CHROMSYMP. 1936

# Sensitive assay system for bile acids and steroids having hydroxyl groups utilizing high-performance liquid chromatography with peroxyoxalate chemiluminescence detection

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ABSTRACT

3$\alpha$- or 3$\beta$-hydroxysteroids, such as bile acids (free and glycine and taurine conjugates), 3$\beta$-hydroxy-5-cholenic acid, pregnanediol, 5-pregnene-3$\beta$,20$\beta$-diol and 5-pregnene-3$\beta$,20$\alpha$-diol, were converted to 3-oxosteroids by enzymatic reaction using immobilized hydroxysteroid dehydrogenase, derivatized with dansylhydrazine to the corresponding dansyl hydrazones and purified by gel permeation chromatography. The dansyl hydrazones were chromatographed on a $C_{18}$ column with a tetrahydrofuran-containing eluent and detected at the level of a few femtomoles by a peroxyoxalate chemiluminescence post-column reaction using bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl] oxalate as a chemilumigenic reagent. The dansyl hydrazones of chenodeoxycholic acid and deoxycholic acid (free and glycine and taurine conjugates) in particular, which coeluted under the chromatographic conditions above, were separated using an eluent including acetonitrile and 2,6-di-O-methyl-$\beta$-cyclodextrin and detected in the same way.

INTRODUCTION

Bile acids, produced from cholesterol in the liver, have one, two or three hydroxyl groups at the C-3, C-7 or C-12 position in the steroid ring. One or two such hydroxyl groups occur in other steroids, steroid hormones and metabolites, such as cholesterol, testosterone and pregnanediol. For the sensitive detection of these steroids, the hydroxyl group has been effectively used. For example, Okuyama *et al.*[1] reported the detection of bile acids by means of post-column derivatization using an immobilized hydroxysteroid dehydrogenase (HSD) column reactor, in which fluorescence detection of NADH [reduced form of nicotinamide adenine dinucleotide (NAD)] produced in the reactor was utilized. Kawasaki *et al.*[2] used an HSD reactor for

the precolumn derivatization of bile acids; the 3-oxo-bile acids thus produced were derivatized with dansylhydrazine to the corresponding dansyl hydrazones, separated and detected fluorimetrically.

Although these fluorimetric methods provided high sensitivity, the detection limits were in the picomole to sub-picomole range. Recently, we reported the sensitive detection of oxosteroids by derivatization of the oxosteroids with dansylhydrazine followed by high-performance liquid chromatographic (HPLC) separation and peroxyoxalate chemiluminescence detection[3,4]. The detection limits were in the femtomole range. Therefore, all hydroxysteroids could be sensitively detected according to the above procedures combined, provide that they can be converted to oxosteroids by the corresponding HSDs.

In this work, 3$\alpha$- or 3$\beta$-hydroxy-bile acids and 3$\alpha$- or 3$\beta$-hydroxysteroids were converted to the corresponding 3-oxosteroids by using co-immobilized 3$\alpha$- and 3$\beta$-HSD, derivatized with dansylhydrazine to the corresponding dansyl hydrazones, separated on a $C_{18}$ column and detected by peroxyoxalate chemiluminescence detection using bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl] oxalate (TDPO) as a chemilumigenic reagent.

EXPERIMENTAL

*Materials*

Fifteen bile acids (cholic acid, deoxycholic acid, chenodeoxycholic acid, lithocholic acid and ursodeoxycholic acid and their glycine and taurine conjugates) were purchased from Technochemical (Tokyo, Japan). 5$\beta$-Pregnane-3$\alpha$,20$\alpha$-diol (pregnanediol), 5-pregnene-3$\beta$,20$\alpha$-diol and 5-pregnene-3$\beta$,20$\beta$-diol were obtained from Sigma (St. Louis, MO, U.S.A.) and 3$\beta$-hydroxy-5-cholenic acid from Steraloids (Wilton, NH, U.S.A.). Dansylhydrazine, nicotinamide adenine dinucleotide (NAD) and hydroxysteroid dehydrogenase (HSD, grade II) were purchased from Sigma. Aminopropyl-CPG (120–200 mesh, 500 Å) was obtained from Electro-Nucleonics (Fairfield, NJ, U.S.A.). Bond Elut $C_{18}$ cartridges were purchased from Analytichem (Harbor City, CA, U.S.A.). Bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl] oxalate (TDPO) and 2,6-di-O-methyl-$\beta$-cyclodextrin were purchased from Wako (Osaka, Japan) and imidazole (zone refined) was a gift from Tokyo Kasei Kogyo (Tokyo, Japan). The other reagents were of analytical-reagent or HPLC grade.

An immobilized enzyme was prepared by immobilizing HSD on aminopropyl-CPG with glutaraldehyde as a coupling reagent[1]. This immobilized enzyme was packed into an open glass column (60 mm × 8 mm I.D.); the height of the packed material was 20 mm.

NAD solution was prepared by dissolving NAD in 0.1 $M$ tris(hydroxymethyl)aminomethane buffer (chloride, pH 9.00)–methanol (9:1) to give a 0.5 m$M$ solution. Dansylhydrazine solution was prepared by dissolving 2 mg of dansylhydrazine in 10 ml of ethanol. Hydrochloric acid (0.022 $M$) was prepared by dissolving 0.45 ml of 36% hydrochloric acid in 250 ml of ethanol.

*Preparation of dansyl hydrazones of hydroxysteroids*

A 20-nmol amount of each hydroxysteroid dissolved in 100 $\mu$l of methanol was applied to the immobilized enzyme column, then three 1-ml portions (for bile acids) or

five 1-ml portions (for other steroids) of the NAD solution were added to elute the oxosteroid produced on the column. The column eluate was mixed with 3 ml (for bile acids) or 5 ml (for other steroids) of water. The mixed solution was then applied to a Bond Elut $C_{18}$ cartridge which had been preconditioned with water. The cartridge was washed with three 3-ml portions of water and then evacuated with an aspirator to remove water in the column. The oxosteroid remaining in the cartridge was eluted with 3 ml of ethanol. The ethanol solution obtained was evaporated to dryness *in vacuo* at room temperature and the residue was dissolved in 1 ml of ethanol. To one tenth of this solution were added 200 μl of the dansylhydrazine solution and 2 ml of the 0.022 *M* ethanolic hydrochloric acid solution. The mixture was allowed to stand overnight at room temperature and evaporated to dryness *in vacuo*. The residue was dissolved in 100 μl of chloroform and a 80-μl portion of the chloroform solution was injected into a Finepak GEL 101 gel permeation column (500 mm × 7.2 mm I.D.) (JASCO). Chloroform was used as the eluent at a flow-rate of 0.7 ml/min.

The column eluate was monitored with a fluorimetric detector (JASCO Model 820-FP) at an excitation wavelength of 350 nm and an emission wavelength of 505 nm. The fraction from 14.2 to 15.2 min (for bile acids) or that from 14.8 to 16.1 min (for other steroids) was collected and evaporated to dryness *in vacuo*. The residue was dissolved in 1 ml of acetonitrile. A portion of the solution was diluted 100-fold with the corresponding eluent and a 10-μl aliquot was injected into the HPLC system.

*HPLC conditions*

The HPLC system consisted of an eluent delivery pump (JASCO Model 880-PU), a column oven (JASCO Model 860-CO), a reagent delivery pump (JASCO Model 880-PU), a chemiluminescence detector (JASCO Model 825-CL) and a mixing device (Kyowa Seimitsu Model KZU-1). The column eluate was mixed at the mixing device with the chemilumigenic reagent solution. The mixture was introduced into the detector, having a PTFE spiral-type flow cell with a volume of 93 μl. The analytical columns were a Finepak SIL $C_{18}$ S (5 μm) (150 mm × 4.6 mm I.D.) (JASCO) and a phenyl-bonded silica gel column packed with Develosil PhA (5 μm) (150 mm × 4.6 mm I.D.) (Nomura Chemical, Aichi, Japan) and the column temperature was 40°C. The eluent was 50 m*M* imidazole buffer (nitrate, pH 6.00)–tetrahydrofuran [95:105 (v/v) for the separation of bile acids or 105:95 (v/v) for that of other steroids] and was delivered at a flow-rate of 1.0 ml/min. In particular, a 25:75 (v/v) mixture of the same imidazole buffer and acetonitrile containing 7.5 m*M* 2,6-di-O-methyl-β-cyclodextrin was used as an eluent to separate chenodeoxycholic acid and deoxycholic acid (free and glycine and taurine conjugates).

The chemilumigenic reagent solution was prepared by dissolving 0.25 m*M* TDPO and 12.5 m*M* hydrogen peroxide in acetonitrile–ethyl acetate (1:1) and was delivered at a flow-rate of 1.7 ml/min.

RESULTS AND DISCUSSION

Hydroxysteroids are converted to oxosteroids in accordance with the oxidation selectivity of HSDs. For example, 7α-HSD converts 7α-hydroxysteroids to 7-oxo-steroids. In this way, selection of suitable HSDs permits the formation of different

kinds of oxosteroids. The oxosteroids obtained are derivatized with dansylhydrazine to the corresponding dansyl hydrazones.

Among several kinds of HSDs, we selected an HSD from Sigma which converts both 3α- and 3β-hydroxysteroids to 3-oxosteroids in the presence of NAD. The enzyme immobilized on aminopropyl-CPG was used repeatedly in the form of an enzyme column reactor to produce 3-oxosteroids. During this experiment, it was found that three times the reactor volume of NAD solution was required to elute 3-oxo-bile acids and five times the reactor volume to elute other 3-oxosteroids, probably because of the greater affinity of the latter to the reactor column.

Compounds derivatized with dansylhydrazine have a higher molecular weight than dansylhydrazine; the former elute faster than the latter in gel permeation chromatography (GPC). Thus, as reported previously[5], dansyl hydrazones of oxosteroids in a reaction mixture were separated from the excess of dansylhydrazine and purified. Fig. 1 shows a gel permeation chromatogram obtained from a reaction mixture of 7α,12α-dihydroxy-3-oxo-5βcholanic acid (an oxidation product of cholic acid) and dansylhydrazine. Peak 1 eluting at 14.7 min corresponds to the dansyl hydrazone of the above oxo-bile acid; the amount corresponds to 1.6 nmol.

A compound derivatized with dansylhydrazine forms *anti* and *syn* conformers; this results in the appearance of two peaks corresponding to these conformers with the
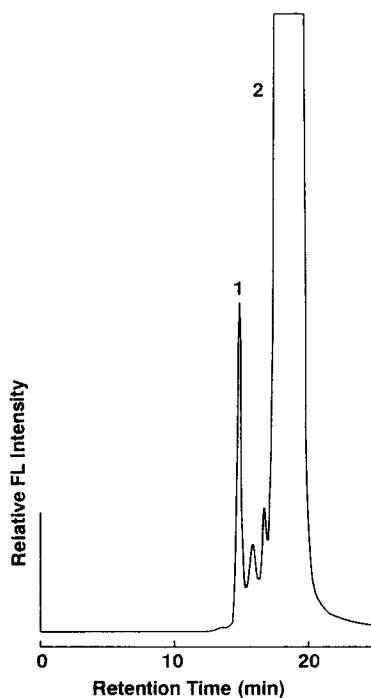


Fig. 1. GPC of a reaction mixture of 7α,12α-dihydroxy-3-oxo-5β-cholanic acid and dansylhydrazine. Peaks: 1 = dansyl hydrazone of cholic acid (1.6 nmol); 2 = dansylhydrazine. Conditions: column, Finepak GEL 101 (500 mm × 7.2 mm I.D.); eluent, chloroform; flow-rate, 0.7 ml/min; detector, 820-FP (excitation wavelength 350 nm, emission wavelength 505 nm).

usual organic constituents such as methanol and acetonitrile in the eluent[6]. As we reported previously[5], the use of tetrahydrofuran as an organic constituent in the eluent caused these two conformers to elute as a single peak with a $C_{18}$ column. This effect of tetrahydrofuran was also demonstrated in this work on the separation of bile acids and other steroids. Fig. 2 shows a chromatogram obtained from a mixture of derivatized bile acids (free form, 160 fmol each). Although chenodeoxycholic and deoxycholic acid remained unresolved, each bile acid exhibited a single peak. In the same way, derivatized glycine- and taurine-conjugated bile acids gave the corresponding single peaks. The detection limits at a signal-to-noise ratio of 2 were between 3 and 8 fmol for all the bile acids. The difference in the detection limits could be due to the difference in reactivity of immobilized HSD with individual bile acids. These detection limits were about 100 times lower than those obtained with fluorescence detection[2].

On the $C_{18}$ column, the separation of the dansyl hydrazone of chenodeoxycholic acid from that of deoxycholic acid was not successful. Another kind of reversed-phase column with phenyl-bonded silica gel was used with tetrahydrofuran as an organic constituent. However, the separation was not achieved. We then investigated the effect of $\beta$-cyclodextrins on the separation between the dansyl hydrazones of these two bile acids, as the addition of $\beta$-cyclodextrin to the eluent (phosphate buffer–acetonitrile) improved the separation of bile acids according to Shimada et al.[7]. Addition of
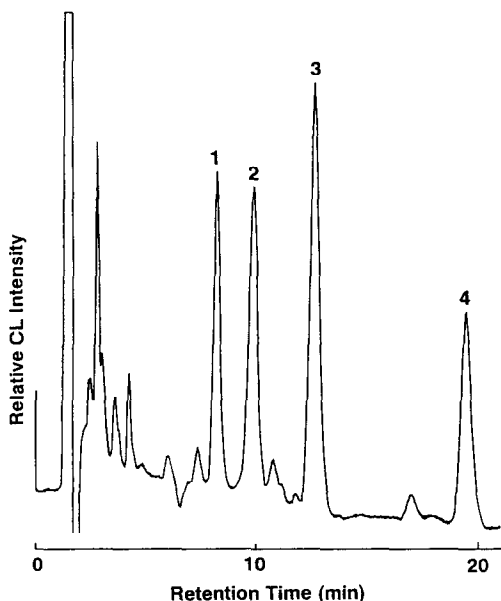


Fig. 2. HPLC of a mixture of bile acids (free form) derivatized with dansylhydrazine. Peaks (160 fmol each): dansyl hydrazones of (1) cholic acid, (2) ursodeoxycholic acid, (3) chenodeoxycholic acid and deoxycholic acid and (4) lithocholic acid. Conditions: column, Finepak SIL $C_{18}$ S (5 μm) (150 mm × 4.6 mm I.D.); column temperature, 40°C; eluent, 50 m$M$ imidazole buffer (nitrate, pH 6.00)–tetrahydrofuran (95:105, v/v); flow-rate, 1.0 ml/min; chemilumigenic reagent, 0.25 m$M$ TDPO and 12.5 m$M$ hydrogen peroxide in acetonitrile–ethyl acetate (1:1, v/v); reagent flow-rate, 1.7 ml/min.

$\beta$-cyclodextrin to the eluent containing 52.5% tetrahydrofuran for the $C_{18}$ column resulted in precipitation of $\beta$-cyclodextrin owing to its low solubility in tetrahydrofuran. Instead, more soluble cyclodextrins such as 2,6-di-O-methyl- and 2,3,6-tri-O-methyl-$\beta$-cyclodextrin were examined. However, the separation between the dansyl hydrazones of the two bile acids was not successful. Next, tetrahydrofuran was replacd with acetonitrile. As shown in Fig. 3, derivatized chenodeoxycholic acid and deoxycholic acid were separated by using an eluent containing 2,6-di-O-methyl-$\beta$-cyclodextrin, acetonitrile and the imidazole buffer, although the peaks were broader than those obtained with an eluent containing tetrahydrofuran without cyclodextrin. The reason why the effect of the cyclodextrin disappeared on addition of tetrahydrofuran is not clear.

In the same way, glycine and taurine conjugates of these two bile acids were separated. The detection limits at a signal-to-noise ratio of 2 were between 8 and 15 fmol. Hence a combination of these two kinds of eluent permits the separation of all fifteen bile acids.

As shown in Fig. 4, derivatized 3$\beta$-hydroxy-5-cholenic acid (a bile acid having a 3$\beta$-hydroxyl group, 160 fmol) was also detected sensitively by the present system. The detection limit at a signal-to-noise ratio of 2 was 4 fmol.
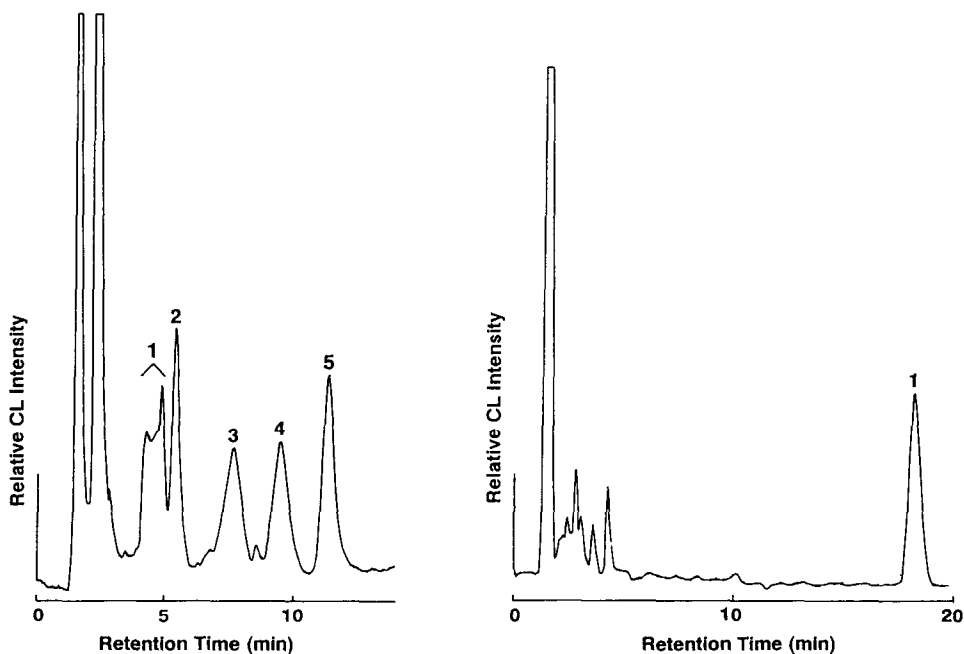


Fig. 3. HPLC of a mixture of bile acids (free form) derivatized with dansylhydrazine. Peaks (160 fmol each): dansyl hydrazones of (1) ursodeoxycholic acid, (2) cholic acid, (3) chenodeoxycholic acid, (4) deoxycholic acid and (5) lithocholic acid. Eluent, 50 m$M$ imidazole buffer (nitrate, pH 6.00)–acetonitrile (25:75, v/v) containing 7.5 m$M$ 2,6-di-O-methyl-$\beta$-cyclodextrin; other conditions as in Fig. 2.

Fig. 4. HPLC of 3$\beta$-hydroxy-5-cholenic acid derivatized with dansylhydrazine. Peak 1 = dansyl hydrazone of 3$\beta$-hydroxy-5-cholenic acid (160 fmol). Conditions as given in Fig. 2.
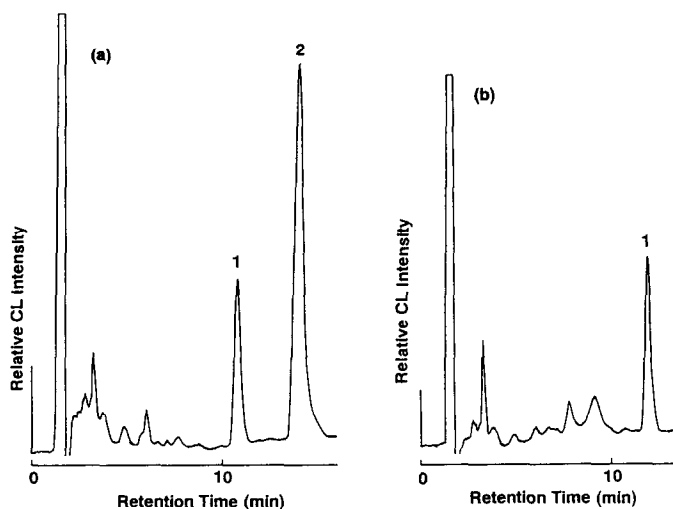
Fig. 5. HPLC of 3β-hydroxysteroids and pregnanediol derivatized with dansylhydrazine. (a) Peaks (160 fmol each): dansyl hydrazones of (1) 5-pregnene-3β,20α-diol and (2) 5-pregnene-3β,20β-diol. (b) Peak 1 = dansyl hydrazone of pregnanediol (160 fmol). Eluent, 50 mM imidazole buffer (nitrate, pH 6.00)–tetrahydrofuran (105:95, v/v); other conditions in Fig. 2.

Fig. 5 shows chromatograms obtained from two derivatized 3β-hydroxysteroids (5-pregnene-3β,20α-diol ad 5-pregnene-3β,20β-diol) and from derivatized pregnane-diol (5β-pregnane-3α,20α-diol). It can be seen that steroids that have a 3β- or 3α-hydroxyl group were also detected sensitively by the present system. The detection limits at a signal-to-noise ratio of 2 were between 2 and 4 fmol.

We are now trying to apply this detection method to the determination of bile acids in biological fluids such as serum and urine. After bile acids have been separated into three groups, namely free, glycine-conjugated and taurine-conjugated, with a piperidinohydroxypropyl-Sephadex LH-20 column[8], each group of bile acids would be analysed by the present detection method.

An immobilized 3α- or 3β-HSD column reactor provides better selectivity than the HSD reactor used in this work and should be used in analysis of bile acids in biological fluids. In the similar way, as in this work, the use of immobilized 7α-HSD would permit the analysis of chenodeoxycholic acid and cholic acid and immobilized 12α-HSD that of deoxycholic acid and cholic acid.

In the present assay system, oxosteroids present in the biological specimens would affect the selectivity. Therefore, steroids having an oxo group should be converted to N-hydroximes with hydroxylamine and removed with a Bond Elut SCX cartridge (strong cation-exchange type) (Analytichem)[9] before the enzymatic conversion of hydroxysteroids. In this way, the hydroxysteroids remaining in the specimens can be subjected to the present assay system.

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