Therapeutic Monitoring of Anticonvulsant Drugs: Gas-Chromatographic Simultaneous Determination of Primidone, Phenylethylmalonamide, Carbamazepine, and Diphenylhydantoin

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We describe a sensitive and precise gas-chromatographic method in which benzylmalonate methylester monoamide is used as the internal standard for the simultaneous determination of primidone, phenylethylmalonamide, carbamazepine, and diphenylhydantoin. The trimethylsilyl derivatives of the anticonvulsants are well separated from each other and from normal serum constituents. The lower limit of detection for each drug is 0.5 mg/liter when 1 ml of serum is analyzed. Within-run precision (CV), established by analysis of 10 replicates, was as follows: primidone (5.4 mg/liter), 2.6%; phenylethylmalonamide (5.5 mg/liter), 1.6%; diphenylhydantoin (6.6 mg/liter), 3.8%; and carbamazepine (10.4 mg/liter), 3.2%. Fifty specimens were analyzed for primidone and 35 for diphenylhydantoin by a standard gas-chromatographic method involving on-column methylation and by the procedure we have developed. The mean value observed for primidone with the on-column alkylation procedure was 9.3 mg/liter and with our procedure was 9.6 mg/liter. When values for our assay were regressed against values for the standard method, the slope of the least-squares line was 0.936, the intercept was 1.00 mg/liter, and r was 0.939. The mean values observed for diphenylhydantoin by on-column methylation and with our procedure were both 12.6 mg/liter. When values for our assay were regressed against the standard method, the slope of the least-squares line was 0.944, the intercept was 0.3 mg/liter, and r was 0.988.

Additional Keyphrases: toxicology • management of seizures • intermethod comparison

Primidone, a drug utilized widely in the management of seizures, is partially metabolized to phenobarbital and phenylethylmalonamide. Because both primidone and phenobarbital possess anticonvulsant activity (1), it is desirable to measure the concentrations of both compounds in serum after therapy with primidone. It remains to be determined to what extent phenylethylmalonamide contributes to the anticonvulsant activity of primidone in man.

Baumel et al. (1) first described a gas-chromatographic procedure for determining primidone and phenylethylmalonamide as their trimethylsilyl derivatives. However, they did not use an internal standard in their assay, and they estimated the amounts of these drugs by measuring absolute peak heights. Moreover, single chloroform extractions of plasma were used, which also extract extraneous substances that interfere with gas-chromatographic measurement of the compounds of interest. Subsequently, these investigators utilized 5-(p-methylphenyl)-5-phenylhydantoin (MPPH) as an internal standard, and reported the day-to-day variation in the slope of the standard curve as <5% (2).

Kananen et al. (3) described a procedure for determining various barbiturates and diphenylhydantoin, based on extraction of serum with toluene and subsequent on-column methylation with trimethylammonium hydroxide. Although this procedure can be used to quantitate primidone, the efficiency of extraction of this compound is only about 20% compared to that of diphenylhydantoin, and the procedure is not applicable to the determination of phenylethylmalonamide, because this compound is not extracted from serum.

Gas–liquid chromatographic methods for determination of carbamazepine, an anticonvulsant drug increasingly used to treat seizure disorders, have been described, in which various techniques are used, including analysis of the free compound (4, 5), measurement of iminostilbene, the decomposition product (6), and quantitation as the trimethylsilyl derivative (7) or after derivatization with dimethylformamide dimethylecatal (8).

Here, we describe a gas-chromatographic method for the simultaneous determination of trimethylsilyl derivatives of primidone, phenylethylmalonamide (PEMA), diphenylhydantoin, and carbamazepine. A single plasma extract constitutes the sample and a new internal standard, benzylmalonate methylester

\[1 \text{Nonstandard abbreviations used: MPPH, 5-(p-methylphenyl)-5-phenylhydantoin; PEMA, phenylethylmalonamide; and BMMA, benzylmalonate methylester monoamide.} \]
monamide (BMMA) is used. This sensitive, specific, and precise method has been employed routinely in our Therapeutic Drug Monitoring Laboratory for nine months.

**Materials and Methods**

**Reagents and Comparison Materials**

- Methanol and dichloromethane (“Chromatoquality”; Matheson, Coleman and Bell, East Rutherford, N.J. 07073).
- Regis Silylation Mix (Regis Chemical Co., Morton Grove, Ill. 60053).
- Primidone and phenylethylmalonamide (PEMA) (Ayerst Laboratories, Montreal 101, P.Q., Canada).
- Diphenhydantoin (Sigma Chemical Co., St. Louis, Mo. 63178).
- Diethyl benzylmalonate and diethyl(2-phenylethyl)malonate (Aldrich Chemical Co., Milwaukee, Wis. 53233).
- Diethyl phenylomalonate (Pfaltz and Bauer Inc., Flushing, N.Y. 11368).

**Instrumentation and Instrumental Conditions**

We used a Hewlett Packard 5710A Gas Chromatograph equipped with dual flame-ionization detectors and a 1-mV Model 7123A recorder. The glass columns were 122 cm × 2 mm i.d., configuration 5 for on-column injection (Hewlett Packard), packed with 3% OV-17 on 100/120 Gas Chrom Q (Applied Science Lab.). Septa were type HT-9 (Applied Science Lab.) high temperature, low bleed. The instrumental conditions were: Injector port temperature, 250 °C; detector temperature, 300 °C; oven temperature, programmed 150–260 °C at 16 °C/min; gas flow rates: nitrogen, 40 ml/min, air, 250 ml/min, and hydrogen 40 ml/min; and recorder chart speed, 13 mm/min.

**Syntheses**

*Benzyllmalonate methylester monoamide.* This was synthesized according to the procedure of Russell (9) for preparation of monosubstituted malonamides.

Diethyl benzylmalonate (10 g) was dissolved in anhydrous methanol (100 ml) containing sodium methyolate (233 mg). The solution was cooled to 0 °C and gaseous ammonia was bubbled through it for 10 min. The mixture was kept in a stoppered flask at room temperature for 6 h, then the solvent was removed under reduced pressure. The product, methyl benzylmalonate monoamide, was recrystallized twice from methanol by gradual addition of water until crystallization commenced. The yield from this synthesis averaged 85% (for four such preparations). The course of the reaction was monitored by periodic gas-chromatographic analysis of samples of the reaction mixture on 3% OV-17 with temperature programming from 140 to 250 °C at 8 °C/min. Within 30 min after adding ammonia, we saw a new gas-chromatographic peak (intermediate 1) with a retention time greater than that of the starting material. As the reaction progressed, the magnitude of this peak increased while that of the dimethyl benzylmalonate decreased, and soon a new peak appeared (intermediate 2) with a subsequent decrease of intermediate 1. Intermediates 1 and 2 were presumed to be benzylmalonate methylester monoamide and benzylmalonamide.

The reaction product at 6 h was isolated, recrystallized as described previously, and analyzed both as the free compound and the trimethylsilyl derivative by gas chromatography/mass spectrometry (by Shrader Analytical and Consulting Laboratories, Inc.). Using electron-impact ionization, a molecular ion of 207 was observed for the free compound and 279 for the silylated derivative, data consistent with benzylmalonate methylester monoamide and its trimethylsilyl derivative. Intermediate 2 was not further characterized but from the synthetic procedure of Russell (9) it was presumed to be benzylmalonamide.

*Monosubstituted malonamides.* From the starting materials diethylbenzylmalonate, diethyl (2-phenylethyl) malonate, and diethyl phenylomalonate, the corresponding diamides—benzylmalonamide, phenethylmalonamide, and phenylomalonamide—were prepared according to the method of Russell (9). Conditions were the same as described above except for the reaction time, which was 24 h at 25 °C.

**Procedure for Extraction and Silylation of Anticonvulsants**

Serum, 1 ml, was combined in a 16 × 125 mm test tube (Teflon-lined screw cap) with 1 ml of phosphate buffer (1 mol/liter, pH 6.8) and 10 ml of dichloromethane containing 15 μg of the internal standard, BMMA. The tubes were shaken for 10 min in an Eberbach shaker at 250 oscillations/min and centrifuged at 1500 rpm for 3 min. The aqueous (upper) phase was aspirated and discarded. The dichloromethane was transferred to fresh 16 × 125 mm test tubes and evaporated under a stream of nitrogen in a water bath at 40 °C. Methanol, 1 ml, was added and the tubes were held in an ultrasonic bath for 10 s, to ensure solution of the residue. Four milliliters of HCl (0.5 mol/liter) was added and the contents of the tubes were mixed on a vortex-type mixer for 5 s. Hexane, 7 ml, was added and the tubes were shaken for 10 min in the Eberbach shaker as described above and centrifuged for 5 min at 2000 rpm. The hexane (upper) layer was aspirated and discarded and the aqueous layer was washed again with 7 ml of hexane. Dichloromethane, 7 ml, was added to the methanol-HCl solution and the tubes were shaken for 10 min in the Eberbach shaker and centrifuged for 5 min at 2000 rpm. The aqueous phase was aspirated and dis-
carded. Anhydrous Na₂SO₄ (0.5 g) was added to each sample, and the contents of the tube were mixed well and then centrifuged for 2 min at 2000 rpm. The di-
chloromethane was transferred into 7-ml screw-cap septum vials and evaporated under a stream of nitrogen in a 40 °C water bath. Regis silylation mix (50 µl) was added to each residue, and each vial was capped immediately thereafter. The vials were mixed on a vortex-type mixer, allowed to stand for 30 min at room temperature, then 1 µl of sample was injected into the gas chromatograph. Results were calculated by the peak-height ratio method, with BMMA as the internal standard.

Standard curves were prepared by analyzing samples of normal human serum containing known amounts of each drug. These serum-based standards were prepared by evaporating under nitrogen appropriate amounts of alcoholic solutions of the anticonvulsants and adding 1 ml of normal serum.

Analysis of diphenylhydantoin and primidone for purposes of correlation with our method were performed by on-column methylation by the method of Kananen et al. (3).

Results

Benzylmalonamide, phenethylmalonamide, phenylmalonamide, and benzylmalonate methylester monoamide were evaluated as possible internal standards in this procedure. The first three compounds were poorly extracted and their trimethylsilyl derivatives did not separate well from chromatographic peaks representing normal constituents in serum. In contrast, benzylmalonate methylester monoamide was well extracted from serum and its trimethylsilyl derivative was well separated from the normal constituents in serum and the anticonvulsants of interest.

Representative chromatograms of serum specimens extracted and silylated according to our procedure are presented in Figure 1. Chromatogram A, with the internal standard omitted, is a pattern typical of the serum of a normal individual not receiving anticonvulsants. Chromatogram B was obtained from serum of a normal individual, to which was added 10 µg of each of the indicated drugs. The trimethylsilyl derivatives of these anticonvulsants and the internal standard are well separated from each other and no significant interferences have been observed in serum from patients not on anticonvulsant therapy. Chromatogram C was obtained from the serum of an individual receiving primidone, 250 mg three times daily, diphenylhydantoin, 100 mg four times daily, and carbamazepine, 800 mg daily. The concentrations determined in this sample of serum were: primidone, 4.6 µg/ml; PEMA, 3.0 µg/ml; diphenylhydantoin; 10.0 µg/ml; and carbamazepine, 6.6 µg/ml.

The specificity of this assay was determined by extracting and preparing trimethylsilyl derivatives of various other anticonvulsant drugs in the presence of primidone, PEMA, diphenylhydantoin, and carba-
mazepine. The following drugs did not interfere when assayed by our method: dimethadione, trimethadione, ethosuximide, mesantoin, metharbital and mephobarbital. However, methsuximide and phensuximide had the same retention times as the trimethylsilyl derivatives of primidone and the internal standard, respectively.

The sensitivity of the method was such that primi-
done, PEMA, diphenylhydantoin and carbamazepine could be readily quantitated at a concentration of 0.5 mg/liter when the sample volume was 1.0 ml.

Table 1 presents precision data for the drugs analyzed by our procedure. Within-run precision was estimated both by analysis of 10 replicate samples and from paired duplicates. Overall precision was estimated from data obtained on samples analyzed over 6 months.

Figure 2 illustrates typical standard curves from primidone, PEMA, diphenylhydantoin and carbamazepine prepared by the peak height ratio technique utilizing BMMA as the internal standard.

Recoveries of the drugs from serum were measured in the following way: 15 µg of each compound, in methanol, was evaporated and to the residue was added 1 ml of drug-free serum. This sample was extracted as described in the Methods section; however, volume transfers were quantitated. A second (non-extracted) standard was prepared concurrently by evaporating 15 µg of the same compounds to dryness, and both sample and standard were derivatized by addition of 50 µl of silylating reagent. One microliter of each sample was chromatographed. The peak heights of the serum sample were corrected for solvent transfers. Absolute recoveries of the drugs from serum were calculated by comparison of the ratio of
Table 1. Precision of Assays for Anticonvulsant Drugs in Serum

<table>
<thead>
<tr>
<th>Drug</th>
<th>Within-run precision by replicate analysis</th>
<th>Within-run precision by analysis of duplicates&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Over-all precision between + within-run&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>mg/liter</td>
<td>CV, %</td>
</tr>
<tr>
<td>Primidone</td>
<td>10</td>
<td>5.4</td>
<td>2.6</td>
</tr>
<tr>
<td>PEMA</td>
<td>10</td>
<td>5.5</td>
<td>1.6</td>
</tr>
<tr>
<td>Diphenylhydantoin</td>
<td>10</td>
<td>6.6</td>
<td>3.8</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>10</td>
<td>10.4</td>
<td>3.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Calculated according to SD = \( \sqrt{\text{d}^2/n} \); \( \text{d} \) = difference between duplicates; \( n \) = number of duplicates.

<sup>b</sup> These data were obtained by three analysts during six-months.

Corrected peak heights of the extracted serum sample to those of the non-extracted standard. The recovery of carbamazepine determined by this procedure approaches 100%. Recoveries of the other compounds of interest based on that of carbamazepine were as follows: primidone 42%, PEMA 47%, and diphenylhydantoin 95%.

Fifty specimens of serum obtained from patients attending the Pediatric Seizure Clinic at The Johns Hopkins Hospital were analyzed for primidone by a standard gas-chromatographic method in which on-column methylation is used (3) and by the procedure we developed. Similarly 35 specimens were analyzed by both procedures for diphenylhydantoin. Serum concentrations of primidone ranged from 1.0 to 34.0 mg/liter. The mean value observed with the on-column alkylation procedure was 9.3 mg/liter and with our procedure was 9.6 mg/liter. When values for our assay were regressed against values for the standard method the slope of the weighted linear least-squares regression line was 0.936, the intercept was 1.00 \( \mu \)g/ml, and the coefficient of correlation was 0.939. Our procedure for the determination of primidone gave a CV of 2.6% at a concentration of 5.4 mg/liter, compared to 7.6% obtained by the method employing on-column methylation. Serum concentrations of diphenylhydantoin ranged from 1.0 to 51.4 mg/liter. The mean value observed for diphenylhydantoin by on-column methylation was 12.6 mg/liter and with our procedure was 12.6 mg/liter. When values for our assay were regressed against values for the standard method, the slope of the weighted linear least-squares regression line was 0.944, the intercept was 0.3 \( \mu \)g/ml, and the coefficient of correlation was 0.988.

Discussion

Determinations of primidone are described that involve extraction of the drug and subsequent gas-chromatographic analysis as either methylated or silylated derivatives. The results presented here demonstrate a good correlation (\( r = 0.939 \)) between our method for primidone and one involving on-column methylation. The improved precision we attained is due in part to the better extraction efficiency (42%) attained in our method.

Baumel et al. (1) did not present information on the reproducibility of their procedure, but it is likely that precision was limited by the injection technique, because no internal standard was included in their assay. Our method for determination of phenylethylmalonamide as its trimethylsilyl derivative gave a CV of 1.6% at a concentration of 5.5 mg/liter. This excellent reproducibility is related primarily to use of BMMA, a compound related in structure to phenylethylmalonamide, as an internal standard. The method we used for extraction of phenylethylmalonamide from serum was that used by Roger et al. (6) for the determination of carbamazepine and other anticonvulsants, including primidone, in human plasma. Extraction efficiency of phenylethylmalonamide with this procedure was 47%. Kupferberg (7) described a method for determination of carbamazepine as the trimethylsilyl derivative in which was used an extraction procedure closely related to that reported by Roger et al. (6). Recovery of carba- mazepine by both extraction procedures was in excess of 90%. Kupferberg (7) included the chromatogram of a plasma extract from a patient receiving carbamazepine and primidone, and it is interesting to note a relatively large unidentified peak with a retention time of approximately 6 min. The position of this peak relative to that of primidone makes it likely that it represents the trimethylsilyl derivative of phenylethylmalonamide.

Patients with severe seizure disorders are fre-
quently treated concurrently with multiple anticonvulsant drugs. Thus, carbamazepine is most often administered in combination with such drugs as diphenylhydantoin, phenobarbital, or primidone. Our procedure permits the simultaneous determination of primidone, phenylethylmalonamide, diphenylhydantoin, and carbamazepine as their trimethylsilyl derivatives. Because of marked variation between individuals in rates of metabolism of these drugs (10) as well as the known interactions between these compounds (11), it is important to measure their concentrations in serum, to avoid both toxic and subtherapeutic effects. Recovery of carbamazepine in our procedure approached 100%, which agreed closely with the results of Kuperberg (7) and Roger et al. (6). The coefficient of variation of our method for carbamazepine at a concentration of 10.4 mg/liter was 3.2%, which indicates that BMMA serves as an adequate internal standard for quantitation of carbamazepine. Kuperberg (7) used cyheptamide as an internal standard in his assay for carbamazepine, but we have confirmed that the trimethylsilyl derivatives of cyheptamide and diphenylhydantoin cannot be separated on OV-1 or OV-17.

Roger et al. (6) determined carbamazepine by an on-column pyrolysis technique that yielded iminostilbene. In attempting to utilize this procedure we observed that the conversion of carbamazepine to iminostilbene was extremely variable and dependent on instrumental conditions and injection technique. The excellent reproducibility for carbamazepine reported by Roger et al. (6) was undoubtedly due to the use of an automatic sample injector.

MPPH cannot be used as an internal standard in our assay because the trimethylsilyl derivatives of carbamazepine and MPPH cannot be resolved on either OV-1 or OV-17. The use of BMMA as an internal standard yielded a within-run coefficient of variation of 3.8% for diphenylhydantoin at a concentration of 6.6 mg/liter. This precision is comparable to that observed in our experience with an on-column methylation procedure for diphenylhydantoin in which MPPH is used as the internal standard (CV = 3.0%, within-run at 20 mg/liter). On comparison of 35 patients' specimens by our silylation procedure with BMMA as the internal standard and on-column methylation with MPPH as the internal standard, the mean values (12.6 mg/liter) were identical and the coefficient of correlation was 0.988. The recovery for diphenylhydantoin from serum was about 95%, which agrees closely with that observed by Roger et al. (6).

A gas-chromatographic procedure that allows simultaneous determination of primidone, phenylethylmalonamide and phenobarbital in a single chromatographic step would be ideal. However, because of the instability of the trimethylsilyl derivative of phenobarbital, this compound cannot be quantitated in the present procedure. For this reason we determine phenobarbital separately by extraction and on-column alkylation with trimethyl-anilinium hydroxide according to the method of Kananen et al. (3). Moreover, this procedure permits an independent confirmation of the results of primidone and diphenylhydantoin obtained by the silylation procedure.

Baumel et al. (1), who also determined primidone, phenylethylmalonamide, and diphenylhydantoin as their trimethylsilyl derivatives, determined phenobarbital underivatized on a separate sample carried through their extraction procedure. Kuperberg (7) separated phenobarbital and diphenylhydantoin from primidone and carbamazepine by extraction and determined the former compounds by on-column methylation and the latter as their trimethylsilyl derivatives. There is still need for an extraction derivatization procedure that permits the simultaneous determination of primidone, phenylethylmalonamide, phenobarbital, diphenylhydantoin, and carbamazepine in a single chromatographic step.

References