Enzyme Immunoassay of Carbamazepine with a Centrifugal Analyzer

David A. Lacher, Roland Valdes, Jr., and John Savory

We describe a rapid enzyme immunoassay for carbamazepine with a centrifugal analyzer (Rotochem IIA-36). Reagent costs are reduced fourfold while good precision and sensitivity are maintained. Sample volume is 10 μL, and as many as 28 patients' sera can be measured during an assay time of 225 s. Assay temperature is 30 °C, the wavelength 340 nm. Linearity is excellent for a carbamazepine concentration range of 1 to 12 mg/L; analytical recovery is quantitative. Results correlate well with those by liquid- and gas–liquid chromatography. Absorbance rates for each carbamazepine concentration are acquired by a multi-point kinetic rate program and a computer program provides a logit-log transformation of absorbance rate vs. concentration data for final calculations in the assay. Hemoglobin interference precludes analysis of severely hemolyzed specimens.

Monitoring antiepileptic drug concentrations is important, to help prevent seizures or drug toxicity in patients. Frequently combined with other anticonvulsant drugs, carbamazepine (Tegretol, Geigy) has been advocated for the control of partial, psychomotor, grand mal, and mixed seizures, as well as for trigeminal neuralgia. Monitoring carbamazepine in serum is especially important because of its narrow therapeutic index. Oral doses of 600 to 1200 mg of carbamazepine per day can give serum values that range from 3 to 16 mg/L. The therapeutic range of carbamazepine is considered to be 2 to 8 mg/L; toxic effects usually are seen when concentrations in serum reach 9 mg/L (1).

We undertook to adapt the carbamazepine enzyme immunoassay to the centrifugal analyzer. We wished to reduce reagent costs, yet maintain adequate sensitivity, precision, and accuracy. Also, we desired to increase analysis rate and decrease sample volume.

Materials and Methods

Apparatus

The centrifugal analyzer we used was a Rotochem IIA-36 (American Instruments Co., Silver Spring, MD 20901). This instrument is under complete computer control, with a 36-space cuvette system. Spectrophotometric precision is ±0.6 milliabsorbance (mA) units with nominal 10 nm or less bandpass and cuvette temperature control of ±0.05 °C according to the manufacturer's specifications. The automatic sample and reagent dispensing device we used was a Rotofill III (American Instruments Co.), which can load a 36-place transfer disc in 3.5 min. A Canola SX-310 programmable calculator (Canon U.S.A., Inc., Long Island, NY 11040) was used for data reduction.

Reagents

Reagents were obtained commercially (EMIT*, Syva Corp., Palo Alto, CA 94304). Reagents A and B, specific for carbamazepine, were in lyophilized form, and were prepared for use with some modifications. They were reconstituted with 3 mL of distilled water and allowed to stand at room temperature for 8 h before further dilutions were made. Reagent A was then diluted with 9 mL of diluted buffer (see below), Reagent B with 45 mL of diluted buffer. Both reagents could be used after they had stood for 1 h at room temperature. Between use, these diluted reagents, stored at 2 to 8 °C, were stable for several weeks. Reagent A contains antibodies directed against carbamazepine, glucose-6-phosphate, and NAD+ substrates, and preservatives dissolved in 55 mmol/L tris(hydroxymethyl)aminomethane (Tris) buffer, pH 5.0. Reagent B has carbamazepine coupled to glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and preservatives in Tris-HCl buffer (55 mmol/L, pH 7.9). The antiepileptic drug buffer solution contains 10 mL of Tris-HCl buffer (55 mmol/L, pH 7.9) and a surfactant. This buffer is further diluted with 128 mL of de-ionized water and is stable at room temperature for at least 12 weeks.

University of Virginia Medical Center, Department of Pathology, Clinical Laboratories, Charlottesville, VA 22908.

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Table 1. Procedure for Carbamazepine EMIT Centrifugal Analyzer Assay

<table>
<thead>
<tr>
<th>Sample ring loading protocol</th>
<th>Sample cup contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample position</td>
<td>Sample cup contents</td>
</tr>
<tr>
<td>0</td>
<td>Empty (600 μL of de-ionized water in transfer disc)</td>
</tr>
<tr>
<td>1</td>
<td>Serum blank (Syva Calibrator #0)</td>
</tr>
<tr>
<td>2–6</td>
<td>Standards (Syva Calibrators #1–5)</td>
</tr>
<tr>
<td>7</td>
<td>Quality control</td>
</tr>
<tr>
<td>8–35</td>
<td>Samples</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Loading-disc protocol</th>
<th>Contents* Volume, μL Well position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent A</td>
<td>50            Inner</td>
</tr>
<tr>
<td>Reagent B</td>
<td>200           Middle</td>
</tr>
<tr>
<td>Sample</td>
<td>10            Inner</td>
</tr>
<tr>
<td>Saline diluent</td>
<td>250           Inner</td>
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</tbody>
</table>

Analytical variables

<table>
<thead>
<tr>
<th>Program name</th>
<th>KRIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>30 °C</td>
</tr>
<tr>
<td>Initial reading</td>
<td>105 s</td>
</tr>
<tr>
<td>Sampling interval</td>
<td>15 s</td>
</tr>
<tr>
<td>Wavelength</td>
<td>340 nm</td>
</tr>
<tr>
<td>Conversion factor</td>
<td>1000 (to obtain mA/min)</td>
</tr>
</tbody>
</table>

* Reagents A and B prepared diluted as indicated in text.

b Kinetic Rate I program is supplied with the centrifugal analyzer, see text.

The calibration kit for the EMIT antiepileptic assay contains five drugs: phenytoin, phenobarbital, primidone, ethosuximide, and carbamazepine. Carbamazepine calibrations are available at 1.0, 2.5, 5.0, 7.5, and 10.0 mg/L, and a serum-based blank control also is provided. In addition, a quality-control specimen with a concentration of 6.0 mg/L is available. The lyophilized calibrators, serum blank and quality control were reconstituted as directed by the manufacturer and stored for 8 h at 4 °C before use.1

Procedure

After initial preparation of reagent and buffer solutions, the centrifugal analyzer transfer disc is loaded automatically as indicated in Table 1. The centrifugal analyzer computer program (KRI) takes nine absorbance readings at 15-s intervals after an initial lag time of 105 s. The program performs a linear least-squares fit of these nine absorbance measurements and calculates the absorbance rate for each sample.

Data Reduction

A programmable calculator obtains a logit-log transformation of the absorbance rates and concentrations of the samples. A best-fit nonweighted linear-regression line is obtained with the following form:

\[
\log \left( \frac{(\Delta m)/(k - \Delta m)}{c} \right) = a \log c + b
\]

where:

- \( c \) = concentration of carbamazepine (mg/L)
- \( \Delta m = m_c - m_{ab} \)
- \( m_c \) = rate of absorbance change (mA/min) at a particular concentration
- \( m_{ab} \) = rate of absorbance change (mA/min) of a serum-based carbamazepine-free blank control

\[ a = \text{slope of regression line} \]
\[ b = \text{intercept of the ordinate axis} \]
\[ k = \text{a constant empirically selected to optimize the curve fit} \]

The standard error of the regression line is used to determine the acceptability of the regression line. Absorbance rates for the patients’ samples are then inserted into the computer program and the concentrations are calculated.

Results

The amount of reagents used was initially reduced by twofold and subsequently by fourfold over the manufacturer’s suggestions. Sensitivity (defined as the absorbance rate change per unit of concentration change, \( \Delta A/\Delta t \) per \( \Delta c \)) was reduced by about half with each twofold dilution. An eightfold reduction in reagents failed to yield adequate sensitivity.

Figure 1 shows the curve for absorbance vs. time for various concentrations of carbamazepine. A nonlinear response was seen between the time of initial mixing of reagents and 45 s.

**Fig. 1**. Absorbance vs. time for carbamazepine concentrations of 1.0, 5.0, and 10.0 mg/L, and a carbamazepine-free serum blank (SE)

\[ a = \text{slope of regression line} \]
\[ b = \text{intercept of the ordinate axis} \]
\[ k = \text{a constant empirically selected to optimize the curve fit} \]

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**Fig. 2**. Absorbance rates (\( \Delta A/\Delta t \)) for carbamazepine concentrations of 1 to 10 mg/L, as measured with the centrifugal analyzer

Increasing absorbance rates but with smaller absorbance rate changes per concentration change (\( \Delta A/\Delta t \) per \( \Delta c \)), reflecting decreased sensitivity, is noted at higher concentrations.

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From 45 to 105 s, sensitivity was insufficient (less than 1 mA/min per mg/L). After 500 s, absolute absorbances of higher carbamazepine concentrations approached 2.0 A, the linear spectrophotometric limit of the centrifugal analyzer. Finally, a nonlinear response was detected by the centrifugal analyzer’s multipoint regression-analysis program (KRI) when a total time interval of 200 s was exceeded. These findings led us to select an optimum time interval of 105 to 225 s. Absorbance rates for different carbamazepine concentrations are shown in Figure 2. At higher carbamazepine concentrations, the absorbance rate change per unit of concentration change (\(\Delta A/\Delta t\) per \(\Delta c\)), which reflects sensitivity, was decreased. The sensitivity of our assay was 2 mA/min per mg/L for a concentration range of 1 to 10 mg/L.

### Precision and Recovery Studies

Precision data are shown in Table 2. We evaluated analytical recovery by adding known amounts of carbamazepine (the pure drug, obtained from Geigy Pharmaceutical Co., Summit, NJ 07901) dissolved in water/methanol (1/1) to a carbamazepine-free serum pool; the results are shown in Table 3. In addition, carbamazepine standard materials obtained commercially (Syva Corp.) were measured by our method. We could account for an average 96% of the added drug for a series of carbamazepine concentrations ranging from 2 to 12 mg/L.

### Correlation Studies

Our centrifugal analyzer method was compared to a “high-pressure” liquid chromatographic procedure used with a modified Kobra et al. method (2). The correlation data for 24 patients’ samples are seen in Figure 3.

In addition, we compared our method to a gas–liquid chromatographic procedure as described by Grove et al. (3). The correlation coefficient was 0.91, with a slope of 0.90, and a y-intercept of 0.75 mg/L for 31 patients’ samples containing carbamazepine.

### Interference Studies

There was no detectable cross reactivity as measured by our procedure with other antiepileptic drugs, including phenobarbital at concentrations up to 60 mg/L, phenytoin at 29 mg/L, primidone at 15 mg/L, and valproic acid at 72 mg/L. However, hemoglobin definitely interferes with our method. Various hemoglobin concentrations, prepared by lysing erythrocytes with distilled water, were added to patients’ sera with known carbamazepine concentrations and the assay results are shown in Table 4.

Lipemic, uremic (serum urea nitrogen, 550 mg/L) and icteric (total bilirubin, 58 mg/L) sera did not interfere with our assay. Also, protein in concentrations as high as 9.0 mg/L did not detectably interfere. These studies were performed by mixing pooled patients’ sera containing known concentrations of carbamazepine, urea nitrogen, total bilirubin, and protein with carbamazepine-free serum containing excessive concentrations of the interfering substance. Specimens containing carbamazepine stored at 4 °C for three months showed no significant change in concentration as measured by our method.

### Discussion

The EMT method adapted to a centrifugal analyzer has several advantages. Much less sample, 10 \(\mu\)L, is used in comparison to the larger volumes required for gas-chromatographic techniques. In addition, our method does not suffer from the poor precision seen in some gas-chromatographic methods, originating from unstable thermal decomposition of carbamazepine to iminostilbene (1). Also, at the present time, there is no reliable internal standard for carbamazepine in gas-chromatographic methods. The enzyme immunoassay technique requires no sample preparation, unlike other conventional methods, and up to 150 samples can be assayed per
hour. The enzyme immunoassay technique avoids the use of radioactive labels of radioimmunoassay methods.

By adapting the EMIT carbamazepine assay from the semi-automatic spectrophotometers to the centrifugal analyzer, reagent costs were reduced fourfold. Precision and recovery compared very well to that with "high-pressure" chromatography and gas-liquid chromatography. We use a multi-point kinetic rate program with appropriate linearity checks on the obtained absorbance measurements. In this analysis nine absorbance readings are made instead of the two readings in the more conventional methods, which should give a better statistical representation of the absorbance rates.

Recently, the Syva Corporation has modified EMIT reagents A and B, allowing them to detect a wider concentration range of carbamazepine, 2–20 mg/L. We found a 20% decrease in sensitivity when we used these new reagents, but this did not seriously affect our assay.

The EMIT assay adapted to the centrifugal analyzer does have some disadvantages. Only one drug can be analyzed at a time, but the potential sample throughput more than compensates for this inconvenience. Hemolysis definitely suppresses carbamazepine concentrations obtained by the enzyme multiple immunoassay technique, a finding similar to that of Finley et al., who reported similar interference patterns when adapting phenytoin and phenobarbital to a centrifugal analyzer (4).

We thank Dr. Joseph Keffner and Ms. Judy Hill (Anderson Memorial Hospital, SC) for performing HPLC on patients' specimens, which aided in our correlation studies. We also thank Dr. Donald Besemer (Syva) and Dr. G. E. Pippenger (Columbia University College of Physicians and Surgeons, New York) for their valuable suggestions. Finally, we are deeply indebted to Ms. Judy Sinn (University of Virginia Medical Center Toxicology Laboratory) for her expert technical assistance throughout this project.

References

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Stability of Dilute Solutions of Gentamicin and Tobramycin

L. Josephson, P. Houle, and M. Haggerty

Dilution of sera containing gentamicin or tobramycin in glass containers results in substantial adsorption of the antibiotic to the surface of the container, which can be prevented if acidic or basic solutions are used to dilute such sera. We describe protocols for radioimmunoassays of gentamicin and tobramycin that include solutions and containers that obviate this problem.

Radioimmunoassays are used to monitor serum concentrations of gentamicin and tobramycin compounds, to ensure that therapeutic concentrations have been achieved and toxic concentrations avoided (1–3). The sensitivity of many radioimmunoassays is such that patients' sera (and standards) must be diluted before incubation with antibody and tracer. The antibiotic in these dilute solutions tends to adsorb to the walls of glass containers, complicating accurate measurement of its concentration in serum.

Adsorption is a function of the nature of the container, duration of incubation in the container, the antibiotic concentration present, and the pH of the medium. Because so many variables affect adsorption, the simpler course is to avoid it. We present here a protocol for radioimmunoassays of gentamicin and tobramycin that is free from such interference.

Materials and Methods

Antibodies to gentamicin and tobramycin were raised in rabbits by administering these compounds attached to bovine serum albumin as described by others (1–3). Antibody was coated on the surface of polypropylene tubes by our usual production process (4). 125I-labeled tracers were prepared as described (2). Serum standards were prepared by augmenting normal human serum with a concentrated stock solution of antibiotic (final concentrations, 1 to 16 mg/L). Adsorption experiments were performed as follows. Adsorption was started by adding 10 µL of standard to 1 mL of buffer in the appropriate containers and then incubating at 24 °C; it was stopped by withdrawing 100 µL of the diluted gentamicin standard or 25 µL of the diluted tobramycin standard from the adsorbing container and placing it in the bottom of the antibody-coated tube. The assay was started by adding 1 mL of phosphate-buffered saline containing bovine serum albumin (1 g/L) and tracer. After 60 min at 37°C, the contents of the tube were aspirated to separate free from bound, and the bound tracer was counted.

Glassware used in the study was from the following sources: Becton-Dickinson (12 x 75 mm) RTU culture tubes, Corning (13 x 100 mm) Pyrex tubes, Elkay Products (12 x 75 mm) polypropylene tubes, and Kimble (10 mL) clear glass vials.

Clinical Assays, Division of Travenol Laboratories, Inc., 620 Memorial Drive, Cambridge, MA 02139.
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