Automated Determination of Drugs in Serum by Liquid Chromatography with Column-Switching.  
I. Separation of Antiepileptic Drugs and Metabolites

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This is a fully automated system for determining six common antiepileptic drugs and two principal metabolites of carbamazepine in serum. It is based on "high-performance" liquid chromatography (HPLC), with column switching. TSKprecolumn BSA-ODS and TSKgel ODS-120A (both from Toyo Soda) were used as the precolumn and analytical column, respectively. The former contains octadeacylil resin treated with bovine serum albumin (BSA), and does not adsorb macromolecules such as serum proteins but retains small lipophilic molecules such as antiepileptic drugs. Serum samples are directly injected onto the precolumn. After washing out the serum proteins from the precolumn with sodium phosphate buffer, we switch the column connections to introduce the retained substances onto the analytical column and elute with a step-gradient of acetonitrile/sodium phosphate buffer. The high analytical recovery (95–102%) and the reproducibilities (CV <5% within-run) indicate that this system is suitable for use in therapeutic drug monitoring in clinical laboratories.

Antiepileptic drugs currently are determined by immunological and chromatographic methods. Immunological methods are very rapid and sensitive, but permit determination of only one compound at a time. Chromatographic methods are more tedious, but they permit determination of various drugs and metabolites in one operation, an advantage particularly important for determination of antiepileptic drugs in clinical laboratories because treatment of epilepsy usually requires concurrent determination of two or more drugs. Furthermore, some of these drugs are metabolized into active species that should also be monitored in assessing the relationship between dose and clinical effects.

Pretreatment of samples by solvent extraction (1-6), deproteinization (7–10), or solid-phase extraction (11) has been applied before to determinations of antiepileptic drugs in biological fluids by HPLC. Recently, fully automated pretreatment procedures that include direct injection of body fluids have been developed for HPLC, aided by the use of a multicolumn system and a column-switching device (12, 13). We have applied these column-switching techniques and a precolumn packed with BSA- precoated octadeacylil (ODS) resin to determine several antiepileptic drugs in human serum: ethosuximide, primidone, phenobarbital, carbamazepine, phenytoin, carbamazepine-10,11-epoxide (CBZ-epo), and carbamazepine-10,11-dihydroxide (CBZ-OH).2

Materials and Methods

Materials

Reagents. Primidone and phenytoin (Dainippon, Tokyo, Japan); ethosuximide (Kodama, Tokyo); sodium valproate (Kyowa, Tokyo); carbamazepine, CBZ-epo, and CBZ-OH (Ciba-Geigy, Basel, Switzerland); and hexobarbital (Tokyo-kasei, Tokyo) were used as external and internal standards. Acetonitrile ("HPLC grade") was from E. Merck, Darmstadt, F.R.G.; other chemicals were A.S grade.

Standard solutions. Standard stock solutions were prepared by dissolving sodium valproate (10 g/L) in distilled water and ethosuximide (10 g/L) and the other drugs (1 g/L) in acetonitrile. We removed the solution from the mixed standard solution under a stream of nitrogen at room temperature, then dissolved the residues in drug-free human serum.

Serum samples. We assayed patients' sera for which measurement of antiepileptic drugs had been requested.

Apparatus. All of the apparatus and all but one column were from Toyo Soda Manufacturing, Tokyo, Japan. The fully automated HPLC system consisted of a TOSO CP-8000 series chromatograph, a Model CCPM pump, a Model UV-8000 variable-wavelength detector, a Model CP-8000 chromatoprocessor, a Model PT-8000 column-switching valve system (two six-point switching valves), and a programmable timer. We also used a Model AS-48 autosampler equipped with a cooling system and a Model CO-8000 column oven.

The columns used were a 40 × 4.0 mm (i.d.) TSKprecolumn BSA-ODS, a 250 × 4.0 mm (i.d.) TSKgel ODS-120A, a 150 × 4.0 mm (i.d.) TSKgel ODS-80TM, and a 250 × 4.0 mm (i.d.) LiChrosorb RP-18 (E. Merck).

Procedures

Conventional HPLC for comparison analysis. Serum samples containing added phenobarbital and phenytoin (each 20 mg/L), carbamazepine (10 mg/L), and sodium valproate (100 mg/L) were deproteinized by mixing with an equal volume of acetonitrile.

To deproteinize patients' sera, we used acetonitrile containing 10 mg of hexobarbital (internal standard) per liter. After centrifuging the mixtures (1000 × 10 min), we analyzed aliquots of the supernates by HPLC on the LiChrosorb RP-18 column. The chromatographic conditions were as follows: column temperature, room temperature; mobile phase, acetonitrile/sodium phosphate buffer (0.1 mol/L, pH 5.7), 30/70 by vol; flow rate, 1.0 mL/min; and detection wavelength, 210 nm.

Immunological method. For comparison, we used the aca III discrete analyzer (DuPont Instruments, Wilmington, DE) and its associated reagents for antiepileptic drugs, without modification.

Automated HPLC with Column-Switching

Pretreatment. We used TSKprecolumn BSA-ODS to deproteinize serum samples. After injecting 20 μL of serum sample onto the precolumn, which had been previously equilibrated with sodium phosphate buffer (0.1 mol/L, pH 5.2), we washed the column for 8 min with the same buffer
at a flow rate of 1.0 mL/min. Under these conditions, excess serum proteins were washed out, whereas antiepileptic drugs and the metabolites were completely adsorbed onto the pre-column. The substances retained on the pre-column were then quantitatively eluted within 4 min with a mixture of acetonitrile and the sodium phosphate buffer (22/78 by vol), at a flow rate of 1.0 mL/min. We used the back-flush mode to elute the drugs and metabolites from the column. This minimized the volume of effluent containing the drugs and metabolites.

After switching the columns so that the drugs and metabolites were eluted onto the analytical column, we returned the six-point valve to its original position. The pre-column was then washed with a mixture of acetonitrile and the sodium phosphate buffer (35/65 by vol) and equilibrated with the same sodium phosphate buffer until the next injection.

Separation of antiepileptic drugs and metabolites. We developed chromatographic methods for two reversed-phase columns: TSKgel ODS-80TM (Method A) and TSKgel ODS-120A (Method B). In both we used a step-gradient elution, slightly different from that in the conventional HPLC, at a flow rate of 1.0 mL/min, to separate antiepileptic drugs and the metabolites. The step-gradient was started just after the pre-column had been washed with the sodium phosphate buffer for 8 min. The step-gradient conditions were as follows: from 0 to 12 min, acetonitrile/sodium phosphate buffer (22/78 by vol); from 12 to 25 min, acetonitrile/sodium phosphate buffer (35/65 by vol) for Method A, or acetonitrile/sodium phosphate buffer (32/68 by vol) for Method B. The column temperatures were room temperature for Method A, 40 °C for Method B.

Results
Chromatograms of Antiepileptic Drugs and Metabolites
Figures 1A and 2A illustrate typical chromatograms for human serum containing added antiepileptic drugs and metabolites. In Method A (Figure 1), the order of elution...
was ethosuximide, primidone, CBZ-OH, CBZ-epe, phenobarbital, carbamazepine, phenytoin, and valproic acid. In Method B, it was ethosuximide, primidone, CBZ-OH, phenobarbital, CBZ-epe, phenytoin, carbamazepine, and valproic acid. Carbamazepine and carbamazepine metabolites were more efficiently retained on TSKgel ODS-120A than on TSKgel ODS-80TM.

When serum of a patient treated with phenobarbital, phenytoin, carbamazepine, and valproic acid was analyzed by the two methods, two peaks other than the four peaks derived from the four administered drugs were clearly detected (Figures 1B and 2B). Retention times of peak 7 in Figure 1B (peak 8 in Figure 2B) and peak 8 in Figure 1B (peak 4 in Figure 2B) were respectively the same as those of authentic CBZ-OH and CBZ-epe.

The chromatographic behavior of valproic acid was affected by the pH of the mobile phases. Valproic acid was eluted after phenytoin (TSKgel ODS-80TM) or carbamazepine (TSKgel ODS-120A) at pH 5.2, but it was eluted between phenobarbital and carbamazepine (TSKgel ODS-80TM) or between CBZ-epe and phenytoin (TSKgel ODS-120A) at pH 5.7. Thus the pH of the mobile phase is critical for the separation of these substances. Because separation of phenobarbital and CBZ-epe was insufficient by Method A, we used Method B in the following experiments.

Analytical Recovery of the Drugs and Metabolites

Recovery of the drugs and metabolites in pooled human serum was calculated by comparing their peak areas with those obtained by injecting authentic standards directly onto the analytical column. Analytical recoveries (n = 3) for all the substances examined ranged between 95 to 104% at three different concentrations (mg/L): phenobarbital (5, 10, 20), 98.3, 96.6, 97.5%; phenytoin (5, 10, 20), 98.4, 99.0, 98.4%; carbamazepine (2.5, 5, 10), 100.8, 98.4, 97.0%; valproic acid (25, 50, 100), 104.4, 95.5, 101.8%; CBZ-epe (1.25, 2.5, 5), 98.4, 98.5, 99.5%; CBZ-OH (1.25, 2.5, 5), 98.0, 94.5, 98.0%, respectively.

We studied absolute recovery of each compound from serum that had been subjected to various deproteinization procedures. Human serum containing 20 μg of phenobarbital and phenytoin, 10 μg of carbamazepine, and 100 μg of valproic acid per milliliter was deproteinized by adding an equal volume of acetonitrile, perchloric acid (50 g/L), or trichloroacetic acid (100 g/L), and centrifuged at 1000 × g for 10 min. We assayed 20 μL of the supernate by the conventional HPLC without internal standard. The results (n = 3) for phenobarbital, phenytoin, carbamazepine, and valproic acid were as follows: acetonitrile, 94, 97, 94, 91%; perchloric acid, 84, 35, 35, 79%; and trichloroacetic acid, 79, 28, 30, 61%, respectively. The recovery achieved by our column-switching procedure with TSKprecolumn BSA-ODS was comparable to that obtained on using deproteinization procedures with 50% acetonitrile and far superior to that with the other two procedures.

### Table 1. Within-Run Reproducibility (n = 10)

<table>
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<tr>
<th>Conc, μg/mL</th>
<th>Phenobarbital</th>
<th>Phenytoin</th>
<th>Carbamazepine</th>
<th>Valproic acid</th>
<th>CBZ-epe</th>
<th>CBZ-OH</th>
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<td>5.0</td>
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<td>Conc, μg/mL</td>
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<td>CV, %</td>
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<td>0.34</td>
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Linearity and Precision

Calibration curves showed a good linear relationship between peak area and analyte concentration in the range 2 to 20 mg of phenobarbital and phenytoin, 10 to 100 mg of valproic acid, 1 to 10 mg of carbamazepine, and 0.5 to 5 mg of CBZ-epe and CBZ-OH per liter (data not shown). Sensitivity (expressed as mV · s· mL/μg) for ethosuximide, primidone, CBZ-OH, phenobarbital, CBZ-epe, phenytoin, carbamazepine, and valproic acid (20 μL injection volume, 210 nm) was calculated to be 13, 75, 165, 108, 229, 117, 162, and 1, respectively. Detection and determination limits of this method were about 10 and 20 mV · a.

Table 1 shows the within-run reproducibility of our method. The coefficients of variation (CV) ranged from 0.34 to 4.42% for all the drugs examined at three different concentrations, except for valproic acid. The between-run CVs (n = 10) for this method were evaluated for single concentrations of the drugs (mg/L): phenobarbital (20), 1.9%; carbamazepine (20), 2.9%; phenytoin (20), 3.1%; and valproic acid (100), 5.6%.

A comparison of the data (our method vs conventional HPLC or EMIT) by regression analysis showed a high correlation. For the present method vs EMIT: phenobarbital, n = 80, r = 0.976, y = 0.977x – 0.11; phenytoin, n = 80, r = 0.989, y = 0.984x – 0.07; carbamazepine, n = 90, r = 0.943, y = 0.732x + 0.23; valproic acid, n = 70, r = 0.936, y = 10.93x – 2.47. For the present method vs conventional HPLC: phenobarbital, n = 80, r = 0.989, y = 0.968x + 0.55; phenytoin, n = 80, r = 0.975, y = 0.958x + 0.33; carbamazepine, n = 90, r = 0.969, y = 1.000x + 0.08; valproic acid, n = 70, r = 0.940, y = 0.972x + 9.26. Carbamazepine values as determined by our method and conventional HPLC were 27% lower than those by the EMIT method. The discrepancies could be caused by the differences of carbamazepine standard preparations used, because, when carbamazepine (product of Ciba-Geigy) was used as a standard for EMIT method, the value was about 30% lower than the value determined by our HPLC method. Moreover, the cross reactivity of CBZ-epe with carbamazepine was about 10% with the EMIT method; however, that of CBZ-OH was negligible.

Discussion

HPLC is not widely used to measure antiepileptic drugs in serum in clinical laboratories, owing to the health-hazardous pretreatment procedures. Column-switching techniques overcome this disadvantage by direct injection of serum onto the chromatographic column. Pretreatment precolumns such as gel permeation (14) or reversed-phase columns (12, 13) have been used for column-switching techniques including direct injection of biological fluids without sample pretreatment. When a gel-permeation column is used as the precolumn, the column-switching program should be carefully set to minimize the volume of effluent from the precolumn if recovery and reproducibility...
are to be adequate. When conventional reversed-phase columns are used as the precolumn, large amounts of serum proteins are retained on the gel surface of the pre-column and may be introduced to the analytical column. A very short cartridge (length 0.5 cm) packed with ODS silica gel was successfully used as the precolumn (13). Because of the low capacity of the column, however, only small sample volumes (less than 50 µL) should be injected, and the flow rate of mobile phase (0.3 mL/min) should be low, to obtain sufficient recovery of the drugs. Therefore, we used the TSKprecolumn BSA-ODS (15), prepared by covalent bonding of BSA to ODS silica gels (20- to 30-µm particle sizes and 8-nm pore size) as a precolumn. Because BSA covers only the surface of the resins, serum proteins easily pass through the precolumn, while small molecules can be adsorbed onto the remaining active ODS on the inside surface of the resins.

In the present study, correction of the values by use of an internal standard was not necessary, because automated pretreatment with TSKprecolumn BSA-ODS permitted determination of the drugs in serum with sufficient reproducibility and quantitative recovery. The theoretical analytical recovery of the drugs indicated that the values obtained by this method represented their total concentrations. Furthermore, not only six kinds of antiepileptic drugs frequently used in the treatment of epilepsy but also CBZ-epo, an active metabolite of carbamazepine, were determined simultaneously in directly injected serum samples.

There were, however, some differences in chromatographic behaviors of the drugs in the two types of reversed-phase column. The resins of TSKgel ODS-80TM are coated with an end-capped monolayer ODS on 8-nm (pore size) silica gel, while those of TSKgel ODS-120A have a non-end-capped polylayer ODS on 12-nm (pore size) silica gel. The differences in the chromatographic behaviors of carbamazepine and CBZ-epo may be caused by unreacted silanol groups. Despite the slight tailing of the peaks and low resolution, TSKgel ODS-120A column was better than TSKgel ODS-80TM for measurement of antiepileptic drugs in the case of concurrent administration of phenobarbital and carbamazepine, because a metabolite, CBZ-epo, is clearly separated from phenobarbital by use of TSKgel ODS-120A.

The values for antiepileptic drugs in patients' sera obtained by our method coincided well with those by conventional HPLC and EMIT methods except for the carbamazepine values by EMIT method. The reason for the different values for carbamazepine by the two methods is caused by the external standards. The determination of valproic acid (<20 µg/mL) was unreliable because of low sensitivity for valproic acid; the injection volume would be better increased to 100 µL. Similar results for analytical recovery and lifetimes of the columns were obtained by injecting 200-µL serum samples. Over 100 serum samples could be assayed with no deterioration of precolumn or analytical column; however, the end-fittings of the columns should be replaced after each 50 samples.

References