COMPETITIVE NEPHELOMETRIC IMMUNOASSAY OF CARBAMAZEPINE AND ITS EPoxide METABOLITE IN PATIENT BLOOD PLASMA

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We have prepared anti-carbamazepine antiserum and developed a competitive nephelometric immunoassay for the determination of carbamazepine and its epoxide metabolite in patient blood plasma.

The antiserum was raised by immunization of a rabbit with a carbamazepine-(bovine serum albumin) conjugate. A carbamazepine-(human serum albumin) conjugate was used as an assay reagent. Carbamazepine and its epoxide inhibited competitively and almost equally the immunoprecipitation of the carbamazepine-(human serum albumin) conjugate. Therefore carbamazepine and its epoxide could be determined by the measurement of the scattered light from the immunoprecipitate in assay solution on a laser nephelometer. Patient plasma specimens were analyzed, and the values correlated well to those determined by the high-performance liquid chromatography. The epoxide concentrations were considerably lower than therapeutic carbamazepine concentrations, which was certified by the chromatographic results. This immunoassay was rapid (incubation time: within 20 min), simple and precise (coefficient of variation: less than 6%), and required as little as 6 μl of plasma, and seemed suitable for routine monitoring of carbamazepine.

**Keywords**—carbamazepine; carbamazepine-10,11-epoxide; antiepileptic drug; anti-carbamazepine antiserum; therapeutic drug monitoring; nephelometric immunoassay; high-performance liquid chromatography

INTRODUCTION

Carbamazepine is used in the treatment of epilepsy and trigeminal neuralgia. It is said that therapeutic carbamazepine concentrations in blood plasma may range between 5 to 10 μg/ml, while concentrations of 20 μg/ml are closely associated with severe toxic effects.1) In addition, no or poor relationship exists between chronic daily dose and plasma carbamazepine concentrations at the steady state.1,2) Therefore, it is desirable to monitor plasma carbamazepine concentrations for each patient for the safe and effective therapy. The concentration of this drug is most often determined by spectrophotometry, gas-liquid chromatography, high-performance liquid chromatography or homogeneous enzymeimmunoassay.2) But these methods have individually disadvantages of technical difficulty, time-consuming, cost or impossibility for the assay of a large number of samples.

We already developed a competitive nephelometric immunoassay of theophylline, phenobarbital and phenytoin.3-5) This method is based on “immunoprecipitation inhibition by hapten,” and has advantages of simplicity, rapidness, low cost and a possibility for serial assay of a large number of samples. Hence, this method is suitable for routine therapeutic monitoring of these drugs in patient body fluids. Therefore, we have developed a competitive nephelometric immunoassay of carbamazepine that seems to be suitable for routine monitoring of carbamazepine.

**MATERIALS AND METHODS**
Instrument — A laser nephelometer (Hyland Laboratories Inc., incident light: He-Ne laser 632.8 nm wavelength, detector angle: 31°) was used to measure the intensity of the scattered light from the immunoprecipitate particles in assay solution. A high-performance liquid chromatograph (Shimadzu PRP-1) equipped with a variable wavelength detector (Shimadzu SPD-1) was used in the determination of carbamazepine and its metabolite (i.e., carbamazepine-10,11-epoxide) (Chart 1).

Reagents — Bovine serum albumin and human serum albumin were obtained from Miles Laboratories Inc. Polyethylene glycol (PEG 6000, average molecular weight 7500) was used as an accelerator of immunoprecipitation. The buffer solution in which PEG was dissolved (25 g/l in 1/15 M Na_2HPO_4·KH_2PO_4, pH 7.4) was used for dilution and dissolution. Carbamazepine-butyric acid was prepared by the reaction of carbamazepine with 4-bromobutyric acid.

Carbamazepine-butyric acid was conjugated with bovine serum albumin and with human serum albumin by the mixed anhydride method. These conjugates were lyophilized and stored. Each male rabbit was immunized with 0.5 mg of the carbamazepine-(bovine serum albumin) conjugate emulsified with complete Freund's adjuvant. Boosters were given every three weeks five times in a manner similar to the first immunization, and the anti-carbamazepine antiseraum was obtained. The antiseraum was stored at 4° after addition of NaN_3 (1 mg/ml antiseraum).

Imмуnoassay Procedure — Just before assay, the antiseraum was diluted 150-fold with the PEG-buffer, allowed to stand at room temperature for 40 minutes, and filtered through a membrane (Nuclepore Co.) with 0.4 μm pore size. The plasma specimen was diluted 8-fold with the phosphate buffer. Into a glass assay cuvette, 50 μl of the diluted plasma and 1.0 ml of the filtered antiseraum were pipetted. The glass cuvette was put into a cuvette holder of the nephelometer, and the nephelometric reading (N_0 min: blank reading) was recorded. Then, 100 μl of carbamazepine-(human serum albumin) in the PEG-
buffer (32 μg/ml, which was equivalent to the added antibodies as described later) was added, the mixture was stirred and allowed to stand at room temperature (about 25°). Nephelometric reading at t minutes incubation time ($N_{t \text{ min}}$) was recorded. The difference ($\Delta N = N_{t \text{ min}} - N_{0 \text{ min}}$) was considered to show the amount of the immunoprecipitates which formed during t minutes.

**High-performance Liquid Chromatography** — To compare the results of the immunoassay, high-performance liquid chromatography was employed to determine carbamazepine in plasma. To 50 μl of plasma was added 200 μl of acetonitrile in which 5-(p-methylphenyl)-5-phenylhydantoin was dissolved at the concentration of 200 μg/ml as an internal standard. The mixture was stirred and centrifuged to remove the precipitated plasma proteins. Then 50 μl of the supernatant was injected into the chromatograph. The chromatographic conditions were: column (30 cm × 3.9 mm, μBondapak C₁₈, Waters Associates), mobile phase (acetonitrile/water=1/2 by volume), column pressure (100 kg/cm²), detection (absorbance at 272 nm), retention time (carbamazepine 6.7 min, the internal standard 10.0 min), quantitation (peak height ratio to internal standard).

Carbamazepine-10,11-epoxide in plasma was determined also by the high-performance liquid chromatography to compare the results according to the slightly modified method of Mihaly et al.²) Because the epoxide metabolite concentrations were generally considerably lower than its parent drug concentrations and because the epoxide had quite different ultraviolet absorption characteristics from those of its parent drug, it was necessary to use extraction and evaporation steps and to detect the epoxide at a shorter wavelength. To 1.0 ml of plasma were added phosphate buffer (pH 4.3) and cyheptamide-containing chloroform. Cyheptamide served as an internal standard. Extraction was performed, and to the chloroform layer was added phosphate buffer (pH 11.0). After mixing and centrifugation, the aqueous layer was discarded and the chloroform was evaporated. The residue was dissolved in acetonitrile and injected into the chromatograph with the absorbance detector operating at 250 nm. The widely used two antiepileptics, phenobarbital and phenytoin, did not interfere with these chromatographic analyses.

**RESULTS**

**Ultraviolet Spectra of the Conjugates**

The ultraviolet spectrum pattern of carbamazepine-(human serum albumin) was similar to that of the mixture of carbamazepine-butyric acid and human serum albumin (Fig. 1). The average number of incorporated carbamazepine moiety into human serum albumin was 20, which was estimated on the assumptions that the molar

![Graph showing ultraviolet spectra of carbamazepine conjugates.](image)

**FIG. 1. Ultraviolet Spectra of Carbamazepine-(human serum albumin) Conjugate, Carbamazepine-butyric Acid and Human Serum Albumin in 1/15M Phosphate Buffer pH 7.4**

Concentration of carbamazepine-(human serum albumin) = 225 mg/l; carbamazepine-butyric acid = 20 mg/l = 62 μM; human serum albumin = 205 mg/l = 3.1 μM.
absorption coefficients of both carbamazepine-butyrinic acid and human serum albumin were unaltered at 286 nm and that the molecular weight of human serum albumin was 66,220. The carbamazepine-(bovine serum albumin) conjugate had 16 carbamazepine moieties per albumin, which was estimated in a similar manner.

**Characterization of the Antiserum**

The conventional agar-immunoelectrophoresis was performed for characterization of the antiserum (Fig. 2). Only one precipitation line appeared between the antiserum and the each conjugate. No precipitation line appeared between the antiserum and bovine serum albumin (*i.e.*, the hapten-carrier of the immunogen). Of course the antiserum formed no precipitation line against human serum albumin. The results indicated that the anti-carbamazepine antibodies were present in the antiserum, and that the antibovine serum albumin antibodies were not present or very few.

![Agar-immunoelectrophoresis Test of Anticarbamazepine Antiserum](image)

**FIG. 2. Agar-immunoelectrophoresis Test of Anti-carbamazepine Antiserum**

Abbreviations: **HSA** = human serum albumin; **BSA** = bovine serum albumin; **CBZ-HSA** = conjugate of carbamazepine butyrinic acid with HSA; **CBZ-BSA** = conjugate of carbamazepine butyrinic acid with BSA. The anti-(human serum) was commercially available antiserum from a goat. The anti-BSA was the antiserum raised by immunization of a rabbit with a theophylline-BSA conjugate (see details in DISCUSSION in the text).

The immunoprecipitation of carbamazepine-(human serum albumin) was performed in a glass cuvette according to the previously described *Immunoassay procedure* with drug-free plasma. The precipitation occurred rapidly, and reached an equilibrium state within 10—20 minutes. Typical precipitation curve was obtained by the reaction of constant amount of the antiserum with varying

![Immunoprecipitation Curve](image)

**FIG. 3. Immunoprecipitation Curve**

Obtained by the reaction of constant amount of the antiserum with varying amount of the carbamazepine-(human serum albumin) conjugate.

![Time Course of Immunoprecipitation Inhibition by Carbamazepine](image)

**FIG. 4. Time Course of Immunoprecipitation Inhibition by Carbamazepine**
amount of the conjugate (Fig. 3). The curve showed antibody excess zone, equivalent (maximum) zone, and antigen excess zone. The addition of equivalent amount of the conjugate was suitable for the assay.

**Dose-response Curve**

The immunoprecipitation was performed with carbamazepine-added plasma according to the previously described *Immuoassay procedure*. The drug inhibited the precipitation quantitatively, and the incubation time 10 to 20 min at 25°C seemed to give precise assay results (Fig. 4). Under the usually employed conditions, $N_0$ min value with the most plasma specimens ranged 1 to 2, and the value with the turbid plasma ranged 2 to 3. Whereas, $N_{20}$ min value with the drug-free plasma and with the plasma of 10 μg/ml was as high as 75 and 35 respectively, giving high test/blank ratio. The standard dose-response curve was linear on log-logit graph (Fig. 5).

The cross-reactivities of the other antiepileptics such as phenobarbital, phenytoin, ethosuximide and valproate were less than 2% by molar ratio to carbamazepine, which indicated that these drugs would not interfere with this immunoassay. However, carbamazepine-10,11-epoxide inhibited the immunoprecipitation almost equally to carbamazepine with cross-reactivity of 90%.

**Analysis of Patient Plasma, and Comparison of the Results by This Immunoassay and by the High-**

![Graph](image_url)

**FIG. 5. Standard Dose-response Curve**

Relative scattered light ($\Delta N/\Delta N_0 \times 100$) was plotted against plasma carbamazepine concentration: $\Delta N = (N_{20 \text{ min}} - N_{0 \text{ min}})$ with each drug dose; $\Delta N_0 = (N_{20 \text{ min}} - N_{0 \text{ min}})$ with zero drug dose.

**TABLE I. Concentration of Carbamazepine and Carbamazepine-10,11-epoxide in Plasma of 33 Epileptic Children, determined by High-performance Liquid Chromatography**

<table>
<thead>
<tr>
<th>Number</th>
<th>Concentration of Carbamazepine (μg/ml plasma)</th>
<th>Concentration of Epoxide (μg/ml plasma)</th>
<th>Ratio of Epoxide/Carbamazepine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.9</td>
<td>0.50</td>
<td>26.3</td>
</tr>
<tr>
<td>2</td>
<td>2.5</td>
<td>0.70</td>
<td>28.0</td>
</tr>
<tr>
<td>3</td>
<td>4.3</td>
<td>0.70</td>
<td>16.3</td>
</tr>
<tr>
<td>4</td>
<td>4.4</td>
<td>1.16</td>
<td>26.4</td>
</tr>
<tr>
<td>5</td>
<td>4.5</td>
<td>0.84</td>
<td>18.7</td>
</tr>
<tr>
<td>6</td>
<td>4.9</td>
<td>0.60</td>
<td>12.2</td>
</tr>
<tr>
<td>7</td>
<td>5.2</td>
<td>0.80</td>
<td>15.4</td>
</tr>
<tr>
<td>8</td>
<td>5.4</td>
<td>1.62</td>
<td>30.0</td>
</tr>
<tr>
<td>9</td>
<td>5.5</td>
<td>0.50</td>
<td>9.1</td>
</tr>
<tr>
<td>10</td>
<td>6.0</td>
<td>0.80</td>
<td>13.3</td>
</tr>
<tr>
<td>11</td>
<td>Range</td>
<td>Range</td>
<td>Range</td>
</tr>
<tr>
<td></td>
<td>6.1−12.8</td>
<td>0.50−1.46</td>
<td>5.6−16.4</td>
</tr>
</tbody>
</table>

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FIG. 6 and FIG. 7. Comparison of Results for Plasma Drug Concentrations determined by the Immunoassay and the High-performance liquid Chromatography
Abscissa in Fig. 6: carbamazepine concentration.
Abscissa in Fig. 7: (carbamazepine + its epoxide) concentration.

Performance Liquid Chromatography

Thirty-three plasma specimens were collected from the chronically carbamazepine-receiving children with epilepsy, and analyzed by this immunoassay. The reproducibility of this immunoassay was excellent with coefficient of within-run variation of 5.7% for 5.1 μg/ml, and 5.4% for 9.9 μg/ml (15 replicate assays). The coefficients of between-day variation were 6.1% for 4.8 μg/ml, and 6.1% for 9.5 μg/ml (10 days, calibration curve was constructed each day).

Table I shows the concentrations of carbamazepine and its epoxide determined by the chromatography. The epoxide metabolite concentrations ranged as low as 0.5 to 1.6 μg/ml, and had a positive but very poor relation to its parent drug concentrations. As the data for specimen Number 1 to 10 in Table I show, lower carbamazepine concentrations were not always associated with lower epoxide concentrations. These results are consistent with the other reported results.1,2)

The values determined by this immunoassay and by the chromatography were compared (Fig. 6 and Fig. 7). The immunoassay values correlated well to both the chromatographic values of carbamazepine alone (r = 0.935) and those of (carbamazepine + its epoxide) (r = 0.940). However, as was expected from the cross-reaction test, (carbamazepine + its epoxide) values agreed more closely with the immunoassay values.

Of these 33 patients, 10 patients received phenobarbital or phenytoin in combination with carbamazepine. Comparison of the data of these test revealed that these two drugs did not interfere with this immunoassay, as was expected from the cross-reaction tests described before.

It could be concluded that this competitive nephelometric immunoassay was accurate for the determination of carbamazepine and its major metabolite, the epoxide, and suitable for routine monitoring of this drug in blood plasma.

Discussion

We previously prepared the antisera against theophylline, phenobarbital and phenytoin by a
method similar to the anti-carbamazepine antiserum preparation method. Of these four antisera, the anti-theophylline antiserum alone contained precipitating antibodies against bovine serum albumin which was commonly used as the hapten-carrier protein of the immunogen. It was not clear as to why some immunogens induced the production of anti-(hapten-carrier) antibodies in rabbits and some immunogens did not induce the production of precipitating anti-(hapten-carrier) antibodies. The carbamazepine-(bovine-serum albumin) may be used as an assay reagent instead of the carbamazepine-(human serum albumin) conjugate, because anti-(bovine serum albumin) antibodies were not detected. However, it may be generally recommended that the hapten-carrier of assay reagent for this immunoassay system is different from the hapten-carrier of the immunogen.

The sum of carbamazepine and its epoxide in the patient plasma was essentially determined by our immunoassay method. The commercially available homogeneous enzymeimmunoaassay of carbamazepine (Syva Co.), which is widely used for the monitoring of this drug, uses the anti-carbamazepine antibodies which also cross-react with its epoxide according to the manufacturer's instruction. It was reported by some authors that the epoxide metabolite was pharmacologically active in experimental animals. Therefore, it may be more useful to determine (carbamazepine + its epoxide) in human plasma than to determine carbamazepine alone. However, the epoxide concentration is generally considerably lower (less than 1.62 μg/ml in our results) than therapeutic carbamazepine concentration. Hence, it may be practically useful enough to determine carbamazepine alone. It is still not clearly known whether the epoxide is responsible for pharmacological and toxicological activities in man, and further precise studies are needed.

We employed nephelometry for a quantitation of immunoprecipitate. Instead of nephelometry, turbidimetry could be employed, since the N value of 80 and 40 corresponded to the absorbance of as high as 0.18 and 0.10 at 340 nm respectively, though turbidimetry of immunoprecipitate may be not so sensitive as nephelometry.

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REFERENCES