Automated Determination of Drugs in Serum by Column-Switching High-Performance Liquid Chromatography. IV. Separation of Tricyclic and Tetracyclic Antidepressants and Their Metabolites

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We describe automated column-switching high-performance liquid chromatography for determining nine tricyclic and tetracyclic antidepressants (TCAs) and their metabolites in human serum. TSKgel ODS-80TM and TSKprecolumn PW (Tosoh Co., Tokyo) are used in the analytical column and the precolumn, respectively. A 200-μL serum sample is directly injected onto the precolumn. After washing the serum proteins from the precolumn with potassium phosphate buffer, the precolumn connection is switched to introduce the retained substances onto the analytical column. The drugs are then eluted within 30 min with an acetonitrile/potassium phosphate buffer mixture containing sodium 1-heptanesulfonate. The analytical recoveries (95–104%), reproducibilities (within-run CV <3%), and detection limits (10 μg/L) indicate that this HPLC system is suited for therapeutic drug monitoring. Correlations were good between the TCA concentrations in serum and administered dose (r = 0.713, n = 41), and between 10-hydroxynortriptyline and nortriptyline in serum (r = 0.691, n = 24).

Tricyclic and tetracyclic antidepressants (TCAs) commonly used in the treatment of depression display marked interindividual differences in the steady-state concentrations of the drugs in serum (1–3). Because of their undesirable anticholinergic effects and cardiac toxicities, the concentrations of TCAs in serum should be kept in the lowest effective range. Furthermore, hydroxylated and desmethylated metabolites of TCAs detected in serum (4) have similar pharmacological activities and side effects. Measurement of TCA concentrations such as imipramine, desipramine, and nortriptyline in serum is helpful for optimized therapy (5).

High-performance liquid-chromatographic (HPLC) methods used to determine drugs and their metabolites (6) include normal-phase (7–10), reversed-phase (11–16), and ion-pair chromatography (17). Reported procedures for sample preparation have been based on solvent extraction from alkali media (7, 8, 10, 14) and further back-extraction into acidic solution (9, 11), ion-pair extraction (17), solid-phase extraction (15), or an automated sample processor (12, 13). One (9–14, 16) or two kinds (7, 8, 15) of internal standards have been used to correct for extraction efficiencies.

We developed a column-switching HPLC method for the automated determination of TCAs in serum. This method shows excellent absolute recovery and precision, and permits determination of any of nine TCAs within 30 min.

Materials and Methods

Reagents: Amitriptyline HCl (Merck-Banyu, Tokyo, Japan), desipramine HCl, maprotiline HCl, imipramine HCl, and clomipramine HCl (Ciba-Geigy Japan, Takarazuka, Japan), nortriptyline HCl (Dainippon, Osaka, Japan), trimipramine maleate (Shionogi, Osaka, Japan), amoxapine base (Lederly Japan, Tokyo, Japan), mianserin HCl (Organon Japan, Tokyo, Japan), and doxepin (Pfizer, New York, NY) were kind gifts from their manufacturers. The TCA metabolites—2-hydroximipramine oxalate, 10-hydroxydesipramine fumarate, desmethy1clomipramine HCl, 2-hydroxycloclomipramine base, 10-hydroxycloclomipramine base, 8-hydroxymianserin maleate, desmethy1mianserin HCl, 10-hydroxynortriptyline base, desmethy1maprotiline mesylate, 7-hydroxyamoxapine base, and 8-hydroxyamoxapine base—were also gifts from the corresponding companies.

Acetonitrile and methanol were of "HPLC" grade (E. Merck-Kanto, Tokyo, Japan). Sodium 1-heptanesulfonate, "ion-pair chromatographic grade," was purchased from Nakarai, Osaka, Japan.

Standard solution of drugs: Stock solutions of each drug were prepared by dissolving each in methanol to give a concentration of 1.0 g/L. Working standard solutions were prepared by appropriate dilution of the stock solutions with the mobile phase or human pooled serum to give concentrations ranging from 25 to 100 μg/L.

Serum samples: Serum samples were collected in the morning before the first daily dose, from patients whose dosages had been the same for at least two weeks.

Apparatus

The components of the column-switching HPLC system were from the TOSO CP-8000 series (Tosoh Co., Tokyo, Japan): a Model CCPM solvent feeder, a Model UV-8000 variable-ultraviolet detector, a Model CP-8000 chromatoprocessor, a Model FT-8000 column-switching device, and a Model AS-88 autosampler. TSKprecolumn PW (40 × 4.0 mm i.d.) and TSKgel ODS-80TM (50 × 4.0 mm i.d.), both from Tosoh Co., were used to fill the precolumn and the analytical column, respectively.
Automated Column-Switching HPLC

A 200-μL serum sample was injected directly onto TSKprecolumn FW. Because of the efficiency with which TCAs are adsorbed on the precolumn and to eliminate proteins and organic acids in serum from the precolumn, we used a potassium phosphate buffer (50 mmol/L, pH 7.5) as the eluent. After washing the serum proteins from the precolumn at a flow rate of 1.0 mL/min for 10 min, we switched the connection of the columns so that the substances retained in the precolumn would be eluted onto the analytical column with acetonitrile/potassium phosphate buffer (100 mmol/L, pH 2.7), 32.5/67.5 by vol, containing 0.2 g of sodium 1-heptanesulfonate per liter, at a flow rate of 1.0 mL/min for 4 min. TCAs were introduced onto the analytical column by back-flushing the precolumn. We washed the precolumn with acetonitrile/water (60/40 by vol) for 6 min, then with potassium phosphate buffer (50 mmol/L, pH 7.5) for 10 min before injecting the next sample. Nine different TCAs were eluted in this system (flow rate 1.0 mL/min) within 30 min. The effluent was monitored at 210 nm.

For simultaneous determination of TCAs and their metabolites, we adjusted the proportions of acetonitrile/potassium phosphate buffer (100 mmol/L, pH 2.7) containing sodium 1-heptanesulfonate, as above. The volume proportions in step-gradient elutions were as follows: amoxapine, 22.5/77.5 (0 to 10 min) to 30/70; mianserin, 22.5/77.5 (0 to 6 min) to 32.5/67.5; imipramine and amitriptyline or nortriptyline, 27.5/72.5 (0 to 8 min) to 32.5/67.5; and maprotiline and clomipramine, 32.5/67.5, isocratic.

Results

Separation of TCAs

Figure 1 illustrates typical chromatograms of human pooled serum and human serum supplemented with 500 μg of each of nine different TCAs. Retention of these drugs on the reversed-phase column was influenced by the pH of the mobile phase, so we compared the retention behaviors of TCAs when we used as mobile phases buffers of different pH, ranging from 2.0 to 7.0. Guided by the results, the pH we finally selected for the phosphate buffer was 2.7. Desipramine could be resolved from an unidentified peak in human serum by adding 0.2 g of sodium 1-heptanesulfonate per liter to the mobile phase. Although nortriptyline and imipramine were not well enough resolved to be determined, the nine different TCAs were separated from one another within 30 min, and interference by peaks produced by components of human serum was negligible.

Analytical Recovery and Precision

Table 1 summarizes our data for analytical recoveries of TCAs in human serum. Peak areas for TCAs added to human serum obtained by use of this system were compared with those in the mobile phase without pretreatment. Vessels made of polyethylene were used in this experiment, because of the significant adsorption of authentic TCAs onto glassware. Analytical recoveries of TCAs in 200-μL serum samples from the precolumn ranged from 95% to 104%, and were not affected by the injection volumes of serum samples in the range from 10 to 500 μL.

Precision data obtained for three different concentrations of TCAs and day-to-day reproducibilities are presented in Table 2. Within-run precision and day-to-day reproducibility for TCAs determined by this method ranged from 1% to 3% and from 2% to 4% in terms of their CVs.

Calibration Curves and Detection Limit

We prepared calibration curves for TCAs in human serum in concentrations ranging from 25 to 1000 μg/L (data not shown). Good linear regressions with negligible constant bias were obtained between the peak areas and TCA concentrations. Detection limits for TCAs by this method were calculated to be about 10 μg/L, or 10 nM·s of peak areas.

Measurement of TCAs in Patients’ Serum

Concentrations of TCAs measured in serum of patients being treated with amitriptyline, amoxapine, maprotiline,

| Table 1. Analytical Recovery of Some Tricyclic Antidepressants |
|------------------|-----------|----------|----------|
|                  | Concentration of TCAs, μg/L | Recovery, % |
|                  | 500               | 250               | 100                  |
| Amoxapine       | 103.2            | 101.3             | 104.1                |
| Doxepine        | 95.8             | 97.1              | 101.1                |
| Desipramine     | 98.9             | 99.2              | 99.1                 |
| Nortriptyline   | 100.0            | 99.7              | 100.6                |
| Imipramine      | 98.8             | 97.3              | 98.6                 |
| Amitriptyline   | 100.4            | 97.3              | 98.6                 |
| Maprotiline     | 96.7             | 98.3              | 97.9                 |
| Trimipramine    | 97.7             | 95.5              | 102.9                |
| Clomipramine    | 95.7             | 98.3              | 100.9                |

| Table 2. Within-Run and Day-to-Day Reproducibility |
|-----------------|---------|---------|---------|
| (n = 10)        |         | Within-run CV, % | Day-to-day CV, % |
|                  | Concentration of TCAs, μg/L | 500               | 250               | 100               | 500               |
| Amoxapine        | 1.93    | 1.95               | 1.34               | 4.17               |
| Dextropramine    | 0.47    | 0.49               | 0.63               | 2.11               |
| Desipramine      | 1.70    | 1.21               | 1.92               | 3.59               |
| Nortriptyline    | 1.49    | 1.06               | 0.91               | 3.51               |
| Imipramine       | 1.23    | 0.70               | 1.80               | 2.94               |
| Amitriptyline    | 1.05    | 1.15               | 1.41               | 2.84               |
| Maprotiline      | 0.31    | 0.46               | 1.40               | 2.26               |
| Trimipramine     | 1.22    | 1.21               | 2.47               | 2.30               |
| Clomipramine     | 1.66    | 1.05               | 1.67               | 2.11               |

Fig. 1. Typical chromatograms for TCAs in serum
(A) human pooled serum, (B) human pooled serum supplemented with 500 μg of TCAs per liter. Peaks: 1, amoxapine; 2, doxepine; 3, desipramine; 4, imipramine; 5, nortriptyline; 6, maprotiline; 7, amitriptyline; 8, trimipramine; 9, clomipramine.
nortriptyline, and (or) clomipramine are shown in Figure 2. These concentrations correlated well with the administered doses expressed as milligrams per kilogram body weight per day, with a regression coefficient of 0.713 (n = 41).

With use of stepwise elutions, TCA metabolites also could be detected in serum of patients. The ratios of 10-hydroxy-nortriptyline to nortriptyline ranged from 0.38 to 3.04 (n = 24, mean 0.98, SD 0.63) with a regression coefficient of 0.691. Desmethylated metabolites of maprotiline, amitriptyline, and clomipramine often were detected in serum of patients in similar or greater concentrations than their parent TCAs. Other TCA metabolites detected in substantial concentrations in serum were 7-hydroxyamoxapine, 2-hydroxydesipramine, and 10- and 2-hydroxyclo mipramine.

Discussion

During pretreatment procedures for the HPLC determination of TCAs by organic solvent extraction from alkaline media and further back-extraction into acidic solution, TCAs were adsorbed onto the glassware; thus analytical recoveries of TCAs were low and different among tertiary and secondary amines (5). In previous reports, the column-switching HPLC methods for the determination of antiepileptic drugs and their metabolites (18), theophylline and its metabolites (19), and antiarhythmic drugs and their metabolites (20) were developed by using a bovine serum albumin (BSA)-coated reversed-phase column, TSKprecol umn BSA-ODS, as the pretreatment column. We found TSKprecolumn PW, a metaacyl polymer gel for size-exclusion chromatography (exclusion limit: 5000, mean particle size: 12 μm), to be more effective than TSKprecolumn BSA-ODS for the pretreatment of hydrophobic TCAs in serum samples.

The nine different TCAs were separated within 30 min when we used TSKgel ODS-80TM, monomeric octadecyl silylated silica gel (pore size 8 nm, mean particle size 5 μm) endcapped by trimethylsilyl chloride, in the analytical column. Insufficient resolution of nortriptyline and imipramine will not cause problems for the clinical assessment of TCAs because these drugs are not often co-administered. Interference by substances in human serum with desipramine was eliminated by use of sodium 1-heptanesulfonate in the mobile phase. Symmetric peaks were obtained with the present system without use of amine buffers (14–16).

TCA metabolites would interfere negligibly with TCA determination under our chromatographic conditions, because desmethylclomipramine, the most hydrophobic metabolite, is eluted between trimipramine and clomipramine, and other hydroxylated metabolites, including 2-hydroxyclo mipramine, are eluted before amoxapine. Although interference by phenothiazines and butyrophenones, which often are co-administered with TCAs, was not examined, we observed no interference in serum of patients being co-medicated with benzodiazepine sedative-hypnotics.

For these low concentrations of TCAs, the reproducibilities (CV: 2% to 3%) and analytical recoveries (95% to 104%) we obtained were sufficient for therapeutic drug monitoring. Direct injection of serum samples onto the precolumn made it possible to eliminate the elaborate sample-preparation procedures and the irreversible adsorption of TCAs onto the glassware. The reproducibilities and analytical recoveries by the present method were superior to those obtained with the automated sample processor (12, 13).

Although in most cases the concentrations of TCAs in serum were correlated to the administered doses (r = 0.713, n = 41), there were several exceptions. The present method nevertheless provides an easy, generally reliable method for monitoring TCAs in serum.

References

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Fig. 2. Relation between the doses administered and concentrations of TCAs in serum

©, nortriptyline; ○, maprotiline; ·, desipramine; △, amoxapine; □, clomipramine


**Screening for Microalbuminuria by Use of a Rapid, Low-Cost Colorimetric Assay**

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We evaluated the pyrogallol red–molybdate(IV) method for quantification of urinary protein as a screening procedure for microalbuminuria by determining the assay’s sensitivity and specificity at different concentrations of urinary albumin as measured by a comparison laser nephelometric immunoassay. The pyrogallol red–molybdate(IV) method has sensitivity and specificity similar to that for other semiquantitative assays, but it is less expensive and sample throughput can be high if microtitre plate techniques are used.

*Additional Keyphrases:* pyrogallol red–molybdate(IV) • diabetic nephropathy

Small increases in albumin in urine of patients with diabetes are associated with subsequent development of clinical nephropathy and retinopathy (1–3). "Microalbuminuria" is generally defined as urinary albumin corresponding to >20 μg/min or approximately 30 mg/L (1). The most appropriate upper limit, however, has not been unequivocally established. Detection of microalbuminuria in patients with diabetes is important, because deterioration of renal function may be reversible if it is detected at the early stages (4).

Low concentrations of albumin in urine can be accurately quantified by immunochromatographic methods. However, these methods are not suitable for screening, because they have a limited calibration range, require trained laboratory staff, and have a high cost per sample. Accordingly, many centers use qualitative or semiquantitative techniques to initially screen urine specimens for subsequent quantitative immunoassay (5–10).

Recently Watanabe et al. (11) reported a colorimetric method for the quantification of urinary protein in the range 10–1000 mg/L. The procedure is simple to perform and the reagents are inexpensive. We have adapted their method to microtitre plates and correlated results with a nephelometric immunoassay for urinary albumin, similar to that reported by Marre et al. (12). This adaptation has produced a rapid, reliable, and low-cost method to select clinic patients for further assessment of microalbuminuria.

**Materials and Methods**

*Materials:* Albumin (Cohn Fraction V) and pyrogallol sulfonphthalein were from Sigma Chemical Co., St. Louis, MO. The protein assay reagent was prepared exactly as described by Watanabe et al. (11). Rabbit antiserum to human albumin (SRL titer: 2.25 mg/L) was from Dakopatts, Gloetrup, Denmark.

*Instrumentation:* Nephelometric measurements were performed with a Behring Laser-Nephelometer (Behringwerke AG, Marburg, F.R.G.). Plates were read with a Titertek plate reader (Flow Laboratorries, Nth Ryde, Sydney, Australia), set at 620 nm.

*Urine specimens:* These were untimed specimens collected from patients attending the diabetic outpatients clinic. Although untimed daytime specimens may not be optimal for investigation of microalbuminuria (however, see Watts et al. (13) and Nathan et al. (14)), they provide a suitable compromise for initial selection when large numbers of clinic patients are to be repeatedly screened. Samples are mixed by hand, then centrifuged at 8930 × g for 2 min before being stored at −20°C for no more than three days before assay.

*Pyrogallol red–molybdate assay* (PR method): Standards consist of albumin in concentrations of 0, 25, 50, and 100 mg/L in distilled water containing sodium azide, 1 g/L. This range of standards was selected to increase the precision of estimation at the critical lower concentrations of protein where interference is most likely.

Controls are selected patients’ urines.

Into the appropriate well of the microtitre plate, 25 μL of urine, standard, or quality-control sample is placed. Urines and quality-control samples are assayed in duplicate; standards are assayed in quadruplicate. The pyrogallol red reagent (200 μL) is then quickly added and the plate allowed to stand at room temperature for 30 min before the absorbance at 620 nm is measured.

The standards response is analyzed by least-squares regression and the derived function parameters are used to calculate the respective concentrations of urinary protein.

The intra-assay CV for a urine quality-control sample was estimated at 5.8% (n = 37; mean = 49.8 mg/L; SD = 2.9). The interassay CV was 4.1% (n = 15; mean = 48.4 mg/L; SD = 2.0). The CV for the total assay is therefore 7.0%.