Automated Determination of Drugs in Serum by Column-Switching High-Performance Liquid Chromatography

V. Separation of Cyclosporins

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Summary An automated determination of cyclosporins in serum by column-switching high-performance liquid chromatography (HPLC) is described. To increase the analytical recoveries of cyclosporins in serum, serum was diluted to 4 times with a solution containing 10% of 1, 1, 3, 3-tetramethylurea and 0.05 % of sodium dodecylsulfate. A 1000-μl of the mixture was directly injected onto a TSK precolumn PW. After washing the precolumn with water for 10 min, the precolumn-connection was switched to introduce the retained substances onto an analytical column, TSKgel OH-100RP. Cyclosporins were separated with acetonitrile/water (40 : 60,v/v) within 30 min. The column temperatures were at room temperature for the precolumn and at 70°C for the analytical column. By the present method, the analytical recovery (70%), detection limit (20ng/ml), and within-run reproducibility (5.7% for 250ng/ml, 3.4% for 500ng/ml, 1.2% for 1000ng/ml) were sufficient for therapeutic monitoring of cyclosporin A.

Introduction

Cyclosporin is a novel immunosuppressant used to prevent the rejection of transplanted organs and to treat diseases of autoimmune origin. Because of wide variability in pharmacokinetics related to state of disease, type of organ transplant, age, and comedicated drugs, monitoring of cyclosporin in serum or blood is essential to prevent a rejection of the transplanted organ and to minimize a drug toxicity. Immunoassay (RIA) or high-performance liquid chromatography (HPLC). RIA method is rapid and sensitive but exhibits considerable cross-reactivity with cyclosporin metabolites. HPLC methods are specific but the pretreatment procedures such as solid-phase or liquid-liquid extraction are complicated and time-consuming. Recently, automated solid-phase extraction methods for HPLC and semi-automated column-switching HPLC methods have been applied to determine cyclosporins in deproteinized serum or blood sample. We developed an automated column-switching HPLC method for the determina-
tion of cyclosporins in the serum treated with a solution containing 10% of 1,1,3,3-tetramethylurea (TMU) and 0.05% of sodium dodecylsulfate (SDS).

Experimental

Reagents: Cyclosporins A, C, and D were kindly gifted from Sandoz Ltd., Basel, Switzerland. Acetonitrile and methanol of HPLC grade, and TMU and SDS of analytical grade were purchased from Wako Pure Chemical, Osaka, Japan.

Materials: Human pooled serum was used for determining analytical conditions. Sera of patients with normal and some kinds of hyperlipidemia were also used without freezing-thawing. Sera of patients administered with cyclosporin were collected and stored at -30°C.

Apparatus: HPLC with column-switching device used was Tosoh Co. (Tokyo, Japan), which was consisted of a Model CCPM pump, a Model UV-8000 variable-wavelength detector, a Model CP-8000 chromatoprocessor, a Model PT-8000 column-switching valve system, a Model AS-48 autosampler, and a Model CO-8000 column oven.

Procedure: A 300-μl serum sample was mixed with 900 μl of 10% TMU and 0.05% SDS solution. A 1000-μl of the mixture was injected directly onto a column of TSK precolumn PW, a metaacryl polymer gel for size exclusion chromatography with exclusion limit of 5000 dalton (35 x 4.6 mm i.d., 12μm of mean particle size, Tosoh Co.). After washing the precolumn with water for 10 min at a flow rate of 1.0 ml/min, the substances retained on the precolumn were eluted for 4 min with the mixture of acetonitrile and water (40:60, v/v) at a flow rate of 1.0 ml/min by the back-flush mode. Cyclosporins eluted from the precolumn were separated with a mixture of acetonitrile and water (40:60, v/v) at a flow rate of 1.0 ml/min on a TSK gel OH-100RP column (150 x 4.6 mm i.d., Tosoh Co.). A reversed phase column introduced a stearoyl group on a silica gel (10 nm of pore size and 5μm of mean particle size) after glycidoxypropyl-trimethoxysilane-treatment and reduction. The column temperatures of the precolumn and the analytical column were room temperature and 70°C, respectively. The effluent was monitored at 210 nm.

Radioimmunoassay for cyclosporin A was performed with 'Cyclosporin RIA' kits supplied by Sandoz Ltd.

Results

Pretreatment Procedure

The recoveries of cyclosporins in human pooled serum from the TSK precolumn PW are shown in Fig. 1. The analytical recoveries of cyclosporins decreased with the increasing injection volume of the serum sample (Fig. 1a). Almost constant and high recovery were obtained when the serum was diluted 4 times or more (Fig. 1b).

As shown in Fig. 2, in any serum used, TMU concentration in the range 10-20% (Fig. 2a) and that of SDS in the range 0.05-0.1% (Fig. 2b) gave the maximum recovery of cyclosporin A. Maximum recoveries of cyclosporin C and D were obtained in slightly lower and higher concentrations of SDS than that for cyclosporin A, respectively.

Analytical recoveries (n=2) of cyclosporins in serum samples diluted 4 times with a solution containing 10% TMU and 0.05% SDS are shown in Table I. The peak areas of cyclosporins in the diluted mixtures obtained by the column-switching HPLC system with the precolumn were compared to those of cyclosporins in the mixture of acetonitrile and water (40:60, v/v) by direct injection onto the analytical column. Values of analytical recoveries of cyclosporins A, C,
a. Effect of injection volume of serum
b. Effect of dilution with water (injection volume, 1000μl)
   ●: cyclosporin A, ○: cyclosporin C, X: cyclosporin D

Fig. 1 Analytical recoveries of cyclosporines in human pooled serum from TSK precolumn PW

48
### Table I Analytical recovery of cyclosporins in human serum (n=2)

<table>
<thead>
<tr>
<th>Serum</th>
<th>Concentration µg/ml</th>
<th>Recovery of cyclosporin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pooled serum</td>
<td>1.0</td>
<td>70.8 70.9 61.8</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>67.5 72.0 58.1</td>
</tr>
<tr>
<td>normolipidemic serum</td>
<td>1.0</td>
<td>68.7 70.6 61.6</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>65.5 70.8 61.7</td>
</tr>
<tr>
<td>hyper α-lipoproteinemic serum</td>
<td>1.0</td>
<td>68.7 70.6 62.9</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>66.3 70.2 61.7</td>
</tr>
<tr>
<td>hyper β-lipoproteinemic serum</td>
<td>1.0</td>
<td>67.9 71.9 62.9</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>70.7 70.6 59.6</td>
</tr>
</tbody>
</table>

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**Fig. 3 Chromatograms of cyclosporins**

- (a): human pooled serum
- (b): human pooled serum spiked with 500 ng/ml each of cyclosporin A, C, and D
- (c): patient serum medicated with cyclosporin A

and D (approximately 70, 70, and 60%) were obtained in both the denatured and the intact serum samples.

**Separation and Determination of Cyclosporins**

Typical chromatograms obtained with a human pooled serum, a human pooled serum spiked with cyclosporins, and a serum of patient medicated with cyclosporin are shown in Fig. 3. Cyclosporins A, C, and D were separated within 30 min. Because the present method can afford a constant recovery of cyclosporin A and a high reproducibility in the determination of cyclosporin A, internal standard (for example, cyclosporin D) was not necessary for the determination of cyclosporin A and actual measurement time was 20 min.

Good linear regressions were obtained between the peak areas (y, mV sec) and the concentrations of cyclosporins (x, ng/ml) ranged from 0 to 2000 ng/ml. Regression equations for cyclosporins A, C, and D were y = 0.51x + 11.1 (R=0.9990), y = 0.37x + 4.15 (R=0.9947), and y = 0.46x - 0.49 (R=0.9999), respectively. The detection limits for cyclosporins were 20 ng/ml respectively, which corresponded to 10 mV sec of peak areas.

Within-run reproducibility (n=10) in this method are shown in Table II. The relative standard deviations for 250, 500, and 1000 ng/ml of cyclosporin A were 5.7, 3.4 and 1.2 %, respectively.

In Fig. 4, correlation between the values obtained by a RIA and the present methods is shown. The values obtained by the RIA method were significantly higher than those obtained by the present method.

**Discussion**

For HPLC determination of cyclosporins, deproteinization and extraction procedures were essential. Semi-automated column-switching HPLC methods have been reported so far, which involved pretreatment procedures of deproteinization and extraction. We developed a fully automated HPLC method which required only dilution of serum sample with a solution containing 10% TMU and 0.05% SDS.

Fully automated column-switching HPLC methods for the determination of antiepileptic drugs, theophylline, antiarrhythmic drugs, and tricyclic antidepressants in serum have been developed in our laboratories. Analytical recoveries of antiepileptic drugs from TSK-preamolumn BSA-ODS and tricyclic antidepressants from TSK preamolumn PW were quantitative and...
independent from sample injection volume ranged from 20 to 500 µl. Although antiepileptic drugs bound mainly on the albumin by a hydrophobic interaction and tricyclic antidepressants bound on the α-acid glycoproteins by an ionic interaction, cyclosporins may be incorporated into the serum lipoproteins. This is the reason why the analytical recoveries of cyclosporins decreased with the increasing injection volumes.

The interaction of cyclosporins incorporated into the lipoprotein particles on the gel surface of precolumn may be much less than those of other drugs. Since TMU and SDS destroy the lipoproteins to form smaller particles, the recoveries of cyclosporins increased significantly by the addition of these detergents. Decreasing recoveries of cyclosporins by excessive addition of detergents suggested that cyclosporins would be also incorporated into micelles of these detergents. Serum sample was diluted with a solution of 10 % TMU and 0.05 % SDS to obtain the same recoveries of cyclosporins in intact serum as those in the serum denatured by repeated freezing-thawing. This method could not be applied to determining cyclosporins in whole blood because of interferences from red cells.

The values obtained by the RIA method were obviously higher than those by the present HPLC method. RIA method is well-known to exhibit cross-reactibility with many kinds of cyclosporin metabolites in serum of patient medicated with cyclosporin A\textsuperscript{21,22}, whose levels reach frequently to several times higher concentrations than cyclosporin A\textsuperscript{23,24}. HPLC method is indispensable to the determination of cyclosporin A and RIA method may be utilizable for monitoring of side-effects such as hepatotoxicity caused by cyclosporin metabolites.

References