Prevention of Co-elution of Steroid Sulfates with Serum Proteins from Pre-column in Column-switching HPLC System

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A method to prevent co-elution of steroid sulfates with proteins in serum from the pre-column in column-switching HPLC was developed. The pre-column, a polymer-coated mixed function column, was used for ion-pair chromatography with 5 mm tetra-n-butylammonium (TBA) ion. As steroid sulfates, estradiol 3-sulfate, dehydroepiandrosterone 3-sulfate and pregnenolone 3-sulfate were used. Human serum (25 μl) was diluted with mobile phases including 5, 100 and 500 mm TBA ion, and then injected directly into the pre-column. The peak areas of the steroid sulfates in serum samples were compared with those of the steroid standards without serum. When 25 μl of serum was diluted with mobile phase including 100 or 500 mm TBA ion, the steroid sulfates in serum were retained in the pre-column; however, the steroid sulfates from the same sample diluted with mobile phase containing 5 mm TBA ion were not retained in the pre-column. Addition of an excess amount of counter ion (TBA ion) into the serum sample made it possible to retain the steroid sulfates in the pre-column. This method was applied to column-switching HPLC for measurement of steroid sulfates in serum using a semi-microcolumn as the analytical column.

Key words steroid sulfate; polymer-coated mixed function column; ion-pair chromatography; column-switching HPLC

Steroid sulfates are important intermediates in both biosynthesis and metabolism of steroid hormones. Numerous methods have been developed for analysis of these sulfates in biological fluids by HPLC,1−3 GC-MS4−6 and RIA.7−9 However, most of these methods require sample preparation steps such as extraction from the biological fluids, enzymatic hydrolysis or acidic solvolysis and derivatization. These procedures are tedious and time consuming, and sometimes disturb the analytical results. For immunoassay, it is necessary to obtain a specific antibody to each steroid of interest. Therefore, it is desirable to develop an accurate and rapid method to determine the serum steroid sulfates with minimal prerequisites.

Many types of packing materials for direct analysis of analytes from serum or plasma by HPLC have been developed.10 All of these are constructed in such a way that large protein molecules pass through while analytes are retained on the packing material. Using the column-switching technique, the analytes that are purified by the packing material can be injected directly into the analytical column.11−13 We used such packing material, the polymer-coated mixed functional phase, as a pre-column. As steroid sulfates bear a negative charge in neutral solution, 5 mm tetra-n-butylammonium (TBA) ion was added to the mobile phase as a counter ion. Under these conditions, steroid sulfates were co-eluted with serum proteins from the pre-column. Then, we observed that direct addition of an excess amount of the counter ion (TBA ion) to the serum samples before injection resulted in retention of steroid sulfates in the pre-column. It was believed that the negatively charged serum proteins prohibited the ion-pair formation between the sulfates and counter ion. Here, we studied the retention of steroid sulfates in the pre-column with variations in serum sample size (6.25, 25 and 50 μl) and the concentration of the counter ion in the sample. As steroid sulfates, estradiol 3-sulfate (3, 160, 17β-tri-hydroxy-1, 3, 5,6,7,8-tri-n-butylammonium, E3 S), dehydroepiandrosterone 3-sulfate (3β-hydroxy-5-androsten-17-one 3-sulfate, DHEA S) and pregnenolone 3-sulfate (3β-hydroxy-5-pregn-20-one 3-sulfate, Preg S) were used; the amounts of these steroids in each size of sample were 2.5 μg, 25 μg and 10 μg, respectively. This method using the pre-column was also applied to the column-switching HPLC system for the measurement of E3 S, DHEA S and Preg S in serum.

MATERIALS AND METHODS

Materials The chemicals used in this study were obtained from the following sources: E3 S, DHEA S, and Preg S (Sigma Chemical Co., Ltd., St. Louis, MO, U.S.A.); TBA hydroxide solution (0.5 m, for HPLC), (Wako Pure Chemical Industries, Osaka, Japan); disodium hydrogen phosphate, potassium dihydrogen phosphate, and acetonitrile for HPLC, (Nacalai Tesque, Kyoto, Japan). Water was purified with a Milli-Q system, (Nihon Millipore Kogyo, Tokyo, Japan). Steroid-free serum was prepared from pooled human serum according to the method of Heyns et al.14

Chromatographic Instrumentation and Pre-column Conditions Primary separation in the pre-column: The LC system was constructed with a Nanospace SI-1 (Shiseido Co., Ltd., Tokyo), model 2001 inert pump, model 2004 column oven, model 2003 autosampler, model 2005 UV-VIS detector set at 210 nm and a model 2010 degassing unit. The pre-column used in this study was Guard cartridge Capcell MF Ph-1® (polymer-coated mixed function pre-column, Shiseido Co., Ltd.). A 10 mm × 4 mm i.d. pre-column was used for DHEA S and Preg S, and a 20 mm × 4 mm i.d. pre-column was used for E3 S. The pre-column was kept at 40°C. The data were integrated with an S-Micro Chrom 4.1 (Shiseido Co., Ltd.) using a personal computer running MS-Windows 95® (Microsoft Corporation, Redmond, WA, U.S.A.). The mobile phases described below were run at a flow rate 0.5 ml/min.

Preparation of the Mobile Phase for the Pre-column The mobile phases for the pre-column with each steroid sul-

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fate were as follows: E3 S, 200 mM phosphate buffer (pH 7.0) containing 5 mM TBA ion; DHEA S, 100 mM phosphate buffer (pH 7.0) containing 5 mM TBA ion-acetonitrile (97:3, v/v); Preg S, 5 mM phosphate buffer (pH 7.0) containing 5 mM TBA ion-acetonitrile (93:7, v/v). The pH was adjusted with phosphoric acid.

**Column-switching System** The column-switching HPLC system is shown schematically in Fig. 1. The system consisted of three pumps, column oven, autosampler, UV-VIS detector set at 210 nm and a degassing unit, and the pre-column was run at 40°C. The system also consisted of a model 2011 six-port high-pressure valve (Shiseido Co., Ltd.), an enrichment column; a Capcell Pak C18 (35 mm × 2.0 mm i.d., Shiseido Co., Ltd.) run at room temperature and a Capcell Pak C18 analytical column (250 mm × 1.5 mm i.d., Shiseido Co., Ltd.) run at 40°C. The mobile phases for the pre-column (Pump 1) were the same as those described above. The mobile phases for elution of steroid sulfates from the pre-column and for sample enrichment (Pump 2) were as follows: E3 S, 5 mM phosphate buffer (pH 7.0) containing 5 mM TBA ion-acetonitrile (90:10, v/v); DHEA S and Preg S, 5 mM phosphate buffer (pH 7.0) containing 5 mM TBA ion-acetonitrile (80:20, v/v). The mobile phases were run at a flow rate of 0.5 ml/min. The mobile phases for the analytical column (Pump 3) were prepared with 5 mM phosphate buffer (pH 7.0) containing 5 mM TBA ion and acetonitrile with the following ratio (v/v): E3 S, 74:26; DHEA S, 55:45; Preg S, 45:55, and were run at a flow rate of 0.1 ml/min. When the column-switching valve was at the A position, the mobile phases for the pre-column were passed through Pump 1, and sample loading and primary separation of steroid sulfates from serum proteins were performed. Subsequently, the valve was switched to the B position and Pump 2 was used to pump the mobile phase solvent for elution of steroid sulfates from the pre-column and for concentration of steroid sulfates in the enrichment column. Position B was maintained for 3.0—9.0, 4.0—9.0 and 5.0—9.0 min for E3 S, DHEA S and Preg S, respectively. Then, the valve position was returned to A and the mobile phase for the analytical column was passed via Pump 3. The steroid sulfates concentrated in the enrichment column were transferred into the analytical column in the backflash mode.

**Preparation of Serum Standards Containing Steroid Sulfates** Stock solutions of 250 μg/ml E3 S, 1 mg/ml DHEA S and 2.5 mg/ml Preg S in methanol were prepared separately. These stock solutions were diluted with the mobile phase containing 5 mM TBA ion for the pre-column of each steroid sulfate (E3 S, 2.5 μg/200 μl; DHEA S, 25 μg/200 μl; Preg S, 10 μg/200 μl). We refer to these solutions as “mobile phase steroid standards”. Because of low resolution of the pre-column, we used high concentrations of steroid sulfates as above. To prepare serum standards containing steroid sulfate, steroid stock solution (14 μl), mobile phase containing 5 mM TBA ion for the pre-column and 8.75, 35 or 70 μl of steroid-free serum were mixed. The final volume of this mixture was 280 μl, which was adjusted by addition of the mobile phase solution. We refer to these standards as “serum steroid standards”. At this point, 200 μl of the serum steroid standards for each steroid sulfate contained 6.25, 25 or 50 μl as the original volume of steroid-free serum, respectively. Aliquots of 200 μl of these standards

![Fig. 1. Schematic Diagram of the Column-switching HPLC System](image)

were injected directly into the pre-column after filtration through a 0.22 μm pore size membrane.

**RESULTS AND DISCUSSION**

A number of pre-columns with specialized packing materials designed on the basis of the concept of restricted access reversed phase are now commercially available. As a result, column-switching HPLC systems using such pre-columns have been developed for the direct analysis of drugs and endogenous compounds in biological fluids. In the primary separation of the column-switching HPLC system using these pre-columns, the important points are to elute the serum proteins at the position of the void volume and to efficiently retain the analytes of interest in the pre-column. The analytes thus retained are transferred to the enrichment or the analytical column using the column-switching technique. However, it was difficult to retain highly hydrophilic samples such as steroid sulfates in the pre-columns, especially in the short columns such as those of the cartridge type of which the length is 10—20 mm. In general, steroid sulfates that bear a negative charge have been analyzed in the presence of a counter ion in the HPLC mobile phase. We used TBA ion as a counter ion to retain steroid sulfates in the cartridge type pre-column.

**Effects of TBA Ion in Mobile Phase on Retention of Steroid Sulfates in the Pre-column** To investigate the effects of TBA ion on retention of steroid sulfates in the pre-column, 200 μl of mobile phase steroid standard containing 5 mM TBA ion (without serum) was injected into the pre-column which was then eluted with the mobile phase containing the same concentration of the counter ion. The steroid sulfates were retained in the pre-column (Figs. 2A, B, C). However, 200 μl of steroid sulfates containing 25 or 50 μl as the original volume of steroid-free serum (serum steroid standards) was not retained in the pre-column under the same mobile phase conditions as above (Figs. 2G—a, H—a, I—a, J—a, K—a, L—a). These findings suggested that ion pair formation
between steroid sulfates and the counter ion in the mobile phase was inhibited by the presence of serum proteins bearing a negative charge under the present conditions (pH 7.0). Instead of 5 mM TBA ion, phosphate buffer containing 100 mM TBA ion was passed through the column as a mobile phase and the same serum steroid standards were injected into the pre-column. However, sulfates were not retained and the sensitivity of the analysis was markedly decreased because of the background absorption (210 nm) due to the high concentration of TBA ion (data not shown). The use of more concentrated TBA hydroxide solution (100 mM) as the pre-column mobile phase disturbed the analysis.

Effects of TBA Ion in Serum Samples on Retention of Steroid Sulfates in the Pre-column As described above, steroid sulfates in serum were not retained in the pre-column despite the presence of a counter ion (5 mM TBA ion) in the mobile phase. Thus, serum steroid standards were prepared with addition of concentrated TBA hydroxide solution (100 mM and 500 mM). The pre-column was eluted with phosphate buffer containing 5 mM TBA ion. Then, 200 µl of the serum steroid standards with concentrated TBA ion (100 mM or 500 mM) which contained 25 µl as the original volume of steroid-free serum was injected directly into the pre-column. The sulfates were retained in the pre-column and separated from the protein observed at the position of the void volume. The retention times and the peak areas of each serum steroid standard were equivalent to those of the mobile phase steroid standard. (Figs. 2G-b, c; H-b, c; I-b, c). When the sample contained 50 µl of serum, the retention was observed at a TBA ion concentration of 500 mM rather than 100 mM (Figs. 2J-c, K-c, L-c). These findings suggested that ion-pair formation of the steroid sulfates with TBA ion was achieved by the addition of concentrated TBA hydroxide solution directly into the sample even for samples containing an excess amount of negatively charged serum proteins.

Column-switching HPLC for Analysis of Steroid Sulfates in Serum According to the results obtained above, 200 µl of the serum steroid standard (250 ng of E3 S, 2.5 µg of DHEA S or 2.5 µg of Preg S) with 100 mM TBA ion which
Table 1. Recovery of Steroid Sulfates in Serum (n=6)

<table>
<thead>
<tr>
<th>Steroid sulfate</th>
<th>Added (ng/ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E3 S</td>
<td>500</td>
<td>101.0±4.6</td>
</tr>
<tr>
<td></td>
<td>2500</td>
<td>97.3±4.4</td>
</tr>
<tr>
<td>DHEA S</td>
<td>1250</td>
<td>95.5±5.2</td>
</tr>
<tr>
<td></td>
<td>2500</td>
<td>96.1±3.4</td>
</tr>
<tr>
<td>Preg S</td>
<td>1250</td>
<td>97.7±2.1</td>
</tr>
<tr>
<td></td>
<td>2500</td>
<td>98.6±1.5</td>
</tr>
</tbody>
</table>

respectively. No interference due to the pre-column or the samples including excess amounts of TBA ion was observed. The durability of the present system was studied with successive injections of the serum steroid standard (E3 S, serum 25 µl). Chromatograms of the 1st and the 50th runs are shown in Fig. 3A-b. Little change in the peak area was observed between the 1st and the 50th run. The lifetime of the pre-column used was over 50 runs (total serum volume, 1.25 ml or more).

The present column-switching method would be applicable to direct analysis by HPLC or LC-MS for steroid sulfates in serum.

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REFERENCES