drugs in plasma. However, most such methods are designed to involve a simple, rapid extraction. Some of these extraction procedures are poor, failing to remove all compounds that might interfere with the assay, contaminate the column and injection system (1–4), or cause low analytical recoveries (5).

Here we describe a rapid, one-step liquid-extraction procedure in which precipitation of plasma proteins is complete and interfering compounds in plasma are eliminated. The result is a rapid HPLC method for simultaneously determining ethosuximide, phenobarbital, phenytoin, and carbamazepine in plasma.

We used a series 600 HPLC system (Kontron AG, Zurich, Switzerland), a visible-ultraviolet variable-wavelength-light detector (Uvikon 720 LC, Kontron AG, a Kontron microprocessor, Model 200, and a Model 3390A integrator (Hewlett-Packard, Avondale, PA). The reversed-phase analytical column was a Spherisorb 5 ODS, 250 × 4.6 mm (Phase Separations Ltd., Queensferry, U.K.), protected with a 50 × 4.6 mm guard-column and packed with CO-PELL ODS (Whatman Inc., Clifton, NJ). Chromatography was at room temperature, with a mobile phase (acetonitrile/methanol/phosphate buffer pH 4.0, 21/24/55 by vol) flow rate of 3 mL/min and a detector wavelength of 195 nm.

The internal-standard solution was prepared daily by diluting in water a stock standard of 5-(p-tolyl)-5-phenylhydantoin (1 g/L in methanol) to give a concentration of 30 mg/L.

Plasma was extracted as follows: In a 10-mL glass-stoppered centrifuge tube place 200 µL of plasma and 200 µL of internal-standard solution, and vortex-mix for 5 s. Add one drop of 5 mol/L HCl and again vortex-mix for 5 s. Then add 2.5 mL of dichloromethane, stopper the tube, and vortex-mix for 1 min. Using a calibrated spatula, add sufficient solid ammonium sulfate to saturate the aqueous layer and vortex-mix for 30 s. Centrifuge (5000 rpm, about 5 min). Aspirate and discard the aqueous (upper) layer with a Pasteur pipette. Carefully separate the precipitate and transfer the lower layer to a 10-mL conical glass tube. Pipette 2 mL of the organic layer into another conical glass tube and evaporate it in a water bath at 42 °C. Redissolve the residue in 50 µL of a mixture of acetonitrile/methanol/water (instead of buffer) with the same volume relationships as the mobile phase and inject 20 µL into the chromatograph.

Quantify by using the peak-height ratios. One obtains a linear response for each of the four drugs in the following ranges: ethosuximide 10–150 mg/L, phenobarbital 5–80 mg/L, phenytoin 2.5–30 mg/L, and carbamazepine 2–20 mg/L. Analytical recoveries varied from 92 to 100%, with within-day CVs from 0.9 to 4.6%, and between-day CVs from 0.9 to 4.2%.

We tested the following drugs for potential interference: theophylline, acetaminophen, acetylsalicylic acid, phenylethylmalonamide, chlorpromazine, primidone, p-hydroxyphenytoin, carbamazepine 10,11-epoxide, pentobarbital, clonazepam, diazepam, methaqualone, and flunitrazepam. Of these drugs, only chlorpromazine co-elutes with ethosuximide, but this is not a serious interference because these two drugs are not co-prescribed.

Extraction with an organic solvent in the presence of added inorganic salt is a suitable method for plasma protein precipitation (6). Dichloromethane is a good extraction solvent because evaporation is fast at 42 °C, thus minimizing losses of the highly volatile ethosuximide. The extraction with dichloromethane at low pH in combination with an excess of ammonium sulfate allows complete precipitation of plasma proteins, which otherwise would contaminate the chromatographic system, and it also eliminates some lipids and other plasma components that generally appear as early-eluting peaks and may interfere with the assay. It is advisable to add the ammonium sulfate before the drugs are extracted into the dichloromethane, to prevent coprecipitation of any drug trapped in the precipitate.

The relatively high concentration of acetonitrile in the mobile phase diminishes back-pressure at room temperature. Moreover, the absence of interfering peaks at the beginning of the chromatogram (Figure 1) allows the use of a high flow rate, which in turn speeds the analysis and improves the shape of the later peaks. The use of an injection solvent with a composition similar to that of the mobile phase also improves the shape of peaks, and the use of water instead of phosphate buffer in this mixture avoids the problem of inorganic salt precipitating in the injection system.

References


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Visual Detection of Abnormal Hemoglobin with a Procedure for Glycated Hemoglobin

To the Editor:

The value of measuring glycated hemoglobin in the management of patients with diabetes mellitus is well documented (1), and the use of cation-exchange chromatography to do so is well accepted (2). Abnormal hemoglobins may give spurious values by these methods (3, 4). We find that visual inspection of the Isolab Fast Hemoglobin Test System (5) columns after the fast hemoglobin (Hb A1) fraction is eluted can lead to detection of abnormal hemoglobins.

Studying glycated hemoglobins in sickle-cell heterozygotes and homozygotes, we observed differences in the appearance of the columns (Figure 1) after the glycated hemoglobins were eluted (6). Glycated hemoglobins S, C, and D are not eluted from the column with the
We suggest that all users of Isolab columns look for the bands, because this is a useful screening procedure for certain abnormal hemoglobins.

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Evaluation of a New Du Pont aca Test Pack for Theophylline

To the Editor:

Patients vary in their responses to a given dose of theophylline. Thus, problems arise both in achieving and maintaining an effective concentration in serum and in under- and overdosage. A large proportion of our requests for theophylline are ordered as urgent procedures throughout the day. Naturally, we were intrigued by the possibility of being able to use our Du Pont aca III to do these tests.

Here we describe our evaluation of the new aca test pack for theophylline and compare the results with those obtained with our routine theophylline procedure (EMRT-aad; Syva Co.).

The homogeneous enzyme immunoassay (EMIT) methodology involves competitive protein binding with an enzyme as label and an antibody as the specific binding protein; enzyme activity is directly related to the concentration of theophylline in the sample (1). The new Du Pont aca methodology, which bears the acronym PETINIA (Particle Enhanced Turbidimetric INhibi- tion Immuno Assay), is also a competitive binding assay. It involves a latex particle tag and theophylline-specific antibody; theophylline is measured turbidimetrically (2).

We used 94 patients’ samples, submitted for urgent theophylline assay. No attempt was made to categorize these samples by age or sex. The comparative statistics showed the mean of aca values to be 13.07 vs 12.78 mg/L for the mean of the EMRT values. Linear regression analysis yielded a slope of 1.02, an intercept of −0.61, and a correlation coefficient of 0.98; within-run precision (1 SD) was 0.3 mg/L; CV was 1.9%. Our regression values and CVs all compare very favorably with the slope of 0.88 to 1.03, correlation coefficient of 0.986, and CV of 2.4% or less reported by the supplier. When theophylline standard was added to these patients’ samples within the range of 10–20 mg/L, analytical recovery varied from 101% to 104%.

Fig. 1. Appearance of the columns after elution of glycated hemoglobin
Column A, sample from a normal AA patient; B, from a heterozygous AS patient; C, from a homozygous SS patient.