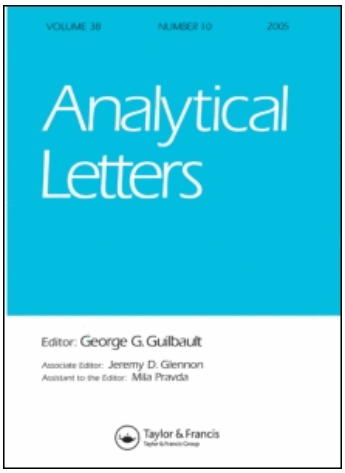

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Enzymatic Fluorimetric Determination of Ursodeoxycholic Acid in Urine Using Clostridium Absonum 7-Hydroxysteroid Dehydrogenase

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ENZYMATIC FLUORIMETRIC DETERMINATION OF URSODEOXYCHOLIC ACID IN URINE USING CLOSTRIDIUM ABSONUM 7β-HYDROXYSTEROID DEHYDROGENASE

Key words: Ursodeoxycholic acid, 7β-hydroxylated bile acids, 7β-hydroxysteroid dehydrogenase, urine analysis, primary biliary cirrhosis, ursotherapy.

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ABSTRACT

A simple and rapid enzymatic fluorimetric method for the determination of ursodeoxycholic acid (UDCA) and its glycine (GUDCA) and taurine (TUDCA) conjugates in urine has been developed. Octadecylsilane-bonded silica cartridges (Sep-Pak C_{18}) are used for the solid-phase extraction of bile acids (BA) from urine samples. The method is based on the fluorimetric monitoring of NADPH formed via the reaction of

7β-hydroxylated BA (7β-BA) with β-nicotinamide adenine dinucleotide phosphate (β-$NADP^+$) catalysed by 7β-hydroxysteroid dehydrogenase (7β-HSD). The 7β-HSD, which is not yet commercially available, was isolated from Clostridium absonum cultures (ATCC # 27555) and purified by affinity chromatography.

The method has a limit of detection of 2 μmol/L (initial sample concentration), within-run precision varied from 8.3% to 5.3% and between-run precision varied from 12% to 1.8% for low and high concentrations respectively. The recovery of ursodeoxycholic acid added to urine samples was about 98% (range 88-110%). The method was successfully applied for UDCA determination in urine samples from patients subjected to UDCA therapy. Randomly collected urine samples from patients and controls were used and the results were expressed as ratio of [UDCA]/[creatinine] to correct for variation in urine flow.

INTRODUCTION

Ursodeoxycholic acid (UDCA), the 7β-epimer of chenodeoxycholic acid (CDCA), is widely used as a medical treatment of choice for cholesterol gallstone disease [1,2], and it has been recently shown that its

administration improves clinical and biochemical signs in patients with primary biliary cirrhosis (PBC) [3-7]

UDCA is normally present in trace amounts in human biological fluids while its concentration rises significantly during UDCA therapy [8,9] .Several methods have been described for the determination of UDCA in serum such as radioimmunoassay [10] enzymeimmunoassay [11,12] high performance liquid chromatography (HPLC) [13] and gas liquid chromatography (GLC) [14] .

The isolation and purification of the 7β-HSD from Clostridium absonum cultures by Macdonald et al [15,16] enabled the first spectrophotometric enzymatic estimation of UDCA in human and bear biles [17] . In a recent report a simple fluorimetric enzymatic method is described for the determination of UDCA in serum using 7β-HSD [18] .

The importance of urine BA analysis for following up liver diseases has already been stated [19,20] . The determination of UDCA in urine may be useful for clinical, pharmacological and metabolic studies [21] . In urine, UDCA and its amidates as well as other BA are excreted mainly in their sulphated and glucuronidated form [9] and can be determined by HPLC [22] , GC-MS [23] or GLC [14] . Most of these methods are sensitive and accurate but usually require many

preparatory steps and thus are difficult to use for routine clinical work.

In the present paper, an enzymatic fluorimetric method for the determination of non-esterified UDCA anc its amidates in urine using 7β-HSD is described. Sep-Pak C_{18} cartridges are used for the solid-phase extraction of BA from urine samples. The measuring step is based on the conversion of 7β-hydroxylated BA to 7-keto BA by β-$NADP^+$ in the presence of 7β-HSD and the fluorimetric monitoring of the produced NADPH. The proposed method is simple, rapid and reliable and can be used for the determination of non-esterified UDCA and its amidates in urine of patients subjected to UDCA therapy.

MATERIALS AND METHODS

Reagents

All solutions were prepared with deionised distilled water (DDW) from analytical-reagent grade materials.

Preparation of 7β-HSD from Clostridium absonum. The 7β-HSD enzyme was prepared according to Macdonald et al[15,16] with some alterations and modifications in order to simplify the procedure[18]. An enzyme solution of about 0.1 U/mL was used for the

measurements. This solution was stored at -20°C and was kept in an ice bath when in use.

β-NADP+. A 0.010 mol/L solution was prepared by dissolving 0.0372 g of β-NADP+ (sodium salt M.W. 743.4, Sigma) in 5.00 mL of DDW. This solution was stored in the refrigerator and was stable for at least two weeks.

Glycine (1.0 mol/L) - hydrazine sulphate (0.40 mol/L) buffer, pH 9.0. Prepared by dissolving 37.6 g of glycine, 26.0 g of hydrazine sulphate and 0.93 g of $Na_2EDTA.2H_2O$ in about 400 mL of water, adjusting the pH to 9.0 with NaOH solution and diluting to 500 mL.

BA solutions. Ursodeoxycholic acid (UDCA) and its conjugates with glycine (GUDCA) and taurine (TUDCA) were obtained from Calbiochem. All bile acids were chromatographically pure (TLC)[24]. Stock solutions (1000 μmol/L) of UDCA, GUDCA, TUDCA separately as well as a mixed stock solution (1000 μmol/L) containing these three BA in equimolar amounts (7β-BA solution) were prepared by dissolving appropriate amounts of these BA in DDW. Urine samples from a healthy person, in which 7β-BA were not detected by the proposed method, were used for the preparation of working standards as well as of spiked urine samples (used for recovery studies). Working standard solutions were prepared in a concentration range of 20-80 μmol/L by

injecting the appropriate amounts of the equimolar 7β-BA stock solution (1000 μmol/L) in these urine samples. These standards were subjected to the whole analytical procedure as further described.

Urine Samples

Urine samples were collected from 11 patients with primary biliary cirrhosis subjected to UDCA therapy and 3 healthy persons. A quantitative 24-hr collection of urine was not necessary as the much simpler random sampling of urine was proposed by Simko et al[18]. In this case urine flow can be corrected by using a urinary [bile acids]/[creatinine] ratio. Creatinine (g/L) was determined on the basis of a reaction with picric acid[25].

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. The fluorimetric signals were recorded on a Knauer potentiometric recorder. Instrumental calibration and settings have been described elsewhere[19].

Procedure for the Determination of UDCA in Urine

The procedure for urine sample preparation and the UDCA measurement in urine consists of two main steps : extraction of BA and enzymatic fluorimetric measurement.

Extraction step. Sep-Pak C_{18} cartridges (Waters Associates) were used for the quantitative extraction of BA from human urine. The cartridges are washed with 3 mL of methanol and 6 mL of DDW prior to their use, then 2.00 ml of a urine sample or working standard is applied and passed through at a rate of about 1 drop.s^{-1}. The cartridge is washed with 2 x 2 mL of dilute acetone solution (20% in DDW), and the BA are then eluted with 2 x 2 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 1.00 mL of glycine-hydrazine buffer (pH 9.0). This extract is used for the measurement of non-esterified UDCA and its amidates in urine.

Measurement step. A 1.70 mL of glycine-hydrazine buffer solution (pH 9.0), 0.100 mL of 7β-HSD solution (0.1 U/mL), 0.100 mL of treated sample or standard, obtained as described above, are placed in the cuvette, and the mixture is stirred. After the fluorescence signal has been stabilized the reaction is initiated by

injecting 0.100 mL of β-$NADP^+$ solution (0.010 mol/L) and the fluorescence intensity is recorded until the signal value reaches its maximum. Each sample is run in duplicate. The unknown UDCA concentration in urine was calculated from calibration graphs (concentrations vs relative fluorescence intensity), taken with working standard solutions.

RESULTS AND DISCUSSION

General considerations concerning optimum pH, temperature, effect of β-$NADP^+$ concentration on the reaction rate, NADPH monitoring, effect of other BA presence and other measurement details were similar to those reported previously[18]

Determination of UDCA in urine

The pretreatment of urine samples with the Sep-Pak cartridges was necessary as it provides a simple and effective way for the reduction of high background fluorescence of urine samples[26] and allows adjustment of the sample concentration to the most suitable level[18]

Linearity. Calibration graphs for UDCA, GUDCA and TUDCA in aqueous solutions were found to be almost identical as previously reported[18] It was experimentally confirmed in this study, that a

calibration graph obtained through use of a mixture of these three BA, called as a 7β-BA solution, was almost identical to the calibration graphs taken for these three BA separately. However, a calibration graph taken with this equimolar mixture should be used, because in urine the amidated fraction of non-esterified bile acids is predominant when compared to the free fraction[9].

Calibration graphs for 7β-BA standard solutions at concentrations of 20, 40, 60,and 80 μmol/L in DDW and in "7β-BA free" urine samples that were subjected to the whole analytical procedure were of the form :

$y = 0.690\ x + 1.5$ (aqueous, $r = 0.999$, $n = 12$),

$y = 0.535\ x + 2.0$ (urine, $r = 0.991$, $n = 12$),

where y is the fluorescence intensity and x is 7β-BA concentration. The recoveries of 7β-BA after Sep-Pak extraction, calculated from the first calibration graph were found to be 77% ± 10. The second graph was used for the unknown UDCA concentration in urine samples, to compensate for losses during the extraction step.

Precision. The within run precision of the proposed method was checked by measuring eight times two pooled "7β-BA free" urine samples, spiked with UDCA at two different concentrations (20 and 80 μmol/L).

TABLE 1

Within run and between run precision for the determination of UDCA in urine.

	Within run*		Between run*			
$[UDCA]_t$, μmol/L	20	80	20	40	60	80
$\bar{x}$ (μmol/L)	18	79.6	16.5	44	58.5	78
SD (μmol/L)	1.5	4.2	1.9	2.6	2.5	1.4
RSD, %	8.3	5.3	11.5	5.9	4.3	1.8
n	8	8	4	4	4	4

$\bar{x}$ = Mean Value, SD = Standard Deviation, RSD = Relative Standard Deviation, n =number of runs per concentration $[UDCA]_t$ = equimolar amounts of UDCA, GUDCA and TUDCA.

* Calculated from the calibration graph.

These samples were subjected to the whole analytical procedure. Between run precision of the proposed method was checked by running sixteen differenty pooled urine samples, spiked with UDCA at four different concentrations (20, 40, 60 and 80 μmol/L), which were subjected to the whole analytical procedure. The results are summarised in Table 1.

TABLE 2

Recoveries of UDCA added to human urine submitted to the whole analytical procedure

Added, μmol/L	Recovered*, μmol/L	Recovery, %
20	18 ± 1.9	88 ± 9.6
40	44 ± 2.6	110 ± 6.7
60	58 ± 2.5	98 ± 4.1
80	80 ± 4.8	100 ± 6.2

* $\bar{x}$ ± SD (n = 4) calculated from the calibration graph

Accuracy. The accuracy of the method was tested with recovery experiments on the whole procedure by adding appropriate amounts of an equimolar mixture of UDCA and its amidates to a "7β-BA free" urine sample of a healthy individual and calculating the recovered UDCA by the proposed method. The losses of UDCA during Sep-Pak extraction are compensated for by subjecting the standards to the same analytical procedure used for the samples. The average recovery of 7β-BA added to urine samples was 98% ± 10 (range 88-110%) and the results are shown in Table 2.

TABLE 3

Determination of UDCA in urine samples of patients under UDCA therapy and healthy individuals.

Urine sample	UDCA, μmol/L	Creatinine, g/L	[UDCA]/Creatinine, μmol/g
1	29	0.708	40.9
2	7.9	0.121	65.3
3	41	0.331	124
4	12	0.791	15.2
5	174	0.845	206
6	9.9	1.04	9.52
7	24	0.878	27.3
8	12	1.66	7.23
9	69	0.932	74.0
10	7.9	0.620	12.7
11	14	1.18	11.9
12*	n.d	-	-
13*	n.d	-	-
14*	n.d	-	-

* Urine samples of healthy individuals.

n.d : not detected, (-) : not measured.

The detection limit of the method, expressed as the analyte concentration that corresponds to the fluorescence signal equal to the blank signal (this can be calculated from the intercept of the calibration graph) plus three standard deviations of the blank (this can be replaced by the value of $S_{y/x}$ from the calibration graph) was found to be 2.0 μmol/L [27]. This limit can be further improved by appropriate preconcentration of the samples during the extraction step.

Application of the Method to Urine Samples

The proposed method was successfully applied for the determination of UDCA in eleven urine samples of patients under UDCA therapy and of three healthy individuals. The results are expressed either in absolute concentrations, μmol/L, or as a ratio of [UDCA]/[creatinine], μmol/g, to correct for variation in urine flow, and they are shown in Table 3.

CONCLUSIONS

The proposed method enables the simple, rapid and specific determination of UDCA and its amidates in human urine, due to the high substrate specificity of the 7β-HSD enzyme. The method can be useful for following up 7β-hydroxylated BA extraction in urine of

patients subjected to UDCA or CDCA therapy, since even in that cases it is reported[4] that a part of administered CDCA is metabolised to the less toxic and more hydrophilic UDCA[28,29]. Ursocholic acid (3α,7β,12α hydroxylated) (UCA) whose presence has also been reported in urine of patients subjected to UDCA therapy[9] may be also determined by the proposed method.

This method does not discriminate between free, glyco and tauro amidates of UDCA and it does not measure the sulphated and glucuronidated fraction of UDCA. For this kind of information more complex chromatographic methods are needed. A newly developed rapid solvolysis procedure for sulphate esters of BA[26] may be applied to the urine samples for the determination of the sulphated fraction of UDCA and its conjugates, when the appropriate sulphated standards will be available to us. Much more information concerning BA levels, following administration of UDCA in patients suffering from liver diseases may be obtained by measuring all forms of UDCA in serum and urine simultaneously.

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