methods for the identification or quantitative analysis of glutethimide in blood, urine, and tissues have been described. Colorimetric methods have been reported which are based on the formation of the hydroxamate derivative of glutethimide (1, 2). These procedures are not very sensitive, not applicable to blood specimens, and are relatively nonspecific.

Glutethimide can be quantitatively determined by ultraviolet spectrophotometry (3, 4). The decrease in absorbance of an ethanolic solution of the drug hydrolyzed by dilute alkali is measured at 235 mμ. This method requires 1 hr. for its completion, but is not specific for glutethimide. Both the metabolites of the drug and the drug itself react similarly. Before the true glutethimide concentrations can be determined, these metabolites must be eliminated.

The presence of glutethimide can be determined qualitatively by thin-layer chromatography (5–9). This procedure will detect as little as 5 μg. of the drug. This high sensitivity permits the detection of glutethimide following its therapeutic use. Thus a positive thin-layer chromatographic result does not indicate whether coma is due to
concomitant disease or trauma after a therapeutic dose of glutethimide has been taken or is the result of the ingestion of toxic amounts of the drug.

Although gas chromatographic procedures have been developed for the detection of glutethimide (10–12), the one quantitative method that has been described was applied only to serum samples. This method does not provide for the separation of glutethimide from barbiturates and does not discuss the possible interference of the metabolites of glutethimide.

A more specific procedure for the determination of glutethimide was developed and compared with other gas chromatographic and ultraviolet methods. This new method was then applied to biologic fluids obtained from patients who had ingested overdoses of glutethimide. The blood glutethimide concentrations were correlated with the patients' clinical conditions. Technics were also developed for the separation and identification of the metabolites of glutethimide. Using these data, the metabolism and excretion of glutethimide were obtained.

**Experimental**

**Reagents**

Only analytic grade reagents are used.

- **Sodium hydroxide, 0.45 N**
- **Potassium hydroxide, 0.2 N**
- **Stock glutethimide solution (50 mg./100 ml.)** Dissolve 50 mg. of glutethimide in 20 ml. of absolute ethanol and dilute to 100 ml. Stable for 1 month.
- **Working glutethimide solutions (0.5, 2, and 5 mg./100 ml.)** Dilute the stock solution with water, blood, or urine, respectively. These solutions must be freshly prepared.
- **α-Phenyl glutarimide solution (100 mg./100 ml.)** Dissolve 100 mg. of α-phenyl glutarimide (Ciba Pharmaceutical Company) in 100 ml. of absolute ethanol.
- **Developing solution for thin-layer chromatography** Mix 80 ml. of cyclohexane and 20 ml. of absolute ethanol.
- **Spray reagents**
  - **Bleach (sodium hypochlorite)** Dilute 20 ml. of household bleach to 100 ml. with water.
  - **Phenol** Dissolve 5 gm. of phenol in 100 ml. of water.
  - **Starch-iodide** Dissolve 2 gm. of soluble starch and 1 gm. of potassium iodide in 100 ml. of water.

The clinical material was serial blood and urine samples obtained from comatose patients admitted to various Cleveland hospitals fol-
lowing the ingestion of an overdose of glutethimide. In no case could the amount of glutethimide ingested be accurately determined.

**Methods**

**Gas Chromatography**

**Extraction of Glutethimide**

Add 5 ml. of blood or urine to 50 ml. of petroleum ether or chloroform. This mixture is shaken for 2 min. and allowed to separate. The organic solvent is removed and filtered through glass wool. Filter paper must not be used, as interfering substances are extracted from the paper by the solvent. A 40-ml. aliquot of the filtered solvent is transferred to an evaporating dish and evaporated to about 2-3 ml. under a stream of air. The concentrated solvent is then transferred to a 10-ml. graduated centrifuge tube using a Pasteur pipet. The evaporating dish is washed three times with 1- to 2-ml. portions of acetone, and the washings are added to the centrifuge tube. The contents of the centrifuge tube are then slowly evaporated to 0.5 ml. with a fine jet of nitrogen. From this solution, 5-µl. aliquots are then gas chromatographed on a 6-ft. glass column containing 90-100 mesh Anakrom AS coated with 3% SE-30. The operating conditions used are: column temperature 200°; injection port temperature 245°; detector cell temperature 260°; helium carrier gas at 3.2/30 lb.; hydrogen at 6.7/20 lb.; air at 8.0/40 lb.; and chart speed 45 in./hr.

After 20 samples have been analyzed the column must be reconditioned overnight.

**Removal of Interfering Substances**

When chloroform is chosen to extract glutethimide from a biologic specimen, the metabolites of glutethimide or any other organic acid that might be present (barbiturate, salicylate) will also be extracted, and thus interfere with the quantitative determination of glutethimide. These interfering substances can be removed by washing the chloroform extract two times with 5 ml. of 0.45 N sodium hydroxide. The washed chloroform is then filtered and the analysis continued as described above.

**Calculation of Glutethimide Concentration**

Each aqueous working standard is analyzed as described above. The resulting disc integration readings are a linear function of the concentration. A graph of this relationship can be used to calculate the glutethimide concentration in test samples. Because of the inherent difficulty of re-establishing exactly the same chromatographic conditions, this graph should be determined each time a series of analyses are performed.
Ultraviolet Spectrophotometry

Occasional blood and urine samples were also analyzed by Goldbaum and Williams' ultraviolet technic (3). These results were obtained so that they could be compared with those obtained by the gas chromatographic technic.

Metabolite Analysis

To isolate the metabolites of glutethimide, large volumes of urine are extracted with chloroform. A 25-ml aliquot of urine is extracted with 100 ml of chloroform. This extracted urine is then discarded, and the same chloroform is used to extract another 25-ml aliquot of the same urine. This process is continued until the entire urine sample is extracted. All the chloroform is transferred to an evaporating dish and concentrated to 2–3 ml. The concentrated chloroform extract is then applied across several thin-layer plates coated with silica gel GF using standard technics. Aliquots of the 50 mg./100 ml. glutethimide solution and the 100 mg./100 ml. α-phenyl glutarimide standard are also applied to each plate as reference compounds. The plates are developed in the cyclohexane-ethanol mixture as described above. They are then removed from the developing tanks, air dried, and viewed with the Mineralite short-wave ultraviolet lamp. If the Rf values of any two spots are within 0.05 of each other, they can be further separated by multiple development in the same system. After each development, the plates are again viewed under the Mineralite lamp until it is apparent that the spots are completely separated. The plates are again viewed under the Mineralite lamp so that each band of spots can be marked. Each of these bands is then scraped off each plate. Similar bands of silica gel from all the plates are pooled and each is eluted with 10 ml. of absolute ethanol. Each of the mixtures is centrifuged. The ethanol is decanted from the silica gel, transferred to an evaporating dish, and concentrated to approximately 3 ml. From these concentrated ethanol extracts 50-μl aliquots are then rechromatographed to determine if each extract yields only one spot. If any aliquot produces more than one spot, the above procedure is repeated. When each extract yields only one spot, it apparently contains only one substance. It is then gas chromatographed to ascertain if the extract contains one or more substances.

Results

Method Evaluation

Aqueous, blood, and urine working solutions containing 0.5, 2.0, and 5.0 mg. of glutethimide per 100 ml. of solution were analyzed according to the gas chromatographic technic described above. Petroleum ether
was the extracting solvent. The average of three extractions of each of these solutions was determined (Table 1).

Aliquots of blood and urine samples obtained from two patients, allegedly intoxicated from glutethimide, were analyzed by the gas chromatographic and ultraviolet technics. A comparison of the results was tabulated (Table 2). Many other blood and urine samples treated in a similar way gave comparable results, but the data have not been included in Table 2 in order to avoid redundancy.

The gas chromatograms obtained from the various extracts of one of the typical urine samples are presented in Fig. 1–3: Fig. 1 is the gas chromatogram obtained when petroleum ether was the extracting solvent; Fig. 2 when chloroform was the extracting solvent; and Fig. 3 when the sample was processed to remove the metabolites by washing the chloroform extract with 0.45 N sodium hydroxide.

Clinical Correlation

Figure 4 shows the change in the respective blood glutethimide concentrations of five patients following their admission to the hospital. The arrows indicate the time and blood concentrations at which each patient regained consciousness. Since the exact time of the ingestion of the drug was not known, admission to the hospital was used as the reference point in time. Figure 5 shows the amount of unmetabolized glutethimide these same five patients excreted in the periods indicated.

Metabolite Study

Figures 6 and 7 show the relative amounts of the metabolites extracted from typical blood and urine samples of one patient. These are compared to the relative amounts of unmetabolized glutethimide. In these figures the concentrations of the substances are expressed as a function of the heights of their peaks resulting from the gas chromatographic analysis. The actual concentrations of the metabolites could not be calculated since standard reference materials are not available to obtain the gas chromatographic peaks corresponding to known concentrations of these products.

A typical thin-layer chromatogram of an aliquot of a chloroform extract of urine showed six distinct spots, A–F (Fig. 8, Columns 2, 7, 12, and 16). The substances corresponding to each of these spots were separated and concentrated using the preparative thin-layer technic described above. Aliquots of these concentrates, of the original chloroform extract of the urine, of glutethimide and of α-phenylglutarimide were thin-layer chromatographed. The results are shown in Fig. 8. The
Table 1. Gas Chromatographic Analysis of Petroleum Ether Extracts of Glutethimide Solutions of Known Concentrations

<table>
<thead>
<tr>
<th>Glutethimide</th>
<th>Sample</th>
<th>Determinations (No.)</th>
<th>Added (mg./100 ml.)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>3</td>
<td>0.5</td>
<td>80 ± 1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>2.0</td>
<td>85 ± 3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>5.0</td>
<td>88 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>3</td>
<td>0.5</td>
<td>80 ± 2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>2.0</td>
<td>85 ± 2.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>5.0</td>
<td>84 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>3</td>
<td>0.5</td>
<td>80 ± 2.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>2.0</td>
<td>80 ± 2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>5.0</td>
<td>88 ± 1.5</td>
</tr>
</tbody>
</table>

Table 2. Results Obtained with Various Extraction Procedures When Applied to Aliquots of Serial Samples

<table>
<thead>
<tr>
<th>Date &amp; time</th>
<th>Gas chromatography</th>
<th>Ultraviolet spectrophotometry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Petroleum ether extract</td>
<td>CHCl₃ extract</td>
</tr>
<tr>
<td></td>
<td>Unwashed</td>
<td>Washed</td>
</tr>
<tr>
<td>BLOOD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8/12 7:05 P.M.</td>
<td>2.8</td>
<td>—</td>
</tr>
<tr>
<td>8/12 9:20 P.M.</td>
<td>2.5</td>
<td>—</td>
</tr>
<tr>
<td>8/12 12:00 M.</td>
<td>2.8</td>
<td>—</td>
</tr>
<tr>
<td>8/13 7:00 A.M.</td>
<td>2.3</td>
<td>—</td>
</tr>
<tr>
<td>Patient 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9/9 A.M.</td>
<td>0.9</td>
<td>1.2</td>
</tr>
<tr>
<td>9/10 A.M.</td>
<td>1.1</td>
<td>1.2</td>
</tr>
<tr>
<td>9/13 (post mortem)</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>URINE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8/14 3:00 P.M.</td>
<td>0.9</td>
<td>1.3</td>
</tr>
<tr>
<td>8/14 5:00 P.M.</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>8/15 6:00 P.M.</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>8/15 2:00 P.M.</td>
<td>0.5</td>
<td>0.7</td>
</tr>
<tr>
<td>8/15 4:00 P.M.</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>Patient 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9/9 0 P.M.–12 P.M.</td>
<td>0.8</td>
<td>—</td>
</tr>
<tr>
<td>9/10 0 P.M.–12 P.M.</td>
<td>1.6</td>
<td>—</td>
</tr>
<tr>
<td>9/11 0 P.M.–12 P.M.</td>
<td>1.4</td>
<td>—</td>
</tr>
<tr>
<td>9/12 0 P.M.–12 P.M.</td>
<td>0.8</td>
<td>—</td>
</tr>
</tbody>
</table>

All values are given in milligrams per 100 ml.
Fig. 1. Gas chromatogram of petroleum ether extract of urine sample obtained 60 hr. after admission to hospital. One substance (retention distance 4.7–5.2 in.) in addition to glutethimide (retention distance 3.6–4.2 in.) is extracted.

Fig. 2. Gas chromatogram of chloroform extract of same urine as in Fig. 1. Six substances besides glutethimide are extracted. Retention distances are: VI, 1.8–2.3 in.; I, 2.9–3.3 in.; II, 3.5–4.1 in.; III, 5.9–6.8 in.; V, 8.3–10.0 in.; IV, 6.9–7.9 in.

Fig. 3. Gas chromatogram of chloroform extract of same urine as in Fig. 1 and 2, washed with 0.45 N NaOH. Washing procedure removed 80–90% of the metabolites.
R, value of glutethimide was 0.53. The R, value of α-phenylglutarimide was 0.38. The urine concentrate gave six spots whose R, values were: A, 0.05; B, 0.13; C, 0.30; D, 0.37; E, 0.42; and F, 0.52. Gas chromatograms of aliquots of these concentrates were also obtained and are presented in Fig. 9 and 10.

Fig. 4. Serial blood glutethimide concentrations in 5 patients (F, H, K, V, W) comatose secondary to acute glutethimide intoxication. Arrows indicate blood concentration at which patients regained consciousness.

Fig. 5. Total unmetabolized glutethimide excreted in urine over the indicated period of time in the same 5 patients (F, H, K, V, W) as in Fig. 4.
Fig. 6 (left) and 7 (right). Relative concentrations of metabolites extracted from blood and urine samples of 3 patients, compared to relative concentrations of unmetabolized glutethimide. Concentrations of substances are expressed as a function of their peak heights as elaborated on gas chromatography.

Discussion

Method

The gas chromatographic method of analysis recovers 80–90% of the glutethimide present. This is adequate for clinical purposes.

Petroleum ether extracts only glutethimide and a small amount of one of its metabolites. This metabolite has a retention distance of 4.7–5.2 in. and, therefore, does not interfere with the quantitative gas chromatographic determination of glutethimide whose retention distance is 3.5–4.1 in. (Fig. 1). Since the amount of metabolite extracted is so small, it also does not add significantly to the glutethimide concentration when the ultraviolet technic is used. Therefore, when petroleum ether is the extracting solvent, comparable quantitative results are obtained using either the gas chromatographic or the ultraviolet method of analysis (Table 2).

Chloroform extracts both glutethimide and its metabolites. In the ultraviolet procedure these metabolites react in a similar manner, as
Fig. 8. Thin-layer chromatogram of substances isolated from urinary chloroform extracts compared with α-phenylglutarimide and the original chloroform extract of the urinary glutethimide. Rf values are: glutethimide (Doriden), 0.53; α-phenylglutarimide (αPG) 0.38; A, 0.05; B, 0.13; C, 0.30; D, 0.37; E, 0.42; F, 0.52. These values are an average of Rf values obtained from several thin-layer plates.
does glutethimide (3). Therefore, high results are obtained when chloroform extracts are analyzed by the ultraviolet technic (Table 2).

Washing this chloroform with alkali reduces the apparent glutethimide concentration by 80-90%. This value still is higher than the

corresponding result obtained by gas chromatography because gas chromatography permits the separate determination of glutethimide and each of the metabolites. When this unwashed chloroform extract is analyzed by the gas chromatographic technic, the two major metabolites interfere because their retention distances are fairly close to that of glutethimide, i.e., 2.9-3.3 in. and 3.5-4.1 in., respectively. When these metabolites are present in high concentration, there is incomplete separation of the three compounds (Fig. 2). The concentration of glutethimide must then be obtained by extrapolation; this limits the accuracy of the results.

Petroleum ether is preferable as the extracting solvent because it
does not extract the metabolites. The petroleum ether extract may then be analyzed by either gas chromatography or ultraviolet spectrophotometry. The gas chromatographic, as well as the recently improved ultraviolet photometric (4), technic both require 1½–2 hr. Since both procedures can be performed with relatively equal facility and both require unusual equipment, the method of choice for a given laboratory is an individual decision dependent upon the local conditions.

Whereas petroleum ether extract is preferable for clinical problems, when a chloroform extraction must be used, information regarding the metabolism of glutethimide is sought.

Clinical Correlation

All but one of the patients included in this study were comatose when admitted to the hospital. Two of the patients were severely intoxicated. One of these two patients required hemodialysis approximately 12 hr. after admission because of acute renal failure. The second was given bemegride because of the depth of his coma and his unresponsiveness to supportive therapy. The remaining patients were mildly intoxicated and were treated symptomatically. One patient regained consciousness when his blood glutethimide concentration became 1.8 mg./100 ml. (Fig. 4). The remaining patients regained consciousness when their blood concentrations were 1.0 mg./100 ml. or less. Those patients who were comatose regained consciousness within 32–61 hr. after their admission to the hospital. Most of the patients excreted the major portion of the unmetabolized drug within the first 60 hr. after admission (Fig. 5), but two patients continued to excrete small amounts for as long as 85 hr.

The correlation between the patients' clinical conditions and blood glutethimide concentrations observed in this study corresponded with those reported by other investigators (13–19). Mildly intoxicated patients whose blood glutethimide concentrations were less than 1.0 mg./100 ml. usually had normal blood pressures, deep tendon reflexes, and could be easily aroused by painful stimuli. Moderately intoxicated patients had blood glutethimide concentrations of 1.0–3.0 mg./100 ml. They manifested hypotension, shallow or abdominal breathing, variable deep tendon reflexes, and some plantar withdrawal. Severely intoxicated patients manifested hypotension, respiratory difficulty, areflexia, deep coma, and an absence of plantar withdrawal and pain response. Blood glutethimide concentrations were usually greater than 3.0 mg./100 ml.

Metabolite Study

A considerable number of animal studies on the absorption, distribution, and excretion of glutethimide, as well as on the metabolism and
the structure of the metabolites, have been done (20–26). The patients in this study, unlike those studied by Keberle and associates (22–24), had all ingested an overdose of glutethimide. Analyses of samples of their bloods and urines gave similar gas chromatograms (Fig. 2 and 11). High concentrations of the metabolites appeared 16 or more hours after admission to the hospital (Fig. 11). Prior to that, approximately 90% of the substances extracted was glutethimide (Fig. 2).

Peaks I and II constituted 80–90% of the metabolites. Peak VI was presumed to be a normal constituent of urine, since this peak was found in extracts of urines obtained from persons known not to have ingested glutethimide.

Gas chromatograms obtained from the chloroform extracts of blood and urine, although similar, did show the following variations: The metabolites were found earlier in the blood than in the urine samples and were never present in as high a concentration as in the urine samples. Peak IV did not appear in extracts of urine samples until several hours after the patients’ admissions to the hospital and was present in very small quantities. This peak, however, never appeared in extracts of blood samples. It was initially thought that perhaps this was also a normal constituent of urine. However, this peak did not appear in extracts of the control urines.

Although the metabolites were apparently separated by the preparative thin-layer chromatographic procedure, four of the six “purified” substances contained more than one peak when gas chromatographed.

From the thin-layer and gas chromatographic data it appeared that
Spot F was glutethimide. \( \alpha \)-phenylglutarimide had an \( R_f \) value of 0.38. This was close to the \( R_f \) values of Compounds D and E which were 0.37 and 0.42, respectively. All the \( R_f \) values reported, however, were average figures obtained from several thin-layer plates. There was much overlapping of the \( R_f \) values of these three compounds. When gas chromatographed, Compound D had three peaks with retention distances of 3.1–3.6 in. (major peak), 4.8–5.2 in., and 6.3–7.2 in. Compound E also had three peaks with retention distances of 3.7–4.2 in., 4.4–5.2 in., and 5.9–7.7 in. \( \alpha \)-Phenylglutarimide had a retention distance of 3.1 in. These data indicate Compound D was probably \( \alpha \)-phenylglutarimide. The chemical structure of the other metabolites was not elaborated because the necessary infrared spectrophotometric and mass spectrometric equipment was not available.

References


