Clinical Applications of Gas Chromatograph/Mass Spectrometer/Computer Systems

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Gas chromatograph/mass spectrometer/computer systems can be used to quantify a wide variety of compounds of clinical interest. A quadrupole instrument operated in the chemical ionization (CI) mode was used in these studies. Because of the sensitivity and specificity of selective ion detection, it is possible to make measurements routinely in the nanogram to picogram range, with 0.1–1.0 ml samples of plasma and 1–5 ml samples of urine. Internal standards, preferably stable-isotope-labeled compounds, were added to the biological samples before isolation was begun. We describe clinical applications of these procedures to problems in toxicology, pharmacokinetics, and perinatal pharmacology.

Gas chromatograph/mass spectrometer/computer (GC-MS-COM) analytical systems can be used to investigate a variety of clinical problems. At the present time such procedures are used in our laboratory to monitor the concentrations in blood of several anticonvulsant drugs, including mephobarbital, phenobarbital, diphenhydantoin, methsuximide, ethosuximide, and primidone (1). Similar analytical systems have been used in other laboratories to monitor tricyclic antidepressants (2), antiarrhythmic drugs (3), prostaglandins (4, 5), and oral contraceptives (6, 7) in blood.

GC-MS-COM techniques are also applicable to other clinical areas. They have been used extensively in our laboratory to study problems in perinatal pharmacology such as the transfer of drug from the mother’s circulation to the fetus and to amniotic fluid and breast milk. These techniques have been used in toxicological studies of overdose patients and of accidental poisoning. Procedures for screening body fluids obtained from overdose patients with GC-MS-COM systems have been described by Law et al. (8) and Costello et al. (9). Pharmacokinetics and pharmacogenetics are other areas of increasing clinical interest where these methods are useful. Although these analytical systems are costly, the systems have high sensitivity and specificity of detection, and several drugs and drug metabolites can be quantified in a single analysis. Because many patients receive multiple drug therapy, it is a distinct advantage to be able to monitor all of the drugs involved.

In this paper, some recent quantitative studies in perinatal pharmacology, pharmacokinetics, and pediatric toxicology are described. Since internal standards, preferably drugs labeled with stable isotopes, are needed in these investigations, some of the problems of quantification and selection of internal standards are reviewed.

Materials and Methods

Caffeine (1-trideuteromethyltheobromine), [2,4,5-13C]-diphenylhydantoin, [2,4,5-13C]-phenobarbital, and [2,4,5-13C]-pentobarbital were obtained from Dr. Monroe Wall of the Research Triangle Institute (Research Triangle, N. C.) through a program sponsored by the National Institute of General Medical Sciences. [1-C2H4]diazepam was generously provided by Hoffmann-La Roche, Nutley, N. J. 07110. Ethosuximide, methsuximide, and phensuximide were obtained from Dr. A. J. Glazko, Parke, Davis and Co., Ann Arbor, Mich. 48106. Silylating reagents were obtained in 1-g ampules from Pierce Chemical Co., Rockford, Ill. 61105. Diazomethane was prepared from "Diazald" and diazoethane was prepared from N-ethyl-N'-nitro-N-nitrosoaniline (Aldrich Chemical Co., Inc., Milwaukee, Wis. 53233). "Nanograde" solvents were obtained from Mallinckrodt Chemical Works, St. Louis, Mo. 63160.

The column packings, 1% SE-30 on 100/120 Gas
Chrom Q and 5% SE-30 on 80/100 Gas Chrom Q, were obtained from Applied Science Laboratories, Inc., State College, Pa. 16801.

Apparatus

Quantitative analyses were based on selective ion detection and done with a Finnigan 1015-PDP 8/1 system operated in the chemical ionization mode (10). The columns used were 270 cm × 2 mm glass coil columns packed with 1% SE-30 (80/100 mesh).

Procedures

The nonacidic drugs and drug metabolites were isolated from plasma, urine, breast milk, and amniotic fluid by using ammonium carbonate/ethyl acetate as a salt/solvent pair (11). The internal standard, usually the drug labeled with a stable isotope, was added to the sample before extraction. For analysis by GC-MS it was necessary to convert most of the drugs and drug metabolites to derivatives. Methylated, ethylated and silylated derivatives were prepared as described (1). The isolation procedure takes 20 min; derivatization may require 30–60 min, and the instrumental analysis time is 10–15 min.

Results and Discussion

For quantitative analysis by GC-MS-COM systems, suitable internal standards are required. Stable-isotope-labeled internal standards are preferred, and several carbon-13 and deuterium labeled compounds are available. When labeled standards were not available, analogs or homologs were used. For example, 2,2,3-trimethylsuccinimide was used as an internal standard for 2-ethyl-2-methylsuccinimide (ethosuximide), and N-methyl-2-phenylsuccinimide was used as the internal standard for N,2-dimethyl-2-phenylsuccinimide (methysuximide) (1).

An N-C<sub>2</sub>H<sub>5</sub>-labeled drug can be used as the internal standard for drugs and their demethylated metabolites, if ethylated rather than methylated derivatives are prepared (1). In our studies, N-C<sub>2</sub>H<sub>5</sub>-caffeine was used as the internal standard for caffeine and for the demethylated metabolites, 1,3-, 3,7-, and 1,7-dimethylxanthines. An analysis of human amniotic fluid is shown in Figure 1 in which N-C<sub>2</sub>H<sub>5</sub>-caffeine was used to quantify caffeine and the ethylated derivative of the metabolite theobromine (1-ethyl-3,7-dimethylxanthine).

When compounds other than stable-isotope-labeled analogs are used as internal standards, it is necessary to determine a response factor. The response factor is defined as the ratio of the peak area per microgram of drug divided by the peak area per microgram of internal standard, and must be determined for each analytical system used. The response factors were constant over the range of concentrations measured. The response factor determined with the Finnigan 1015-PDP 8/1 system for 2-ethyl-2-methylsuccinimide (ethosuximide), with 2,2,3-trimethylsuccinimide as the internal standard, was 1; the response factor for ethylated theobromine, with N-C<sub>2</sub>H<sub>5</sub>-caffeine as the standard, was 0.84. When ethylated [2,4,5-<sup>13</sup>C]phenobarbital (N,N-diethylphenobarbital) was used as the standard for ethylated meprobamate (N-ethyl-N-methylphenobarbital) the response factor was 1.55.

Quantification of hydroxylated metabolites is a more troublesome problem, because many of these metabolites are not available as pure reference standards and they are frequently difficult to synthesize. Thus it would not be possible to obtain a calibration curve or a response factor. Under these circumstances, a hydrocarbon or drug with suitable gas-chromatographic properties can be added as an internal standard. Quantification is then based on an area relationship, assuming a response factor of 1. The area relationship of the metabolite to the internal standard should be linear over the range of concentrations measured. In Figure 2, three monohydroxyl me-

![Fig. 1. Analysis of an ammonium carbonate/ethyl acetate extract of 1 ml of amniotic fluid, by selective ion detection, with use of a Finnigan 1015-PDP 8/1 system. The ions at m/e 194–196 (caffeine), 197–199 (N-C<sub>2</sub>H<sub>5</sub>-caffeine) and 208–210 (1-ethyltheobromine) were monitored.](image1)

![Fig. 2. Analysis of an ammonium carbonate/ethyl acetate extract of 1 ml of plasma by selective ion detection. The ions at m/e 244 [MH<sup>+</sup>], 2-ethyl-3-hydroxy-2-methylsuccinimide, 3-OH, and 2-(1-hydroxyethyl)-2-methylsuccinimide, 1-HO-ETHYL and 258 (MH<sup>+</sup>, [2,4,5-<sup>13</sup>C]-N,N-dimethylpentobarbital, 13C-PENTO) were monitored.](image2)
Table 1. Urinary Excretion of Ethosuximide and Hydroxyethosuximide

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time of collection</th>
<th>Ethosuximide, mg/liter</th>
<th>Hydroxyethosuximide, mg/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0900 h(^b)</td>
<td>13.16</td>
<td>5.3</td>
</tr>
<tr>
<td>2</td>
<td>1118 h</td>
<td>23.91</td>
<td>3.9</td>
</tr>
<tr>
<td>3</td>
<td>1300 h</td>
<td>28.0</td>
<td>4.3</td>
</tr>
<tr>
<td>4</td>
<td>1500 h</td>
<td>21.2</td>
<td>3.4</td>
</tr>
<tr>
<td>5</td>
<td>1700 h</td>
<td>21.7</td>
<td>5.4</td>
</tr>
<tr>
<td>6</td>
<td>2010 h(^c)</td>
<td>23.1</td>
<td>2.3</td>
</tr>
</tbody>
</table>

\(^a\)Sum of three unconjugated monohydroxyethosuximides; calculations are based on an assumed response factor of 1.
\(^b\)Before morning dose of 250 mg.
\(^c\)After evening dose of 250 mg.

tabolites of ethosuximide (2-ethyl-3-hydroxy-2-methylsuccinimide and two diastereoisomeric 2-(1-hydroxyethyl)-2-methyl-succinimides) were measured as the methyltrimethylsilyl derivatives, with [2,4,5-\(^{13}\)C]pentobarbital (N,N-dimethyl derivative) as the internal standard. [2,4,5-\(^{13}\)C]Pentobarbital rather than the unlabeled drug was used because many patients receive pentobarbital during treatment. The three metabolites have the same molecular ion (MH\(^+\) = 244) and the peak areas were integrated and summed by the computer. Table 1 lists some results obtained when quantification was done in this manner, and also the concentrations of ethosuximide in plasma, which were determined in a separate analysis (1). The concentration of the three metabolites together varied from 2-5 mg/liter. The patient had received ethosuximide for only a few days and the plasma concentrations of the drug (13 to 28 mg/liter) were still below the therapeutic concentration. It is interesting that the concentration of unconjugated hydroxylated metabolites (2-5 mg/liter) of ethosuximide is considerably higher than the reported concentrations of the hydroxylated metabolite of diphenylhydantoin (0.01-0.03 mg/liter) in plasma (12).

These results represent only the ratio of the areas of metabolites to the standard. However, the area relationship can be converted to mg/liter subsequently by multiplying by a constant (k), the response factor, which can be determined whenever the metabolites become available as pure compounds. Because many biological samples are unique and can never be replaced, it is better to measure them in this fashion than to discard the sample.

At present, these analytical techniques are probably more useful in clinical research than in routine clinical analysis. The methods are particularly valuable in the area of perinatal pharmacology because of the small size of the biological samples that are available for analysis. In our study of the total exposure of the fetus/neonate to pharmacologically active agents, we found that most drugs ingested by the gravid female were transferred across the placenta to the fetus (13). As a result, the urinary drug profiles of the mother and her newborn infant were qualitatively similar; however, there were quantitative differences, as illustrated in Figures 3 and 4. The mother who received 250 mg of primidone four times daily, and her newborn infant were both excreting primidone and primidone metabolites, including phenobarbital, hydroxyphenobartil, phenethylmalonamide, and hydroxyprimidone. Comparable quantities of primidone and phenethylmalonamide were present in the maternal urine, while phenethylmalonamide was the major metabolite in the infant’s urine.
Samples of the cord blood, breast milk, and maternal and neonatal plasma were also obtained from this mother/infant pair. Phenobarbital was measured in all of the samples; the results are listed in Table 2. From the analysis of maternal plasma collected 31 days before delivery and of cord blood, it is clear that the fetus was exposed to the drug and some of the metabolites in utero.

Because drugs are transferred from the mother’s circulation to the fetus, they should be excreted into amniotic fluid by the fetus. In a related study, samples of amniotic fluid, collected before delivery and before administration of drugs during labor, were analyzed for the presence of drugs and their metabolites. Caffeine and theobromine were identified in all of the samples (see Figure 1). Paired samples of blood and amniotic fluid were obtained from several patients, and concentrations of caffeine and theobromine in amniotic fluid and the mother’s plasma were compared. It was found that the concentration of caffeine in the mother’s plasma was usually higher (0.4–5.6 mg/liter) than the concentration in amniotic fluid (0.2–1.6 mg/liter). Because the fetus swallows 500 ml of amniotic fluid daily during the last trimester, the ingestion of caffeine by the fetus may be several milligrams per day, depending on the concentration of caffeine in the mother’s plasma.

When the transfer of drugs from the maternal circulation to breast milk was investigated, it was found that all of the drugs ingested by the mother were transferred to breast milk. Table 3 lists the drugs identified and quantified by GC-MS-COM procedures in our laboratory. Because the neonate consumes 600–1000 ml of milk daily, the amount of drug ingested (for example, phenobarbital, diphenylhydantoin, primidone, and caffeine) may reach amounts that are therapeutic for the infant. From the quantitative analysis of the amniotic fluid and breast milk, it is evident that the fetus/neonate may ingest surprisingly large quantities of drugs. If the quantities ingested are calculated on a milligram per kilogram body weight basis, the neonate frequently receives an adult dose.

The half-lives of drugs in breast milk and plasma were compared. The samples (2–5 ml) were obtained from mothers who were no longer nursing their infants. Figure 5 shows a comparison of the analyses of plasma and breast milk after ingestion of a single 100-mg dose of diphenylhydantoin. Although the concentration of the drug was lower in breast milk than plasma, the half-life of diphenylhydantoin was longer (7 h, 20 min) in breast milk than in plasma (5 h, 20 min). The respective half-lives calculated by the use of regression analysis were 7 h, 36 min, and 5 h, 19 min.

GC-MS-COM methods are particularly useful in pharmacokinetic studies because of the specificity of detection. The original drug can be distinguished readily from its metabolites, and as a result the measurements represent the concentration of the parent drug, not the combined concentrations of one or more compounds having the same spectrophotometric properties. But the concentration of the individual metabolites can also be measured, as exemplified in Figure 6. After ingestion of mephabarbitol, the plasma concentrations of mephabarbitol and its metabolite, phenobarbital, were followed in two individuals and half-lives of mephabarbitol determined. In the subject for whom the half-life was shorter (34 h) the concentration of phenobarbital in plasma became constant between 48 and 72 h. In the subject for

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### Table 2. Concentration of Phenobarbital in Plasma, Urine, and Breast Milk after Ingestion of Primidone

<table>
<thead>
<tr>
<th>Sample</th>
<th>Days after delivery</th>
<th>Phenobarbital, mg/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma (M)</td>
<td>(31 days before birth)</td>
<td>6.5</td>
</tr>
<tr>
<td>Urine (M)</td>
<td>at delivery</td>
<td>13.9</td>
</tr>
<tr>
<td>Urine (B)</td>
<td>1</td>
<td>6.0</td>
</tr>
<tr>
<td>Cord Blood (M)</td>
<td>at delivery</td>
<td>10.5</td>
</tr>
<tr>
<td>Plasma (M)</td>
<td>5</td>
<td>9.8</td>
</tr>
<tr>
<td>Plasma (B)</td>
<td>5</td>
<td>3.4</td>
</tr>
<tr>
<td>Colostrum</td>
<td>6</td>
<td>9.2</td>
</tr>
<tr>
<td>Breast milk</td>
<td>47</td>
<td>9.0</td>
</tr>
<tr>
<td>Breast milk</td>
<td>116</td>
<td>6.3</td>
</tr>
</tbody>
</table>

*250 mg, four times daily.  
*M, mother; B, baby.

### Table 3. Drugs Identified in Human Breast Milk and Colostrum by GC–MS–COM Procedures

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration, mg/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenobarbital</td>
<td>2.7*</td>
</tr>
<tr>
<td>Pentobarbital</td>
<td>0.17*</td>
</tr>
<tr>
<td>Butobarbital</td>
<td>0.37*</td>
</tr>
<tr>
<td>Diazepam</td>
<td>0.10*</td>
</tr>
<tr>
<td>Diphenylhydantoin</td>
<td>1.5–4.5*</td>
</tr>
<tr>
<td>Caffeine</td>
<td>3.2–8.6*</td>
</tr>
<tr>
<td>Theobromine</td>
<td>0.24–1.6*</td>
</tr>
<tr>
<td>Primidone</td>
<td>4.6–10.5*</td>
</tr>
<tr>
<td>Phenyethylmalondiamide</td>
<td>1.3–2.4*</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>+</td>
</tr>
<tr>
<td>Salicyluric acid</td>
<td>+</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>+</td>
</tr>
<tr>
<td>Ethosuximide</td>
<td>+</td>
</tr>
<tr>
<td>Codeine</td>
<td>+</td>
</tr>
<tr>
<td>Methadone</td>
<td>+</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>+</td>
</tr>
<tr>
<td>Secobarbital</td>
<td>+</td>
</tr>
</tbody>
</table>

* [2,4,5-13C1] Phenobarbital added as internal standard.  
[1-C'H3] Valium added as internal standard.  
[2,4,5-13C1] Diphenylhydantoin added as internal standard.  
[1-C'H3] Caffeine added as internal standard.  
Drug present but not quantified.
whom the half-life was longer, it took more than 96 h for the phenobarbital concentration to become constant. The dose ingested by the two subjects was not significantly different when calculated on a milligram per kilogram body weight basis. The concentrations of free and conjugated hydroxyphenobarbital are also being determined for these samples in an attempt to explain the plateauing of the phenobarbital values. All of these measurements can be made on 1–2 ml of plasma.

GC-MS-COM methods have also been used in toxicological studies. A very effective GC-MS-COM system has been developed by Costello et al. (9) for rapidly identifying drugs in urine, blood, and gastric juice obtained from overdose patients. One or more toxic agents were identified in 75% of the 600 cases studied.

In our studies, adverse reactions to drugs—including withdrawal symptoms—have been investigated in infants. Neonates frequently exhibit withdrawal symptoms shortly after birth if their mothers have chronically ingested barbiturates, narcotics, or tranquilizers during the gestational period. An infant born to a mother who received mephobarbital (700–1000 mg/day) for control of seizures exhibited characteristic withdrawal symptoms 2 h after birth; the symptoms persisted for six months. An untimed urine sample was obtained from the mother at delivery and timed urine samples were collected from the infant for 22 days. The infant was not breast fed. When GC methods were used, it was possible to follow the excretion of phenobarbital for only eight days and mephobarbital was only detected on day 1. When selective ion detection was used, mephobarbital was identified and quantified in urines collected through day 8 and phenobarbital was identified and quantified in urines collected through day 22 (Table 4). The excretion of phenobarbital could have been followed even longer if samples had been available, because the limit of detection of the analytical system had not been reached.

The time of each urine collection was recorded, so it was possible to calculate the half-life of mephobarbital and phenobarbital from the rate of urinary excretion.

<table>
<thead>
<tr>
<th>Postnatal days</th>
<th>Mephobarbital</th>
<th>Phenobarbital</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.54</td>
<td>13.15</td>
</tr>
<tr>
<td>5–6</td>
<td>0.31</td>
<td>6.83</td>
</tr>
<tr>
<td>8</td>
<td>0.29</td>
<td>4.78</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>3.22</td>
</tr>
<tr>
<td>14–15</td>
<td>0</td>
<td>0.98</td>
</tr>
<tr>
<td>22</td>
<td>0</td>
<td>0.13</td>
</tr>
</tbody>
</table>

![Graph showing plasma and breast milk concentration over time](image)

Fig. 5. Comparison of the analyses of ammonium carbonate/ethyl acetate extracts of 200-μl samples of plasma and 500-μl samples of breast milk by selective ion detection

The ions at 267 (M⁺, diphenylhydantoin) and 270 (M⁺, [2,4,5-¹³C]diphenylhydantoin) were monitored. The results were plotted on semi-log paper and the t₁/₂ values read from the graph.

![Graph showing urinary excretion of mephobarbital and phenobarbital](image)

Fig. 6. Comparison of the analysis (by selective ion detection) of ammonium carbonate/ethyl acetate extracts of 200-μl samples of plasma from two subjects

The ions at 275 (M⁺, N-ethyl-N'-methylphenobarbital), 289 (N,N-diethylphenobarbital), and 292 ([2,4,5-¹³C]-N,N-diethylphenobarbital) were monitored. The half-lives were determined as described in Figure 5. When regression analysis was used, the half-lives were 46.5 and 37.6 h.

Table 4. Urinary Excretion of Mephobarbital and Phenobarbital by the Neonate
cretion of the two drugs. The half-life of mephobarbi-
tal was 30 h (days 1–5) and the half-life of phenobarb-
itral was 48 h (days 8–14) during the log-linear phase. The excretion rate for phenobarbital decreased between days 14 and 22, resulting in an increased half-life, indicating that trace amounts of phenobarbital persisted in neonatal tissues for several
weeks after the last urine sample was collected on
day 22. In a study involving administration of pheno-
obarbital to a premature infant, it was possible to
identify and quantify the drug in neonatal urine 60
days after ingestion of the last dose.

From the examples that have been described, it is
evident that these analytical systems can be used ef-
effectively in many areas of clinical research. In addi-
tion to specificity and sensitivity of detection, GC-
MS-COM systems can be used to monitor any drug
or compound of biological interest without changing
the analytical system as long as (a) the compound is
or can be made volatile and (b) the molecular weight
of the compound lies within the mass range of the
mass spectrometer.

The chemical-ionization mode is preferred to the
electron-impact mode because very little fragmenta-
tion is observed in CI spectra. As a result, the proba-
bility that fragment ions from other compounds in
the biological sample will contribute to the intensity
of the ion(s) being monitored is markedly reduced.
The GC-MS-COM system we used is operated only
in the CI mode without a separator and at 1 Torr.
This type of analytical system is easier to operate and
maintain than are magnetic sector instruments oper-
ated in the electron-impact mode.

In our experience the precision of these methods is
2–6%; under the most favorable circumstances (for
example, using [2,4,5-13C]phenobarbital in the mea-
surement of phenobarbital in biological samples) the
precision is 1–2%. The accuracy is of the same magni-
tude but varies with specific applications.

Although it would be difficult to introduce GC-
MS-COM systems into most hospital clinical chemis-
try laboratories in their present configuration, they
are being used in a few of these laboratories currently
monitoring concentrations of drugs in the blood.
Their use would undoubtedly increase if their opera-
tion were automated.

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