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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF HYDROXYSTEROIDS DETECTED WITH POST-COLUMN IMMOBILIZED ENZYME REACTORS

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SUMMARY

Assaying the low concentrations of steroid hormones in extracts of body fluids requires detectors that are both highly sensitive to the steroid and relatively insensitive to interfering compounds usually present in much higher concentrations. To explore the use of moderately specific enzymes in post-column reactors, we immobilized 3α- and β-hydroxysteroid dehydrogenase on controlled pore glass beads, 37 μm in diameter, and constructed 4.6-mm diameter reactor columns, 3-cm long, packed with one of the two kinds of these beads. Hydroxysteroids eluted from the analytical column were mixed with the coenzyme, nicotinamide adenine dinucleotide (NAD), before passing through the reactor. The effluent from the reactor was passed through the 70-μl flow cell of a fluorometer in which the fluorescence of the NADH produced in the enzyme-catalyzed oxidation of the hydroxysteroid was monitored.

At the conventional high-performance liquid chromatography flow-rates used, oxidation of the steroids was almost complete. The yield depended on both the residence time of steroids in the reactor column and the concentration of organic modifier in the reaction mixture. Maximal yield was obtained with buffer having a low organic solvent concentration and passing through the reactor slowly. In assays of mixtures of epimeric hydroxysteroids, the 3α-hydroxysteroids were detected with the 3α-hydroxysteroid dehydrogenase reactor; the β-hydroxysteroids were not, confirming the specificity of the enzymatic detection. With the fluorometer used, picomole quantities of steroids could easily be distinguished from noise.

INTRODUCTION

In body fluids steroid hormones are generally found in low concentrations accompanied by a variety of chemically similar compounds that follow the steroids through most extraction and clean-up procedures. Assaying them by chromatography therefore requires detectors that are highly sensitive to the steroids of interest and that do not respond to the potentially interfering compounds that emerge from the chromatographic system simultaneously.

The JASCO high-performance liquid chromatography (HPLC) system for the analysis of bile acids and certain of their metabolites involves the use of a post-

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column reactor containing immobilized 3α-hydroxysteroid dehydrogenase. A reagent stream, containing nicotinamide adenine dinucleotide (NAD) in buffer, is mixed with the column effluent and delivered to the reactor, in which the steroids react to produce NADH. The fluorescence of the NADH is monitored. This combines the high sensitivity of fluorescence detection with the group specificity of the enzymatic reaction.

Our success in assaying bile acids in serum with this system[1] encouraged us to extend this approach to the somewhat more difficult task of assaying steroid hormones. In the work described here, we prepared post-column reactors containing either 3α- or β-hydroxysteroid dehydrogenase immobilized on glass beads and studied the conditions for quantitative analysis with this method of detection.

EXPERIMENTAL

Reagents and materials

Methanol and acetonitrile were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). The enzymes, 3α-hydroxysteroid dehydrogenase and β-hydroxysteroid dehydrogenase, the coenzymes, NAD and NADH and all the steroids were purchased from Sigma (St. Louis, MO, U.S.A.). Controlled-pore glass beads, CPG/aminopropyl, which were specified to be silylated with a primary amino function at the terminal end, were bought from Pierce (Rockford, IL, U.S.A.).

Preparation of the post-column reactor

Enzymes were immobilized on the glass beads by the procedure described by Weetal[2]. CPG/aminopropyl glass beads (5 g; pore diameter 500 Å; diameter 125–171 μm), were washed twice with 10-ml portions of 10 m*M* potassium dihydrogenphosphate (pH 7.8) in an ultrasonic bath and the washing solution was decanted. An aldehyde derivative of the amine function was prepared by adding 4.5 ml of 10 m*M* potassium dihydrogenphosphate (pH 7.8) and 0.5 ml of 25% glutaraldehyde and incubating for 1 h with stirring by ultrasonic vibration intermittently (for 5 min every 15 min), to avoid overheating. The reaction was then stopped by decanting the reagent, and the beads were washed twice 10-ml portions of the phosphate buffer. They were then packed in a 30 × 4.6 cm stainless-steel column by the technique used to pack the usual HPLC column. The beads were then coated with dehydrogenase by pumping a solution of the enzyme through the column. Twenty-five units of the steroid dehydrogenase were dissolved in buffer and recirculated through the glass bead column with the HPLC pump at a flow-rate of 0.3 ml/min for 1 h. The beads were then washed with phosphate buffer at 1 ml/min for 30 min.

HPLC of steroids

A JASCO HPLC system (JASCO, Tokyo, Japan), designed for HPLC of bile acids[1,3], was used to chromatograph the steroids. This system includes a microprocessor-controlled gradient programmer, a tri-rotor pump for delivering the mobile phase and a bi-rotor pump for the substrate buffer. In place of the fluorometric detector supplied with the system, we used an Aminco Fluoromonitor, Model J4.7461 (American Instruments, Silver Spring, MD, U.S.A.) with a 70-μl flow cell. For simultaneous UV absorbance and fluorescence detection, a Kratos Spectroflow 757 absorbance detector (Kratos, Ramsey, NJ, U.S.A.) was placed in the line before the

fluoromonitor. Steroids were separated on a 150 mm × 4.6 mm C_{18} reversed-phase column by mobile phases containing different concentrations of methanol or acetonitrile in water. As the effluent left the column, a reagent solution was added and mixed in a 30 cm × 0.2 mm stainless-steel capillary that led the combined solution to the reactor column. The reagent solution, which was freshly prepared, contained 0.3 mmoles of NAD, 10.0 mmoles of potassium dihydrogenphosphate, 1.0 mmole of disodium ethylenediaminetetraacetate and 2 mmoles of N-acetylcysteine in 1 l of water. The production of NADH in the reactor was monitored by its fluorescence at 470 nm, excitation at 340 nm.

Measurement of enzyme activity in the reactor

The activity of the 3α-hydroxysteroid dehydrogenase immobilized in the reactor column was estimated by pumping reagent solution (see above), containing androsterone added in relatively high concentration, through the reactor at different flow-rates and monitoring the absorbance of the effluent at 340 nm. A LDC pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.) was used to meter the flow and a Perkin Elmer (Norwalk, CT, U.S.A.) LC-55 spectrophotometer to monitor the absorbance. The substrate solution was prepared by mixing 15 mg of androsterone in 40 ml of methanol with 60 ml of the reagent buffer. For assaying the activity of β-hydroxysteroid dehydrogenase in the corresponding "beta" reactor, 15 mg of testosterone in 20 ml of methanol was mixed with 80 ml of reagent buffer. The flow-rate was increased until the rate of production of NADH, calculated by multiplying the absorbance by the flow-rate, was maximal. At this flow-rate, the enzymatically active sites were assumed to be saturated. The rate of production of NADH was then taken as an indication of the enzyme activity.

The enzymatic activity of one of the JASCO reactors, containing 3α-hydroxysteroid dehydrogenase, assayed the same way, measured 60% of that of the reactor prepared in our laboratory.

Measurement of conversion efficiency

The efficiency of oxidation of steroid in the enzyme reactor was measured by direct introduction of steroid solution without an analytical column. Known quantities of 3α,21-dihydroxy-5α-pregnance-20-one were injected into the reactor containing the α-enzyme and of testosterone into the reactor containing the β-enzyme. The fluorescence of the NADH produced was compared with that of known concentrations of NADH injected similarly.

RESULTS

The maximum rate of oxidation of androsterone and testosterone, pumped at concentrations of 150 mg/l, through reactors containing 3α- or β-hydroxysteroid dehydrogenase respectively indicated that approximately 20 I.U. of enzyme activity was available in the reactor columns. This estimate was based on observing an absorbance of 0.4 in a 0.6-cm path length when reagent was pumped through a column containing 0.5 ml void volume at 1 ml/min. (The substrates were considered to have been in contact with the enzyme for approximately 0.5 min, under these conditions). We did not estimate the fraction of the nominal 20 I.U. added (from the package

insert) that this activity represented. We assumed that we had presented the solid support with enzyme in excess and that it bound as much as it could.

The β-reactor was very sensitive to organic solvent content of the buffer. As the concentration of methanol in the buffer was increased from 10% to 50%, the enzyme activity decreased, and at 50% methanol the reactor was irreversibly deactivated. Below 30% of methanol, the reduction was reversible.

As the substrate concentration was increased well beyond the range expected in the biological samples, smaller fractions of the substrate were oxidized, suggesting saturation of the enzyme at these concentrations. Increasing the reactor length and the quantity of enzyme by placing a second reactor in series allowed a higher maximum reaction rate to be achieved.

The completeness of the reaction was estimated by comparing the peak area with that obtained by injecting a measured quantity of NADH. Oxidation of 3α-hydroxysteroids was estimated to be 84% complete when the substrate flowed through the α-reactor at 1.0 ml/min, and was complete at 0.5 ml/min. At 1.0 ml/min, two reactor columns in series increased the yield from 84 to 100%. In the β-reactor, oxidation of testosterone was complete with a buffer that contained 20% methanol at a flow-rate of 2.0 ml/min. After several months of use, the completeness of reaction decreased at all flow-rates.

When different 3α-steroids were chromatographed on a reversed-phase column with a mobile phase containing 30% of acetonitrile and 30% methanol, the completeness of the reaction of different compounds varied only slightly (Table I), while that of the β-hydroxysteroids in the β-reactor was somewhat more variable (Table II).

The fluorescence response was proportional to the quantity of steroid injected over at least the 0.15–4 nmole range (Fig. 1).

At somewhat higher concentrations, some steroids could be detected by their absorbance in the UV as well as by the fluorescence of the NADH produced in the reactor. Ketosteroids absorb at 240 nm while hydroxysteroids do not, although both could be detected after passing through the reactor, provided they contained reactive hydroxyl groups. Simultaneous UV detection, ahead of the reactor, and fluorescence detection after it gave specific information about the kind of compounds being detected, although the sensitivity of UV absorbance detection was comparatively poor (Fig. 2).

TABLE I

CAPACITY RATIO (k') AND CONVERSION EFFICIENCY OF 3α-HYDROXYSTEROIDS

Steroid	*k'*	*Conversion (%)*
3α,21-Dihydroxy-5α-pregnan-20-one	12.50	96
3α,17α-20β-Trihydroxy-5α-pregnan	15.43	100
3α,17α-Dihydroxy-5α-pregnan-20-one	16.76	89
3α,20α-Dihydroxy-5α-pregnan	27.25	91
3α,20β,Dihydroxy-5α-pregnan	33.69	85
3α,17β-Dihydroxy-5α-androstan	11.43	100
3α-Hydroxy-5β-androstan-17-one	12.68	97
3α-Hydroxy-5α-androstan-17-one	14.09	91

TABLE II

CAPACITY RATIO (k') AND CONVERSION EFFICIENCY OF β-HYDROXYSTEROIDS

Steroid	k'	*Conversion (%)*
3β-17α,20β-Trihydroxy-5α-pregnan	7.47	56
3β,20α-Dihydroxy-5α-pregnan	17.49	100
3β,20β-Dihydroxy-5α-pregnan	22.64	100
3β,17β-Dihydroxy-5β-androstan	8.00	78
3β,17β-Dihydroxy-5α-androstan	8.55	78
17β-Hydroxy-5α-androstan-3-one	8.32	93
3β-Hydroxy-5β-androstan-17-one	7.56	40
3β-Hydroxy-5α-androstan-17-one	7.65	73
17β-Hydroxy-4-androsten-3-one	5.49	65

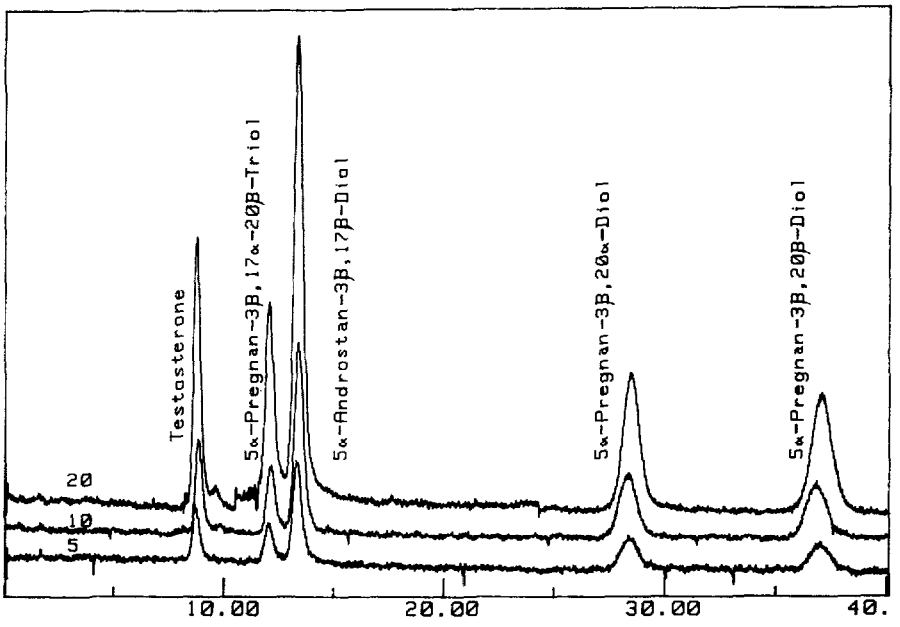

Fig. 1. Chromatogram of 3β-hydroxysteroids, separated on a Nucleosil-5, C_{18} column. The five-component standard mixture contained 5 ng/μl of each steroid. The chromatograms from bottom upwards correspond to 5-, 10-, and 20-μl samples. The mobile phase contained 70% of methanol at a flow-rate of 1.0 ml/min. The mixing ratio of effluent to reagent buffer was 1:2.

DISCUSSION

With all flow-rates and reactor lengths described, the oxidation reaction did not always go to completion. The yield of product seemed most affected by the time allowed for reaction. Slowing the flow-rate from 1 ml/min to 0.5 ml/min, thus doubling the residence time of the substrate in the reactor produced more product. Further slowing did not increase the product any more, suggesting that the reaction was complete. Adding a second reactor in series, thus also doubling the reaction time,

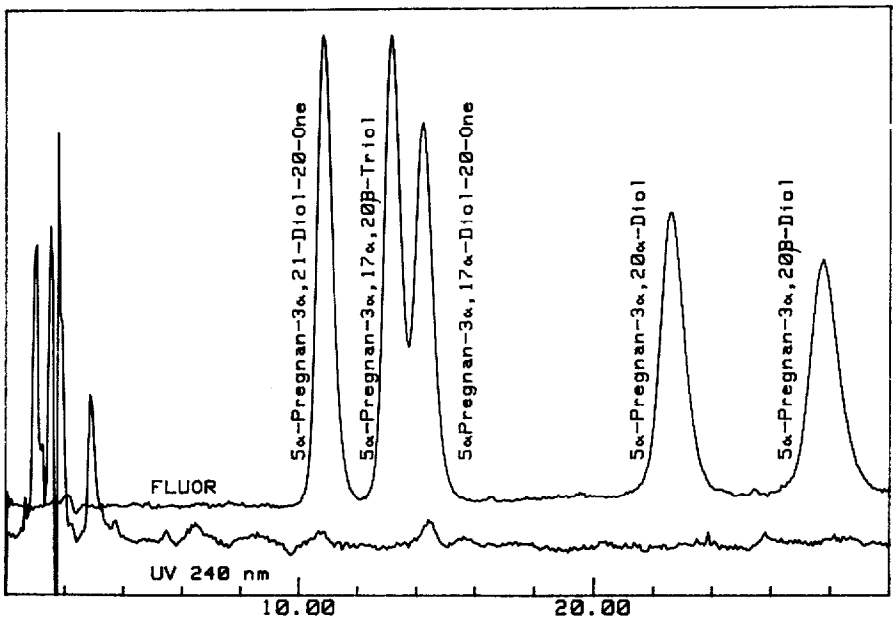

Fig. 2. Chromatogram of 3α-hydroxysteroids, separated on a Nucleosil-5, C_{18} column. The fluorescence peaks (upper tracing) are those of the NADH formed from the oxidation of the steroids by NAD in the enzyme reactor, and the UV peaks (lower tracing) are due to the absorbance at 240 nm by ketosteroids. The mobile phase contained 30% each of methanol and acetonitrile at a effluent to reagent buffer was 1:1.

also brought the reaction to completion. Since we were limited by starting with prepared glass beads with a propylamine function, we could not study the effect of increasing the enzyme-coated surface area of the same length of reactor, which would be predicted to have the same effect as increasing the reactor length.

Similarly complete reactions were observed with both the α- and β-enzymes, although the latter was more sensitive to the organic content in the buffer: increasing the methanol content slowed the reaction and decreased the yield. If the concentration of methanol was kept low, this effect was reversible, suggesting either that some of the enzyme was more subject to reversible inactivation than the rest, or that the effect of the methanol was to decrease the affinity of the enzyme for the substrate.

Since high methanol concentrations inactivated the enzyme, we found it advisable, when shutting down the system, to stop the flow of mobile phase through the analytical column several minutes before stopping the flow of reagent buffer through the enzyme reactor.

Oxidation of hydroxy to keto groups in reactions catalyzed by dehydrogenases are generally promoted by increasing pH. Increasing the pH in the reactor from 7.8 to 9.8 did not affect the yield, suggesting that the reactions were close to complete even at the lower pH. For reasons yet to be determined, at higher pH, bands emerging from the reactor were higher and narrower, as though the elution of compounds from the reactor was speeded.

Post-column reactors containing hydroxysteroid dehydrogenase have previously been used quite effectively for detecting bile acids in extracts of serum[1,4,5].

Wu *et al.*[3] described a similar approach for assaying steroid hormones. In our bile acid studies with the JASCO system, which employed gradient elution as well as post-column reactors, we were impressed with the sensitivity, speed and the group specificity of the approach. We observed, however, that different bile acids appeared to react to varying extent, requiring calibration of the system for each compound to be assayed. Although we postulated several reasons for this, we were unable to verify any of these experimentally.

In the work described here, we observed less variation in response from compound to compound, but have so far not had the opportunity to determine whether the steroid hormones behaved differently in the enzyme-catalyzed reaction than the bile acids, or whether the increased yield was caused by increased quantity of enzyme and length of reactor column. Careful comparison of the conditions used here with those recommended by JASCO and those described by Wu *et al.* showed only that we used a somewhat higher ratio of post-column reagent to mobile phase, and therefore could use a mobile phase with higher content of organic solvent without inactivating or supressing the activity of the enzyme. We also used a fluorometer with a larger flow cell which may have contributed to the higher sensitivity we obtained. The larger volume flow cell, 70 μl, had little effect on resolution because of the 3 ml/min combined flow-rate of mobile phase and post-column reagent. These factors, faster chromatography, more complete reactions in the post-column reactor, and overall more sensitive detection of the NADH, all contributed to what we believe were analyses that were improved over what were reported previously.

The high sensitivity of fluorescence detection of NADH, coupled with the group specificity offered by the dehydrogenase reactors, should simplify many assay procedures for steroids in body fluids. It is predicted that even greater sensitivity should be attainable by allowing the NADH produced to react further, *e.g.*, with resorufin in the presence of the enzyme, diaphorase, to produce an even more easily detectable fluorescent compound. It should also be possible to couple the NADH formed in the reactor to a chemiluminescent reaction with still further increase in sensitivity.

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