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Determination of Ursodeoxycholic Acid in Serum by a New Fluorometric Enzymatic Method Using 7β-Hydroxysteroid Dehydrogenase from *Clostridium absonum*

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A fluorometric enzymatic method for the determination of ursodeoxycholic acid (UDCA) and its glycine and taurine conjugates in human serum has been developed. A simple and fast purification and preconcentration procedure using Sep Pak C_{18} cartridges was employed for the UDCA extraction from human serum. UDCA and its conjugates were determined in the extracted sample by an equilibrium method based on the enzymatic conversion of the 7α-hydroxy group into 7-oxo group by β-nicotinamide adenine dinucleotide phosphate in the presence of 7β-hydroxysteroid dehydrogenase (7β-HSD) and the produced NADPH was monitored fluorometrically. The 7β-HSD, which is not yet commercially available, was isolated from *Clostridium absonum* cultures (ATCC No. 27555) and purified by affinity chromatography. The method has a limit of detection of 0.8 μM in serum and the precision varied from 6.1 to 2.0% for low and high concentrations, respectively. The recovery of UDCA from serum samples was about 99% (range 85–105%). The method was successfully applied to UDCA determination in serum samples from patients treated with UDCA for primary biliary cirrhosis.

Ursodeoxycholic acid (UDCA),[2] the 7β-epimer of chenodeoxycholic acid (CDCA), is the major bile acid in bear bile. Recent interest in UDCA has risen from its use as a medical treatment of choice for cholesterol gallstone disease (1,2), as well as for loading tests since it is not hepatotoxic (3). Recently it was shown that UDCA administration improves clinical and biochemical signs in patients with primary biliary cirrhosis, but the therapeutic mechanisms of this action are still under investigation (4–7).

During ursotherapy the concentration levels of UDCA in serum rise significantly while in normal conditions are very low (8–9). Therefore, the estimation of UDCA in serum during ursotherapy is potentially beneficial for clinical, pharmacological, and metabolic studies.

Several methods for the determination of UDCA in serum have been described. GLC (10) and HPLC (11) are sensitive and accurate methods particularly useful for metabolic studies but they are time consuming and require many preparatory steps. Immunoassays such as EIA (12,13) and RIA (14) are very sensitive and relatively simple techniques. However, they require reagents which are not commercially available and are difficult to prepare.

The first spectrophotometric enzymatic estimation of UDCA in human and bear biles in the range of 20–100 μM (15) was enabled after the isolation and purification of the 7β-HSD from *Clostridium absonum* cultures by Macdonald *et al.* (16–19).

In this report we describe a simple, rapid, and sensitive fluorometric enzymatic method for the determination of UDCA and its glycine and taurine conjugates in serum using 7β-HSD. The 7β-HSD was prepared by us according to Macdonald *et al.* (16–19) after various modifications of the isolation and purification steps. The determination is based on the conversion of 7β-hydroxy bile acid to 7-oxo bile acid by β-$NADP^+$ in the presence of 7β-HSD and the fluorometric monitoring of the produced NADPH NAPH.

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[2] Abbreviations used: 7β-HSD, 7β-hydroxysteroid dehydrogenase; UDCA, ursodeoxycholic acid; BHIB, brain heart infusion broth; BA, bile acids; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; GUDCA, glycoursodeoxycholic acid; TUDCA, tauroursodeoxycholic acid; EIA, enzyme immunoassay; RIA, radioimmunoassay; RSD, relative standard deviation.

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METHODS AND MATERIALS

Reagents

All solutions were prepared with deionized–distilled water from analytical reagent grade materials unless otherwise noted. Sodium salts of cholic acid (CA), CDCA, and deoxycholic acid (DCA) were purchased from Sigma. UDCA and its conjugates with glycine (GUDCA) and taurine (TUDCA) were obtained from Calbiochem. All bile acids were chromatographically pure (TLC) (20). β-$NADP^+$ and NADPH sodium salts were from Sigma. Sep Pak C_{18} cartridges (Waters Associates) were used for the UDCA extraction from human serum samples.

Preparation of 7β-Hydroxysteroid Dehydrogenase from C. absonum

C. absonum strain (ATCC No. 27555) was kindly donated by J. D. Sutherland, collaborator of the late Professor I. A. Macdonald (Dalhousie University Nova Scotia, Canada). The 7β-HSD enzyme was prepared according to Macdonald *et al.* (16–19) with some alterations and modifications in order to simplify the procedure.

Lyophilized bacteria were inoculated directly in Robertson's cooked meat medium and grown overnight (37°C) under anaerobic conditions in nitrogen atmosphere. One volume of this stock culture was inoculated into 10 vol of brain heart infusion broth (BHIB) medium and grown anaerobically overnight at 37°C for the preparation of the starter culture. Then, 1 vol of the starter culture was transferred into a 10-fold volume of BHIB medium containing 0.40 mM DCA. The inoculated preparation was cultivated under anaerobic conditions for 6 h at 37°C. The produced harvest culture was centrifuged at 6000*g* for 20 min at 4°C for the separation of the *C. absonum* cells. The cells were lysed in an ultrasonic bath and the crude cell-free enzyme extract (containing both 7α- and 7β-HSD) was obtained by centrifugation at 6000*g* for 20 min at 4°C. The crude preparation was immediately lyophilized and stored at −80°C.

The 7β-HSD was purified from the crude lyophilized extract using Procion Red HE3B (Pierce Eurochemie) affinity chromatography (19). The 7β-HSD eluate was immediately lyophilized and stored at −80°C. The purified enzyme had a specific activity of about 0.11 U/mg.

Sources of Human Serum Samples

Seventeen serum samples were obtained from Hippokration General Hospital, Athens, in a code number form from cirrhotic patients subjected to ursotherapy and from healthy individuals. All serum samples were kept at −10°C until use.

Instruments

Fluorescence measurements were performed using a Perkin–Elmer M512 fluorescence spectrophotometer supplied with a 150-W xenon lamp and a specially designed magnetic stirrer for the cuvette, and the fluorometric signals were recorded on a Sargent–Welch XKR potentiometric recorder. Instrumental calibration and settings have been described elsewhere (21). The specific activity of 7β-HSD preparations was checked with a Spectronic 20 Bausch & Lomb spectrophotometer.

Procedure for Kinetic Studies

A 1.70-ml volume of glycine buffer, 0.100 M (pH 9.00), containing EDTA (5.0 mM), 0.100 ml of bile acid solution, and 0.100 ml of 7β-HSD solution (0.04 U/ml) are placed into the cuvette. The stirrer is started and after the fluorescence signal has stabilized the reaction is initiated by injection of 0.100 ml of β-$NADP^+$ (50.0 mM). The fluorescence signal is recorded for about 2 min. Kinetic parameters are determined from initial rate measurements using Lineweaver–Burk plots (22).

Determination of UDCA, GUDCA, and TUDCA in Aqueous Solutions

A 1.50-ml volume of glycine 1.0 M/hydrazine sulfate 0.4 M buffer (pH 9.00), containing EDTA (5.0 mM), 0.200 ml of bile acid solution (in the concentration range 20–80 μM), and 0.200 ml of 7β-HSD solution (0.09 U/ml) are placed into the cuvette, and the mixture is stirred. After the fluorescence signal has stabilized the reaction is initiated by injection of 0.100 ml of β-$NADP^+$ solution (30.0 mM) and the fluorescence intensity is recorded until the signal value reaches its maximum (about 10 min). Calibration graphs for UDCA, GUDCA, and TUDCA were obtained by plotting BA concentration (final concentration range 2.0–8.0 μM) vs relative fluorescence intensity, *F*.

Determination of UDCA in Serum

The various steps for sample preparation and the UDCA determination in human serum are shown in the diagram of Fig. 1. Sep Pak cartridges were used for the quantitative extraction of UDCA and its conjugates from human serum (23). Two milliliters of a serum sample that is well mixed by a Vortex mixer with 10 ml of phosphate buffer 0.100 M, (pH 7.50) is applied to the cartridge and allowed to pass through at a rate of about 1 drop $\cdot s^{-1}$. The cartridge is washed with 3 ml of water and the BA are then eluted with 2× 1 ml of absolute methanol. The eluate is evaporated to dryness at about 60°C under a stream of nitrogen and the residue is dissolved in 1.70 ml of glycine 1.0 M/hydrazine sulfate 0.4 M buffer, (pH 9.00). The Sep Pak extract (1.70 ml) is placed in the cuvette and UDCA is measured according to the procedure for the determination of UDCA in aqueous solutions.

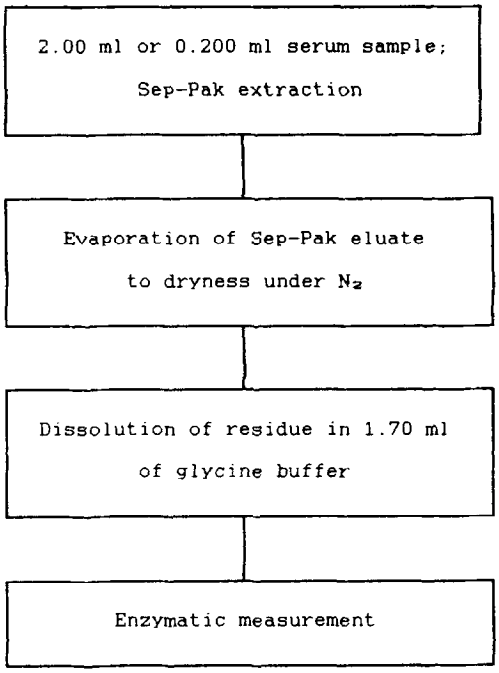

FIG. 1. Analytical procedure for the enzymatic fluorometric determination of UDCA and its conjugates in serum.

The above procedure is used when UDCA concentration in the serum sample is in the range of 2.0 to 8.0 μM. When UDCA concentration levels are higher than 8 μM, the same procedure can be used, but only 0.200 ml of serum sample is applied to the Sep Pak cartridge, the dry residue is redissolved in 1.70 ml of glycine/hydrazine buffer, and UDCA is measured as described previously. Calibration graphs (UDCA concentration vs fluorescence intensity) in a final concentration range of 2.0 to 8.0 μM were obtained using UDCA standards added to UDCA-free pooled serum, treated according to the entire analytical procedure.

RESULTS AND DISCUSSION

7β-Hydroxysteroid Dehydrogenase Purity and Activity Study

The purity of the produced 7β-HSD was checked fluorometrically by the reaction of a 3α-hydroxybile acids such as lithocholic acid and 7α-hydroxybile acids such as CA and CDCA with β-$NADP^+$, in the presence of the enzyme. Clearly the enzyme does not catalyze the reaction of β-$NADP^+$ with any of the above mentioned substrates nor with their glycine and taurine conjugates. The specific activity of the purified enzyme (reconstituted lyophile) determined spectrophotometrically according to Macdonald *et al.* (18) was found to be 0.11 U/mg.

Effect of pH on 7β-HSD Activity

The effect of pH on 7β-HSD activity was studied spectrofluorometrically. A glycine buffer 0.100 M containing

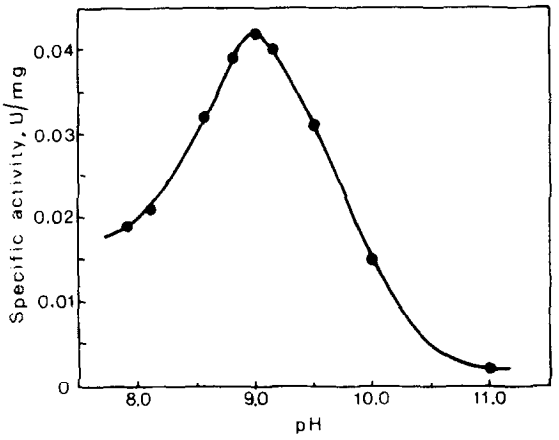

FIG. 2. Effect of pH value on the specific activity of 7β-HSD.

EDTA 5.0 mM, in the pH range of 7.75 to 11.75 was used for the study. The measure of the reaction rate was the change in relative fluorescence intensity (ΔF/min) expressed in specific activity units (U/mg). The obtained results are shown in Fig. 2. The optimal pH range for the purified and lyophilized 7β-HSD was 8.50–9.50, the optimal value being 9.00, while Macdonald and Roach (16) reported a pH optimum of 9.50 for the crude cell-free preparation. A glycine buffer, 1.0 M of pH 9.0, containing hydrazine sulfate 0.4 M as a trapping reagent and EDTA 5.0 mM, was chosen for the determination (21).

Effect of β-$NADP^+$ Concentration

The effect of β-$NADP^+$ concentration on the initial reaction rate was studied in the range 0.20 to 2.8 mM as shown in Fig. 3. For final β-$NADP^+$ concentrations greater than 1.50 mM the initial reaction rate remains constant, while the background fluorescence increases with increasing β-$NADP^+$ concentration. Therefore, the β-$NADP^+$ concentration of 1.50 mM was chosen as optimum for the endpoint determination of UDCA. A K_m of

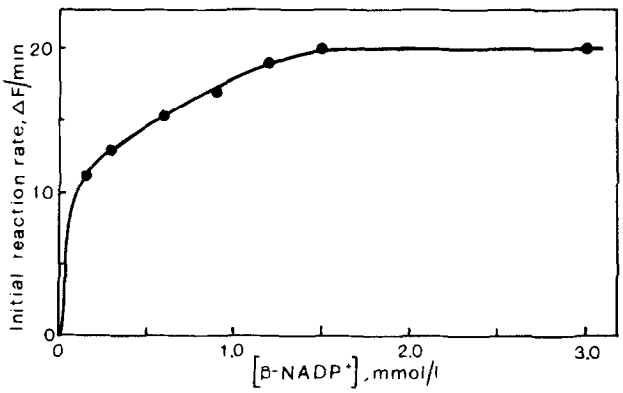

FIG. 3. Effect of β-$NADP^+$ concentration on the initial reaction rate.

TABLE 1

Kinetic Parameters of 7β-HSD from *Clostridium absonum*

Substrate	K_m (μM)	V_{max} (U/mg)
UDCA	20[a]	0.060[a]
	9.5[b]	0.420[b]
	18[c]	0.170[c]
GUDCA	35[a]	0.025[a]
	93[c]	0.074[c]
TUDCA	290[a]	0.023[a]
	140[c]	0.062[c]

[a] Present work.
[b] Ref. (16).
[c] Ref. (24).

150 μM for the β-$NADP^+$ as substrate was calculated from Lineweaver–Burk plots under constant UDCA concentration. For the kinetic measurements a final concentration of 2.5 mM β-$NADP^+$ was used in order to obtain a pseudo-zero-order reaction as required for the two substrate enzymatic reactions (22).

Kinetic Studies on 7β-HSD

Kinetic parameters, K_m and V_{max}, for the 7β-HSD using UDCA, GUDCA, and TUDCA as substrates are presented in Table 1. The calculated K_m and V_{max} values, compared with those reported by Macdonald and Roach (16) and Sutherland *et al.* (24), can be considered as of the same order of magnitude despite some different experimental conditions (pH, determination method, source of enzyme, etc.).

Effect of Other Bile Acids Presence

The effect of the presence of CDCA, CA, and DCA at various concentrations on the reaction of UDCA with 7β-HSD was studied and the results obtained are shown in Table 2. Recovery of the fluorescence signal is not influenced when UDCA represents the majority (50% or more) of the total bile acid concentration, as is the case with patients subjected to ursotherapy where UDCA is the predominant bile acid (8,9). However, the determination of UDCA, when present at a low percentage, is affected by the presence of other bile acids; these findings are in accordance with those reported by Macdonald *et al.* (15). It is well known that the normal values for UDCA in serum vary from 0.15 to 0.27 μM (12,25–27), but in ursotherapy, the UDCA level in serum increases drastically (2,3,26,27). Nevertheless, further work is needed to elucidate the role of other bile acids present in the reaction of UDCA with 7β-HSD.

Determination of UDCA and Its Conjugates in Aqueous Solutions

Calibration graphs for UDCA, GUDCA, and TUDCA in aqueous solutions, which were not subjected to the Sep Pak procedure, in a final concentration range of 2.0 to 8.0 μM were found to be almost identical, had excellent linearity, and were of the form $y = 6.32x + 1.45$ (UDCA, $r = 0.992$), $y = 6.50x + 0.00$ (GUDCA, $r = 0.996$), and $y = 6.30x + 0.70$ (TUDCA, $r = 0.995$). Recoveries for UDCA, GUDCA, and TUDCA ranged from 85 to 110% for added concentrations in the range 2.0–8.0 μM ($n = 24$), with a mean relative standard deviation (RSD) of 7.7%.

Determination of UDCA in Serum

For the UDCA measurements in serum the pretreatment of serum samples with the Sep Pak cartridges was necessary for the following reasons: (i) deproteinization and removal of various interferences, (ii) reduction of the basic fluorescence of the sample, and (iii) dilution of the sample at the most suitable concentration size. Furthermore, the step of solid-phase extraction is not easy to avoid with the existing sensitivity of the method. The Sep Pak cartridges used for the quantitative extraction of UDCA and its conjugates from human serum can be regenerated after each use by washing with 5 ml of methanol and 5 ml of water (23).

Linearity. Calibration graphs can be obtained through the use of UDCA solutions alone because, as was experimentally confirmed in this study, the same concentrations of UDCA, GUDCA, and TUDCA give rise to equivalent amounts of NADPH. Calibration graph equations for UDCA standards, in a final concentration range of 2.0 to 8.0 μM, in aqueous and UDCA-free pooled serum samples subjected to the entire analytical procedure were of the form $y = 5.32x + 0.90$ (aqueous, $r = 0.994$) and $y = 5.20x + 1.50$ (serum, $r = 0.995$).

Recovery of UDCA after Sep Pak extraction. Recovery studies were carried out on 20 aqueous and 20 serum UDCA samples treated by the Sep Pak procedure. When the recoveries were calculated from a calibration graph obtained with untreated UDCA aqueous solutions the obtained values ranged from 65 to 88% and from 70

TABLE 2

Influence of the Presence of CDCA, CA, and DCA on the Determination of 4.0 μM UDCA with 7β-HSD

UDCA (%)	Recovery of fluorescence signal (%) in the presence of		
	CDCA	CA	DCA
100	100	100	100
50	100	99	98
7.4	45	99	23
4.8	13	—[a]	—[a]

[a] Not measured.

TABLE 3

Recoveries of UDCA Added to Aqueous Solutions and Human Serum Subjected to the Entire Analytical Procedure

	Aqueous			Serum		
Added (μM)	Recovered[a] (μM)	Recovery (%)	n	Recovered[b] (μM)	Recovery (%)	n
2.0	1.7	85	5	1.7	85	5
3.0	3.0	100	2	3.0	100	1
4.0	4.3	107	3	4.2	105	2
5.0	5.2	104	2	5.2	104	2
6.0	6.2	103	2	6.1	102	3
7.0	7.3	104	1	7.0	100	2
8.0	7.7	96	5	7.8	98	5
$\bar{x} \pm$ SD		99.9 ± 7.4			99.1 ± 6.7	

[a] From calibration graphs of standard UDCA aqueous solutions subjected to the entire analytical procedure.

[b] From calibration graphs of UDCA standards in human serum subjected to the entire analytical procedure.

to 86% for aqueous and serum samples, respectively, for added concentrations in the range 2.0–8.0 μM (n = 40).

Accuracy. The accuracy of the method was tested with recovery experiments of the entire procedure by adding appropriate amounts of UDCA in UDCA-free pooled serum and calculating the recovered UDCA by the proposed method. The small losses of UDCA during Sep Pak extraction are compensated for by subjecting the standards to the same analytical procedure as that used for the samples. Recovery values calculated from these calibration graphs are presented in Table 3. The average recovery for aqueous samples was 99.9% (range 85–107) and that for serum samples was 99.1% (range 85–105).

Precision. The precision of the proposed method was checked by determination of UDCA in replicate standard aqueous solutions and pooled serum samples containing UDCA at two different concentrations (2.0 and 8.0 μM). These samples were subjected to the entire analytical procedure described in Fig. 1. The RSD was 4.8% (n = 5) and 2.0% (n = 5) in aqueous solutions and 6.1% (n = 5) and 2.0% (n = 5) in spiked serum samples for UDCA concentrations of 2.0 and 8.0 μM, respectively.

Sensitivity. The detection limit of the method (estimated as the UDCA concentration producing a fluorescence signal equal to the blank signal (normal serum) plus three standard deviations of the blank) was found to be 0.8 μM. This can also be the minimum detectable initial UDCA concentration in serum when the volume of the serum sample is 2.00 ml.

Application of the Method to Patient Sera

The proposed method was successfully used for UDCA determination in 17 coded serum samples. The diagnosis of each sample was reported after the determination of UDCA by the proposed method. As can be seen in Table 4, all results correspond to the patients' situations. The UDCA concentrations in serum samples of patients under ursotherapy are scattered within the wide range of 1.4 to 33.4 μM. This is due to differences in UDCA doses and duration of treatment.

CONCLUSIONS

The proposed method is simple and suitable for routine clinical use, especially for the determination of UDCA in sera of patients subjected to ursotherapy. The method can also be used to obtain information concerning bile acid tolerance and metabolism following administration of UDCA to patients with liver diseases. Much more information concerning bile acid levels, following administration of UDCA to patients suffering from liver diseases, may be obtained by the simultaneous detection of UDCA in serum and urine. However, further work is needed to apply the present method to urine samples as well as to improve the sensitivity of the method by employing an immobilized 7β-HSD enzyme and other more sensitive monitoring techniques.

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TABLE 4

Determination of UDCA in Sera of Patients under Ursotherapy and of Healthy Individuals

Sample number	UDCA (μM)	Diagnosis
1	1.4	T[a]
2	8.3	T
3	<0.8	H[b]
4	30.9	T
5	<0.8	H
6	25.2	T
7	<0.8	H
8	<0.8	H
9	33.4	T
10	<0.8	H
11	<0.8	H
12	15.6	T
13	7.0	T
14	<0.8	H
15	<0.8	H
16	<0.8	H
17	<0.8	H

[a] Treated.

[b] Healthy.

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