Determination of 17-Oxosteroid Sulfates and Glucuronides
II. Improved Pre-labeling with Dansylhydrazine and Analytical Condition by HPLC

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SUMMARY To determine 17OS sulfates and glucuronides, which separately extracted by ion pair extraction as described in the previous report, by high-performance liquid chromatography using a reversed phase Capcell-Pak C8 column, we conducted experiments on labeling condition of the steroids with dansylhydrazine and on chromatographic condition for the separation of the labeled steroids.

As a result, we found a method that obtained much more labeled steroids under lower temperature than already reported methods, and obtained three mobile phase solutions that can separate individual steroid. The labeled 17OS sulfates were perfectly separated by using methanol-0.5% (w/v) sodium acetate-50% (v/v) acetic acid (57:42:1, v/v) at 30°C. The labeled 17OS glucuronides were also clearly separated by using methanol-0.03% acetic acid (v/v) (46:54, v/v) and methanol-0.01% acetic acid (v/v) (56:44, v/v) at 55°C.

Introduction

We reported a method for separative extraction of 17-oxosteroid (17OS) sulfates and glucuronides by ion pair extraction in the previous report (part I). To determine the individual four sulfates and seven glucuronides by high–performance liquid chromatography (HPLC), we examined on the condition for prelabeling of the 17OS conjugates with dansylhydrazine and on chromatographic condition. Dansylhydrazine has been used as a highly sensitive prelabeling reagent for the determination of 17OS by thin layer chromatography1 and HPLC2-4. However, when the ion pairs of 17OS sulfates or 17OS glucuronides with tetraptentylammonium ions were labeled by these methods, we occasionally experienced marked variation on the values measured simultaneously using the sample. And furthermore, on the direct determination of individual 17OS sulfates and 17OS glucuronides without hydrolysis, the only...
two methods for the determination of three 17O\(\text{S}\) and two 17O\(\text{S}\) glucuronides\(^3\), and for the determination of four 17O\(\text{S}\) sulfates\(^4\) in main eleven 17O\(\text{S}\) conjugates have been reported. We accordingly conducted experiments on the reaction procedure, acid and solvent for the reaction, and reaction temperature and reacting time for labeling reaction using dansylhydrazine, and then examined on chromatographic condition using reversed-phase Capcell-Pak C\(\text{S}\) column for the separation of the all main 17O\(\text{S}\) conjugates.

**Materials and Methods**

**Apparatus and reagents**

The same apparatus and reagents described in the previous report (part I.) were used.

**Preparation of samples for labeling**

Three milliliters of TPA solution and 2 ml of borate buffer (pH 9.0) were added to 10 ml of aqueous solution containing 200 µg of each steroid. The 17O\(\text{S}\) sulfates in the solution were extracted with 30 ml of benzene. Benzene layer was transferred to another test tube. Sodium sulfate (4 g) was added to the aqueous layer, and the 17O\(\text{S}\) glucuronides in the aqueous layer were extracted with 30 ml of dichloromethane. The benzene layer (0.3 ml) and 0.3 ml of dichloromethane were evaporated, respectively.

**Labeling procedures.**

To three test tubes containing the same volume of the sample prepared as above, 200 µl of the dansylhydrazine solution and 50 µl of acetic acid were added and mixed well. The solvent in the first tube was evaporated for 1 min under reduced pressure with a water jet pump in a water bath at 50 °C and left to stand in bath for 20 min (17O\(\text{S}\) sulfates) or 10 min (17O\(\text{S}\) glucuronides) (pre-evaporation method). The second tube was stoppered, left to stand in the water bath at 50°C for 20 min (17O\(\text{S}\) sulfates) or 10 min (17O\(\text{S}\) glucuronides), then evaporated for 1 min under reduced pressure in the water bath at 50°C (post-evaporation method). The third tube was stoppered, and left to stand for 21 min (17O\(\text{S}\) sulfates) or 11 min (17O\(\text{S}\) glucuronides) in the bath at 50 °C (non-evaporation method). The labeled residue in the first and second tubes was dissolved in 250 µl of acetonitrile. The same volume of the solution in each tube was injected into the chromatograph system.

**Acid concentration.**

1. 0.2 % (w/v) dansylhydrazine dissolved in acetonitrile and acetic acid solution (10 %, v/v, dissolved in acetonitrile) or acetic acid were added to the sample (prepared as above), as shown in Table I.). 200 µl of the dansylhydrazine solution and 50 µl of 0.25, 0.5, 1.25 or 2.5 % (w/v) trichloroacetic acid dissolved in acetonitrile were added to the sample (final acid concentration: 0.05, 0.1, 0.25 and 0.5 % [w/v]). The solvent in the mixture of the sample and the reagents were evaporated for 1 min under reduced pressure using water jet pump in a water bath at 50 °C, then the residue was left to stand for 20 min (17O\(\text{S}\) sulfates) or 10 min (17O\(\text{S}\) glucuronides). The labeled residue was dissolved in 100 µl of acetonitrile, and the same volume of each solution was injected into the chromatograph system.

**Solvent for dansylation or dansylated samples.**

A sample prepared as above was dissolved by adding 200 µl of dansylhydrazine dissolved in ethanol, acetonitrile, 1,2-
Table I  Volumes of acetic acid (or acetic acid solution) and
dansylhydrazine solution added the sample.

<table>
<thead>
<tr>
<th>Reagent (µl)</th>
<th>Final concentration of acetic acid (% v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Dansylhydrazine solution</td>
<td>180</td>
</tr>
<tr>
<td>10% Acetic acid</td>
<td>20</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>10</td>
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dichloromethane or benzene, then 50 µl of
acetic acid was added. The solvent in the
mixture was evaporated for 1 min under
reduced pressure in a water bath at 50°C,
then the residue was left to stand for 20
min (17OS sulfates) or 10 min (17OS glucur-
onides). The labeled residue was dissolved
in 100 µl of ethanol or acetonitrile, and
the same volume of each solution was
injected immediately and after four hours
into the chromatograph system.

Reaction temperatures and times.

The dansylhydrazin solution (200 µl)
and 50µl of acetic acid were added to the
sample prepared as above, mixed well, and
the solvent in the mixture was evaporated
for 1 min under reduced pressure at either
30, 40, 50, 60 or 70°C in a water bath. Six
samples were evaporated at each of the
five temperatures. After the evaporation,
one residue obtained by evaporation at
each temperature was dissolved in 100 µl
of acetonitrile, and the other residues were
left to stand in the bath for 5, 10, 15, 20
or 30 min. The residues left in the water
bath were dissolved in 100µl of acetonitrile.
The acetonitrile solutions (5 µl each) were
injected into the chromatograph system.

Preparation of mobile phase solutions for
separation of 17OS sulfates.

550, 560, 570, 580 and 600 ml of meth-
anol were mixed with 445, 435, 425, 415
and 395 ml of 0.4, 0.5 or 0.6% (w/v) sodium
acetate solution, respectively. 5 ml of acetic
acid was added to the each mixture.

For separation of five 17OS glucuronides.

440, 460, 480 and 500 ml of methanol
were mixed with 560, 540, 520 and 500 ml
of 0.01, 0.03 or 0.05 % (v/v) acetic acid
solution, respectively.

For separation of two 17OS glucuronides
(Ad glucuronide and Ec glucuronide).

540, 560 and 580ml of methanol were
mixed with 460, 440 and 420 ml of 0.01,
0.03 or 0.05 % (v/v) acetic acid solution,
respectively.

Results and Discussion

Labeling reaction

Labeling procedure. Dansylhydrazine
has been used as a highly sensitivity pre-
labeling reagent for the determination of
free 17OS, obtained by hydrolysis of 17OS
sulfates and glucuronides, by thinlayer
chromatography1 and for the determina-
tion of the 17OS or 17OS sulfates and 17OS
glucuronides by HPLC4). In these method,
17OS sulfates and 17OS glucuronides are
labeled by incubation with dansylhydrazine
in benzene added trichloroacetic acid(final
centration: 0.4 %, w/v) for 20 min at
60°C3), and the ion pairs of the 17OS sulfates
with benzyltributylammonium ions are
labeled in the mixture of ethanol and
benzene added trichloroacetic acid (final concentration: 0.33%, w/v) for 30 min at 70°C). Each labeling reaction ends by evaporation of the reacting solvent after the incubation. However, when the ion pairs of 17OS sulfates or 17OS glucuronides with tetrapentylammonium ions were labeled by these methods which are a post-evaporation method, we occasionally experienced marked variation on the values measured simultaneously using the same sample. We noted that the labeling reaction proceeded by evaporation of reacting solvent under a lower temperature, and caused much more labeled steroids those obtained by non-evaporation in our pre-experiment. To confirm the effect of the evaporation and improve the labeling method, we labeled the ion paired steroids by the non-evaporation method the pro-evaporation method and the post-evaporation as described above, and compared the peak area values each labeled steroid on the chromatogram obtained by HPLC.

The ratio of the average values measured by the pre-evaporation method, the post-evaporation method, the non-evaporation method, and other method which is a post-evaporation using benzene as reacting solvent (HPLC was conducted under the condition of our method) were 1.00 : 0.67 : 0.09 : 0.28 for four 17OS sulfates (Fig. 1) and 1.00 : 0.73 : 0.07 : 0.27 for two 17OS glucuronides (Ad glucuronide and Ec glucuronide). The CVs (n=10) of the values obtained by the pre-evaporation method and post-evaporation method were from 2.3 to 3.0 and 5.3 to 7.0 % for the four 17OS sulfates, and 4.4 to 5.0 and 8.1 to 8.9 % for the two 17OS glucuronides. The results indicate that the labeling reaction of ion paired 17OS sulfates and 17OS glucuronides proceeds markedly by the evaporation procedure of reacting solvent,

![Chromatograms of labeled steroids obtained by four methods](image)

**Fig. 1** Chromatograms of labeled steroids obtained by four methods I: non-evaporation method, II: post-evaporation method, III: pre-evaporation method, IV: other post-evaporation method (reacting solvent: benzene; incubation temperature: 60°C). 1: DHEA sulfate, 2: EA sulfate, 3: Ec sulfate, 4: Ad sulfate.
the pre-evaporation method produces much more labeled steroids than that by the other methods, and the precision of the pre-evaporation method is better than the post-evaporation method. The optimal evaporation times were about 1 min at 40–50°C.

**Optimal acid concentration in the reacting solution.** To determine the optimal final acid concentration for dansylation, the labeling reaction was conducted using reacting solutions with various concentrations of acetic acid or trichloroacetic acid as described above. As shown in Fig. 2 the optimal final concentrations of acetic acid and trichloroacetic acid were 20% (v/v) and 0.25% (w/v), respectively. There was no difference between the peak area values obtained with the use of acetic acid and trichloroacetic acid.

![Fig. 2 Effect of acid concentration in the reacting solution](image)

**Fig. 2** Effect of acid concentration in the reacting solution. ○: DHEA sulfate, □: Ad sulfate, ◇: Ad glucuronide, △: 11 OHA glucuronide, ⊿: 11 COE glucuronide.

![Fig. 3 Chromatograms](image)

**Fig. 3** Chromatograms obtained using ethanol or acetonitrile solvents for dansylated steroids. E-0 and E-4 used ethanol. A-0 and A-4 used acetonitrile. E-0 and A-0 were obtained immediately after dansylation. E-4 and A-4 were obtained after E-0 and A-0 remained four hours at room temperature. 1: DHEA sulfate, 2: EA sulfate, 3: Ec sulfate, 4: Ad sulfate.
Selection of solvent for dansylation and dansylated sample. To select an optimal solvent for dansylation of the ion paired 17αS sulfates and 17αS glucuronides and for injecting the dansylated sample into the chromatograph system, the sample was dansylated using ethanol, acetonitrile, 1,2-dichloroethane or benzene as the reacting solvent. The dansylated sample was dissolved in ethanol or acetonitrile, and the solution was injected immediately or after four hours into the chromatograph system as described above. The peak area values (mV·min) of each steroid on the chromatogram were compared. The ratio of the average values obtained by use of ethanol, acetonitrile, 1,2-dichloroethane, and benzene as solvent for dansylation were 1.00 : 1.00 : 0.11 : 0.22 for the 17αS sulfates, 1.00 : 0.98 : 0.12 : 0.47 for five 17αS glucuronides (11OHE, 11OHA, DHEA, 11COE and 11COA glucuronides), and 1.00 : 1.00 : 0.17 : 0.57 for the two 17αS glucuronides. Ethanol and acetonitrile were more excellent than benzene and 1,2-dichloroethane as reacting solvent. However, when the dansylated sample was dissolved in ethanol and leaved at room temperature, some non-steroidal peaks were present on the chromatograms, and those peaks increased with time. These peaks did not appear with acetonitrile (Fig.3). Accordingly, a selected acetonitrile as the solvent for dansylation and dansylated sample.

Optimal temperature and times for dansylation. To bring the dansylation to an end after evaporation of the solvent in the reacting solvent at the selected temperatures, the optimal times to be maintained at the same temperatures were studied. As shown in Fig.4, the optimal times at the selected temperatures were 25 min for the 17αS sulfates and 10~20 min for the 17αS glucuronides at 40°C; 20 min for the 17αS sulfates and 10 min for 17αS glucuronanides at 50°C.
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Chromatographic condition.

Optimal components of the mobile phase solutions. The mobile phase solutions prepared as described above on the basis of the results of preliminary screening, which obtained by changing the proportion of methanol, sodium acetate solution (0.5 and 1.0 %, w/v) or water and acetic acid, were examined with the following results.

Each 17OS sulfate separated each other with a decrease in methanol concentration, but the capacity factor (k') increased and the retention time was prolonged (Fig. 5). However the k' values decreased with an increase in column temperature (Fig. 6, A). And the k' value decreased slightly with a reduction in the pH of the mobile phase solvent (Fig. 7, A). The optimal proportion of a mobile phase components obtained by the examination was 0.5% methanol (v/v), 50% sodium acetate (w/v), acetic acid (57:42:1, v/v; pH 5.25) at 30°C (Fig. 5-7, A).

Five 17OS glucuronides (Fig. 5-7, B) and two 17OS glucuronides (Fig. 5-7, C) showed a similar separative tendency with changes in the methanol concentration, pH and column temperature. The peaks of 11OHA glucuronide and 11OHE glucuronide, and 11COA glucuronide and 11COE glucuronide overlapped, making complete separation difficult. The separation was improved by decreasing the methanol concentration and lowering the mobile phase pH. But as the k' values increased (Fig. 5, B : 6, B), the retention time was prolonged, and the column pressure was increased. The k' value and column pressure decreased with an increase in column temperature (Fig. 6, B). Hence the methanol concentration was adjusted as low as possible, and the column temperature was settled as high as possible. The optimal proportion of a mobile phase components for the five 17OS glucuronides obtained by the examination was 0.03% methanol (v/v), acetic acid (46:54, v/v; pH 4.35) at 55°C, and those for two 17OS glucuronides was 0.01% methanol (v/v), acetic acid (55 : 44, v/v; pH 4.15) at 55°C (Fig. 5-7, B and C).

Fluorescence spectrum. To obtain the fluorescence spectrums of DHEA sulfate and Ad glucuronide extracted as ion pairs and labeled with dansylhydrazine, the
fractions of the labeled steroids were separated by HPLC, collected, and the spectra of these fractions were monitored with a fluorescence spectrophotometer (Hitachi Model 4010). The dansylated DHEA sulfate had an excitation maximum at 332 nm and an emission maximum at 538 nm in the mobile phase solvent A. The dansylated Ad glucuronide had an excitation maximum at 332 nm and an emission maximum at 532 nm in solvent C.

**Standard curve and sensitivity.**

We prepared curves for the normal range (0–2 mg/l in original urine samples) and high range (2–20 mg/l in the same). The working curves were linear. The limits of detection were 360 pg for DHEA sulfate and 400 pg for Ad glucuronide (S/N=2).

**Stability.** The fluorescence of the dansylhydrazones obtained by dansylation of the ion pairs of 17S sulfates with TPA or 17S glucuronides with TPA following evaporation of the solvent was stable for 30 days under refrigeration.

**References**