Electron-Capture Gas–Liquid Chromatographic Determination of Ethosuximide and Desmethylmethsuximide in Plasma or Serum

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We describe the determination of ethosuximide and desmethylmethsuximide, simultaneously or separately, in 50 to 100 μL of plasma or serum. Derivatives of ethosuximide and desmethylmethsuximide formed by reaction with pentafluorobenzoyl chloride are extremely sensitive to the electron-capture detector of a gas–liquid chromatograph. The sample, with added internal standard and ammonium sulfate as a pH-adjusting and salting-out agent, is extracted with ethyl acetate/benzene (20/80 by vol). The extract is evaporated and the derivatives are formed. Analytical recoveries of ethosuximide and desmethylmethsuximide exceed 99%, and the relative standard deviation (CV) between analyses is usually <4.0%. α-Methyl-α-propylsuccinimide is used as the internal standard for ethosuximide, 2-phenylsuccinimide as the internal standard for desmethylmethsuximide.

This report describes an electron-capture gas-chromatographic analysis of ethosuximide (2-ethyl-2-methyl succinimide) and (or) N-desmethylethosuximide in serum or plasma. Several gas-chromatographic methods have been described for the determination of ethosuximide and other succinimide analogs in serum (1–9). Many of these methods require derivatization—either by methylation (5, 8), silylation (9), or butylation (2, 3)—and both flame ionization and nitrogen/phosphorus detectors have been used. Several liquid phases have been used for the assay of the derivatized succinimides (1, 4). With these methods, however, several drugs and normal serum constituents have a retention time that is the same or near that for ethosuximide.

Here, we describe the use of pentafluorobenzoyl chloride for derivatization of the two succinimides and analysis of the derivatives by electron-capture gas-chromatography, either simultaneously (with temperature programming) or separately (under isothermal conditions). We use a highly specific extraction solvent, which provides a high analytical recovery and is more selective in extracting the anticonvulsants than other solvents that have been used for this purpose. The internal standards are structural analogs of the drugs, and thus are closely similar to them in extraction, derivatization, and chromatographic characteristics.

Materials and Methods

Apparatus and Operating Conditions

We used a Model 5830A gas chromatograph (Hewlett Packard, Avondale, PA 19311) equipped with a 63Ni electron-capture detector and a 1 m × 2 mm (i.d.) glass column packed with 3% OV-17 on 100/120 mesh Gas Chrom Q (Supelco, Inc., Bellefonte, PA 16823). Column temperature for simultaneous determination of ethosuximide and desmethylmethsuximide was 170 °C for 4 min, followed by temperature programming at 25 °C/min for 2 min to a temperature of 220 °C. When the drugs were to be separately assayed, ethosuximide and desmethylmethsuximide were determined at column temperatures of 170 and 220 °C, respectively. The injector temperature was 300 °C; detector temperature was maintained at 350 °C. Methane in argon (5/95 by vol) was used as the carrier gas at a flow rate of 30 mL/min.

Reagents

Pentafluorobenzoyl chloride (cat. no. 58115; Pierce Chemical Co., Rockford, IL 61105).

Ammonium sulfate (granular), ACS grade (cat. no. 3512; Mallinckrodt Inc., St. Louis, MO 63147), saturated solution in de-ionized water.

Benzene, ethyl acetate, and methanol were purchased in "pesticide" grades from Fisher Scientific Co., Fair Lawn, NJ 07410 (cat. no. B-426, E-191, and A-450, respectively).

N-Desmethylethosuximide (α-methyl-α-phenylsuccinimide) (Aldrich Chemical Co., Milwaukee, WI 53233, cat. no. 86058-1), a stock 1 g/L solution in methanol.

Ethosuximide (cat. no. CI-366; Parke, Davis & Co., Detroit, MI 48200), stock 1 g/L solution in ethanol.

α-Methyl-α-propylsuccinimide (cat. no. 19495-6; Aldrich Chemical Co.), stock 1 g/L solution in ethanol.

2-Phenylsuccinimide (Parke, Davis & Co.), stock 1 g/L solution in methanol.

Procedure

Specimens for the standard curve are prepared by placing known amounts of the stock solutions of ethosuximide and desmethylmethsuximide into 16 × 125 mm screw-cap tubes. The solvent is removed under a stream of dry air at room temperature, 10 mL of blank plasma is added, and the tube contents are vortex-mixed for 10–20 s to ensure a uniform concentration. For standard curves, 0.1-mL aliquots of the appropriate standards are used. The standards in plasma can be stored at 4 °C for two or three months.
For analysis, 0.1 mL of a saturated ammonium sulfate solution is added to 0.1 mL of plasma or serum in a 16 × 125 mm screw-cap tube and the contents of the tube are briefly vortex-mixed. Extraction is accomplished by adding 2 mL of ethyl acetate/benzene (or toluene), 20/80 by vol, containing \( \alpha \)-methyl-\( \alpha \)-propylsuccinimide, 2 mg/L, and 2-phenyl succinimide, 3 mg/L, to the previously diluted plasma, capping with Teflon-lined caps, shaking the tubes for 15 min on an Eberbach shaker, and centrifuging at 2000 rpm for 2 min. The organic layer is decanted into 16 × 125 mm screw-cap tubes and evaporated under a stream of dry air at room temperature. The tubes are removed immediately upon drying. Pentfluorobenzyl chloride, 50 \( \mu \)L, is added to each tube. The tubes are again capped with Teflon-lined caps, vortex-mixed, and placed in a heating block at 160 °C for 30 min, allowed to cool to room temperature, and their contents dried in a stream of dry air, in a heating block at 55 °C. After about 10 min of drying time, any remaining liquid can be rapidly dried by dispersing the liquid about the tube with the tip of the drying-manifold pipette. The residue is reconstituted in 5 mL of ethyl acetate and 2 \( \mu \)L of the resulting solution is injected into the electron-capture gas-liquid chromatograph.

Results

Optimal conditions for derivatization of ethosuximide and desmethylmethsuximide and their respective internal standards, \( \alpha \)-methyl-\( \alpha \)-propylsuccinimide and \( \alpha \)-phenylsuccinimide, were established experimentally by obtaining constant peak-height ratios. At a reaction temperature of 160 °C, the peak-height ratios of both ethosuximide and desmethylmethsuximide did not change appreciably after 30 min. Ethosuximide and desmethylmethsuximide can be simultaneously analyzed most effectively by means of temperature programming. An initial short isothermal program allows ethosuximide to be assayed and a subsequent program at 25 °C/min up to 220 °C permits the analysis for desmethylmethsuximide. Figure 1 illustrates the chromatographic separations for ethosuximide, desmethylmethsuximide, and their respective internal standards under these conditions. A 5-min extension of the chromatographic run ensures that there will be no interferences from primidone or other drugs during subsequent assays.

Both ethosuximide and desmethylmethsuximide can be measured individually under isothermal conditions (Figure 2). Such analysis for ethosuximide may require a 40–50 °C temperature program after the internal standard has eluted, particularly if the biological extracts contain high concentrations of other anticonvulsants such as primidone or carbamazepine. If desmethylmethsuximide is measured isothermally, no temperature program is required at the end of the chromatographic analysis.

Analytical recoveries were 101% for ethosuximide and 99% for desmethylmethsuximide. Within-day estimates of precision (coefficient of variation) for ethosuximide and desmethylmethsuximide over a concentration range of 10–120 mg/L were 4.1% and 2.8%, respectively (Table 1). The estimated day-to-day precision of analysis is 4.6% for ethosuximide and 10.1% for desmethylmethsuximide. Each of the values represents a single analysis, as would occur under routine analytical conditions. In Table 2, we compare values obtained from various samples prepared for a nationwide antiepileptic drug quality-control program. For ethosuximide the average difference between the expected values and our analytical values was 6.8%.

![Fig. 1. Simultaneous analysis of ethosuximide (ES) and desmethylmethsuximide (DMMS)](image1)

![Fig. 2. Isothermal analysis of ethosuximide and desmethylmethsuximide](image2)

### Table 1. Standard Curves for Ethosuximide and Desmethylmethsuximide Analysis

<table>
<thead>
<tr>
<th>Serum concn, mg/L</th>
<th>ES/MPS a (peak-height ratio ± SD)</th>
<th>DMMS/PS a (peak-height ratio ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.259 ± 0.019</td>
<td>0.301 ± 0.004</td>
</tr>
<tr>
<td>20</td>
<td>0.477 ± 0.011</td>
<td>0.598 ± 0.012</td>
</tr>
<tr>
<td>40</td>
<td>0.978 ± 0.023</td>
<td>1.240 ± 0.010</td>
</tr>
<tr>
<td>80</td>
<td>1.790 ± 0.095</td>
<td>2.470 ± 0.145</td>
</tr>
<tr>
<td>120</td>
<td>2.705 ± 0.087</td>
<td>3.483 ± 0.140</td>
</tr>
<tr>
<td>Av CV = 4.1%</td>
<td></td>
<td>Av CV = 2.8%</td>
</tr>
</tbody>
</table>

*Triplicate analyses. ES, MPS, DMMS, and PS are abbreviations for ethosuximide, \( \alpha \)-methyl-\( \alpha \)-propylsuccinimide, desmethylmethsuximide, and \( \alpha \)-phenylsuccinimide, respectively.*
The lower limit of sensitivity for both ethosuximide and desmethylmethsuximide was determined to be 0.5 mg/L for 0.1 mL of plasma. This limit was established by analyzing blood-bank plasma, unselected samples from hospitalized patients, and pre-dose serum samples from patients being treated with anticonvulsant drugs. Extraneous peaks based on over 20 analyses represented less than 0.2 mg/L of ethosuximide and desmethylmethsuximide. Thus, under analysis conditions, the lower limit of sensitivity of the assay was set at about twice the background response. At concentrations of 10 mg/L, none of the following anticonvulsant compounds interfered with the proposed method: phenytoin, phenobarbital, primidone, or carbamazepine. Phenytoin and phenobarbital do not react with the derivatizing reagent; primidone and carbamazepine yield products that have significantly longer retention times.

Figure 3 shows chemical structures of ethosuximide, desmethylmethsuximide, and their internal standards, along with those of the corresponding pentfluorobenzamide derivatives as verified by gas chromatography–mass spectrometry. These products of ethosuximide and desmethylmethsuximide are stable for at least a month in ethyl acetate.

**Discussion**

The method proposed in this report offers several advantages over many of the current gas-chromatographic methods. Sensitivity is significantly enhanced with the electron-capture detector. As a result, smaller sample volumes are needed, e.g., 0.05 mL vs. 1.0 mL or more. The amounts of extraction solvent are correspondingly reduced, affording a short evaporation time. For ethosuximide, temperature programming is not required for analysis of the derivative, but is required for those flame-ionization procedures that involve no derivatization (2–5). The proposed procedure utilizes one reagent and requires fewer steps than procedures in which tetramethylammonium hydroxide, dimethylacetamide, or iodobutane is used as the derivatizing reagent (2). Use of internal standards helps lower CV’s. A recent report by Solow et al. (10) also demonstrates the feasibility of using α-methyl-α-propylsuccinimide as internal standard in the analysis for ethosuximide. Our procedure has considerable applicability for the simultaneous analysis for ethosuximide and desmethylmethsuximide, considering the nonspecificity of the appropriate enzyme immunoassay reagent for these two anticonvulsants.

Pentafluorobenzoyl chloride has been used for the analysis of carbamazepine (11) and primidone (12), and the current procedure for ethosuximide extends the number of anticonvulsant drugs that can be derivatized to pentfluorobenzamide derivatives for analysis by this technique. The perfluoroaryl groups that contain an adjacent C=O or C=N have greater electron affinity than do compounds containing only the perfluorophenyl groups (13). The formation of an amide or Schiff’s base to which is conjugated a highly electronegative resonating pentafluorophenyl ring accounts for the greater electron-deficient character of pentafluorobenzamides and pentafluorobenzylidines than with other polyfluorinated drug derivatives.

**References**

1. Bonitati, J., Gas-chromatographic analysis of succinimide anti-


