Comparison of Enzyme Immunoassay and Gas Chromatography for Determination of Carbamazepine and Ethosuximide in Human Serum

Lilla Sun¹ and Izrail Szafir²

Patients’ sera were analyzed for carbamazepine and ethosuximide by enzyme immunoassay (x) and gas chromatography (y), and the results were compared. The correlation coefficients were: for carbamazepine, x vs. y 0.94 (n = 93); for ethosuximide, x vs. y 0.99 (n = 30). These results suggest that the two methods could be used interchangeably. Ten serum samples supplemented with carbamazepine (2.5 to 12.5 mg/liter) and ethosuximide (20.0 to 130.0 mg/liter) were analyzed by both methods. The correlation coefficients were: x vs. y 0.99 (n = 10) for carbamazepine and x vs. y 0.99 (n = 10) for ethosuximide.

Carbamazepine and ethosuximide have come to replace the more commonly used anticonvulsant drugs, because they seem to be more efficient and better avoid side effects induced in some patients by other anticonvulsants such as phenobarbital, phenytoin, and primidone.

Methods have been published for determination of carbamazepine and ethosuximide by gas-chromatographic techniques (1-10) as well as by high-pressure liquid chromatography (11). Thin-layer chromatography has also been used (12, 13). Recently, enzyme immunoassay of anticonvulsant drugs (14, 15) has become available for both carbamazepine and ethosuximide as EMIT-aed³ kits. Here, we report a comparison of results by enzyme immunoassay vs. gas chromatographic methods, including precision and accuracy.

Materials and Methods

Sera from 93 patients who had been treated with carbamazepine treatment and 30 patients who had received ethosuximide were assayed by enzyme immunoassay and gas chromatography.

Serum for precision and recovery studies was prepared by adding a known amount of carbamazepine and ethosuximide to drug-free human serum.

Both control serum and sera supplemented with carbamazepine and ethosuximide were obtained from Syva Corp., Palo Alto, Calif. 94304.

Enzyme Immunoassay

Reagents for the enzyme immunoassay (EMIT) of carbamazepine and ethosuximide were obtained from Syva Corp. and used according to their suggestions. Absorbances were measured at 340 nm with a Model 25 double-beam spectrophotometer, with a heated sipper cell, and a Model 701 printer/calculator (all from Beckman Instruments, Inc., Irvine, Calif. 92713).

Gas Chromatography

Carbamazepine. To 1 ml of serum, add 1 ml of phosphate buffer (1 mol/liter, pH 2.7), 5 ml of ether/toluene (1/1 by vol), and 1 ml of cyheptamide (10 mg/liter) as internal standard. Shake the mixture for 5 min and centrifuge; transfer the organic layer to a conical tube. Evaporate the organic solvent under a stream of air or nitrogen. Add 30 μl of dimethylformamide/dimethyacetel, cap the tube tightly, and place it in a boiling water bath for 10 min. Cool the tube and inject 1 μl of the solution into a gas chromatograph. We used a Model 402 instrument (Hewlett-Packard, Avondale, Pa. 19311). Figure 1 shows a typical example of a chromatogram for carbamazepine and internal standard.

Ethosuximide. To 1 ml of serum, add 1 ml of phosphate buffer (1 mol/liter, pH 2.7), 5 ml of ether/toluene (1/1 by vol), and 1 ml of 2,2,3-trimethylsuccimide (100 mg/liter) as internal standard. Shake the mixture for 5 min and centrifuge; transfer the organic layer to a conical tube. Add 50 μl of isomyl acetate and evaporate the organic solvent to 50 μl at room temperature under a gentle stream of air or nitrogen. Inject 1 μl into the gas chromatograph. Figure 2 shows a typical example of a chromatogram for ethosuximide and internal standard.
Gas Chromatographic Conditions

Column packing: PC 3210 (Pierce Chemical Co., Rockford, Ill. 61105). The use of this packing material is essential for the chromatography of carbamazepine. It is a mixture of 2.8% SE 30 and 3.2% OV 210.

Column temperature: carbamazepine, 240 °C, ethosuximide, 100 °C.

Detector: FID.

Gas flows: standard.

Statistical Analysis

Concentrations of the two drugs found by enzyme immunoassay were compared to the concentrations found by gas chromatography. Errors were estimated from the least-squares parameters (slope of the least-squares line, y-intercept, standard error of estimates, $S_{xy}$) as recommended by Westgard and Hunt (16).

Results

Patients' Sera

Carbamazepine. For enzyme immunoassay vs. gas chromatography (line a, Table 1) the slope of the line is 0.98, a proportional error of 2%. Constant error is estimated at 0.09 mg/liter from the intercept. Random error is estimated at 0.99 mg/liter from the standard error of the estimate, $S_{xy}$.

Ethosuximide. For enzyme immunoassay vs. gas chromatography (line b, Table 1) the slope of the line is 0.96, a proportional error of 4%. Constant error is estimated at -0.40 mg/liter from the intercept. Random error is estimated at 4.73 mg/liter from the standard error of the estimate, $S_{xy}$.

Supplemented Samples

Ten supplemented serum samples, each containing a known amount of pure carbamazepine and ethosuximide, were used to make up 10 different concentrations, which were assayed by enzyme immunoassay and gas chromatography (Table 2).

Assay values were compared, by least-squares regression, to the amount of carbamazepine or ethosuximide added. For carbamazepine-supplemented serum by enzyme immunoassay (line c, Table 1) the slope of the line is 1.05, a proportional error of 5%. Constant error is estimated at -0.04 mg/liter and random error at 0.03 mg/liter. For carbamazepine-supplemented serum by gas chromatography (line d, Table 1) the slope of the line is 1.17, a proportional error of 17%. Constant error is estimated at -0.13 mg/liter and random error at 0.08 mg/liter.

For ethosuximide-supplemented serum (line e, Table 1) the slope of the line is 0.97, a proportional error of 3%. Constant error is estimated at -0.25 mg/liter and random error at 0.28 mg/liter. For ethosuximide-supplemented serum by gas chromatography (line f, Table 1) the slope of the line is 1.09, a proportional error of 9%. Constant error is estimated at -1.12 mg/liter and random error at 0.48 mg/liter.
Table 1. Statistical Comparison of Enzyme Immunoassay (EIA) and Gas Chromatography (GC) for Carbamazepine and Ethosuximide

<table>
<thead>
<tr>
<th>Patients' sera</th>
<th>(a) EIA vs. GC (carbamazepine)</th>
<th>(b) EIA vs. GC (ethosuximide)</th>
<th>Supplemented sera</th>
<th>(c) Supplemented, carbamazepine vs. EIA</th>
<th>(d) Supplemented, carbamazepine vs. GC</th>
<th>(e) Supplemented, ethosuximide vs. EIA</th>
<th>(f) Supplemented, ethosuximide vs. GC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m</td>
<td>b</td>
<td>$s_{xy}$</td>
<td>r</td>
<td>n</td>
<td>m</td>
<td>b</td>
</tr>
<tr>
<td>(a) EIA vs. GC (carbamazepine)</td>
<td>0.98</td>
<td>0.09</td>
<td>0.99</td>
<td>0.94</td>
<td>93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b) EIA vs. GC (ethosuximide)</td>
<td>0.96</td>
<td>-0.40</td>
<td>4.73</td>
<td>0.99</td>
<td>30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Where m = slope of least-squares regression line; b = its intercept; $s_{xy}$ = standard error of the estimate; r = correlation coefficient; and n = number of samples.

Table 2. Results for Drug-Supplemented Samples

<table>
<thead>
<tr>
<th>Supplemented with carbamazepine</th>
<th>Found by EIA</th>
<th>GC</th>
<th>Supplemented with ethosuximide</th>
<th>Found by EIA</th>
<th>GC</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/liter</td>
<td></td>
<td></td>
<td>mg/liter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>4.2</td>
<td>3.2</td>
<td>50.0</td>
<td>42.0</td>
<td>39.0</td>
</tr>
<tr>
<td>12.0</td>
<td>14.0</td>
<td>13.7</td>
<td>45.0</td>
<td>41.0</td>
<td>36.0</td>
</tr>
<tr>
<td>5.0</td>
<td>5.2</td>
<td>4.8</td>
<td>95.0</td>
<td>86.0</td>
<td>88.0</td>
</tr>
<tr>
<td>2.0</td>
<td>2.0</td>
<td>1.8</td>
<td>55.0</td>
<td>54.0</td>
<td>43.0</td>
</tr>
<tr>
<td>2.5</td>
<td>2.7</td>
<td>2.6</td>
<td>45.0</td>
<td>39.0</td>
<td>36.0</td>
</tr>
<tr>
<td>5.0</td>
<td>5.0</td>
<td>4.3</td>
<td>20.0</td>
<td>15.0</td>
<td>14.0</td>
</tr>
<tr>
<td>12.5</td>
<td>14.0</td>
<td>13.0</td>
<td>110.0</td>
<td>108.0</td>
<td>110.0</td>
</tr>
<tr>
<td>3.0</td>
<td>3.3</td>
<td>1.0</td>
<td>45.0</td>
<td>42.0</td>
<td>40.0</td>
</tr>
<tr>
<td>8.0</td>
<td>8.4</td>
<td>6.4</td>
<td>130.0</td>
<td>123.0</td>
<td>138.0</td>
</tr>
<tr>
<td>10.0</td>
<td>11.0</td>
<td>11.1</td>
<td>31.0</td>
<td>32.0</td>
<td>32.0</td>
</tr>
</tbody>
</table>

Table 3. Day-to-day Replications (n = 20)

<table>
<thead>
<tr>
<th>Given control value</th>
<th>Av</th>
<th>SD mg/liter</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbamazepine, 6.0 mg/liter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme immunoassay</td>
<td>5.9</td>
<td>0.17</td>
<td>2.9</td>
</tr>
<tr>
<td>Gas chromatography</td>
<td>6.2</td>
<td>0.34</td>
<td>5.5</td>
</tr>
<tr>
<td>Ethosuximide, 75 mg/liter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme immunoassay</td>
<td>77</td>
<td>4.6</td>
<td>5.9</td>
</tr>
<tr>
<td>Gas chromatography</td>
<td>75</td>
<td>5.0</td>
<td>6.7</td>
</tr>
</tbody>
</table>

Controls

A control serum provided by Syva Corp. was assayed in duplicate on each of 20 successive days. Table 3 summarizes the results.

Discussion

For enzyme immunoassay, no extraction or concentration is required; therefore, the assay can be completed within 15 min of receipt of sample. However, because enzyme immunoassay involves an enzyme kinetic reaction, the assay will be affected by the time of the kinetic measurement as well as variations in temperature. For gas chromatography, extraction, concentration, and derivatization are required; thus sample preparation is time-consuming. The gas chromatography assay can be completed in about 1 h after receipt of the sample.

According to the manufacturer's protocol, severely hemolyzed samples of carbamazepine and ethosuximide should not be used for enzyme immunoassay, because hemolysis may cause poor precision and accuracy. Because extractions are used in the gas chromatography technique, the effect of hemolysis on final results is eliminated.

Optimal therapeutic concentrations in serum, as given in the enzyme immunoassay protocol, are 4–10 mg/liter for carbamazepine and 40–100 mg/liter for ethosuximide. Based on all results, both enzyme immunoassay and gas chromatography methods are linearly related above therapeutic concentrations (up to 10 mg/liter for carbamazepine, and up to 150 mg/liter for ethosuximide). Both enzyme immunoassay and gas chromatography methods are sensitive below the therapeutic concentrations down to about 1 mg/liter for carbamazepine, and 10 mg/liter for ethosuximide.

Results by both procedures are not statistically different; therefore either technique could be used with equivalent results.

References


*Syva, EMIT-aed test protocol 6A164-3.*
Improved Direct Determination of Serum Cholesterol in Low-Density Lipoproteins with Use of Polycations

C. C. Heuck, G. Middelhoff, and G. Schlierf

The previously described procedure of direct determination of LDL-cholesterol after selective extraction of VLDL and HDL with polycations and a polycation-exchange resin has been improved by using dodecylated poly(ethyleneimine) instead of poly(ethyleneimine). The acyl derivative has a higher affinity for lipoproteins. Disc-electrophoretic patterns of lipoproteins separated by this procedure correspond to patterns of apolipoproteins in VLDL and HDL, a finding that corroborates the conclusion that the mechanism of the selective binding of certain lipoproteins to lipophilic water-soluble polycations is based on hydrophobic interaction between the polymer and lipids located at the surface of lipoprotein particles. The procedure offers a simple possibility for direct LDL-cholesterol and LDL-triglyceride determination and for the isolation of VLDL and HDL. The mechanism of the reaction has a biochemical correlate in the action of phospholipase A$_2$ on phospholipids.

Additional Keyphrases: heart disease · polycation resin · hyperlipoproteinemia

Certain cationic polymers exhibit a high affinity towards lipids. This behavior has been recently studied with respect to lipoproteins. Under certain conditions the polymer selectively binds to VLDL and HDL. They can be extracted from serum in the presence of a cation-exchange resin. We have described a procedure for direct determination of cholesterol in LDL with poly(ethyleneimine) (1). From chemical investigations (2) we predicted that polycations with lipophilic side-chains should be even better reagents, increasingly so as the number of lipid-binding sites is increased. The purpose of this investigation was therefore to test acylated poly(ethyleneimine) for LDL cholesterol determination and thereby to confirm the assumption that the principle of the reaction is based both on hydrophobic and ionic interactions between the polymer and polar lipids located at the surface of VLDL and HDL.

Material and Methods

General procedures for lipoprotein isolation, lipid and apolipoprotein analyses. Serum was sampled from apparently healthy volunteers with normallipemia and from untreated persons with hyperlipoproteinemia of type IIa, type IIb, and type IV after an overnight fast. The specimens were analyzed within 12 h.

Triglyceride and cholesterol concentrations were determined with a Technicon AA II AutoAnalyzer, using isopropanol extracts treated with zeolite. The quantitation was exactly according to the procedure of the U.S. Lipid Research Clinical Program (3). The determination of cholesterol and triglyceride concentrations in LDL was performed after serum lipoproteins were fractionated by ultracentrifugation ($d = 1.006, 4^\circ\mathrm{C}, 20$ h) and precipitation with heparin/MnCl$_2$ as previously described (3). Analytical recovery of cholesterol from isolated lipoproteins was $94 \pm 4\%$ (SD). For triglyceride it was $85 \pm 6\%$. The purity of lipoproteins after ultracentrifugation and polyanion precipitation was controlled by agarose gel electrophoresis.

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1 Nonstandard abbreviations used: LDL, low-density lipoproteins; HDL, high-density lipoproteins; and VLDL, very-low-density lipoproteins.

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2 Technicon AutoAnalyzer II (1972), clinical method No. 24, Technicon Instruments, Tarrytown, N.Y., 10591.