ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
24 (2001) 937–946

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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High-performance liquid chromatographic determination of ursodeoxycholic acid after solid phase extraction of blood serum and detection-oriented derivatization

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Received 16 May 2000; received in revised form 14 November 2000; accepted 16 November 2000

Abstract

Ursodeoxycholic acid (3α, 7β-dihydroxy-5β-cholanoic acid, UDCA) is a therapeutically applicable bile acid widely used for the dissolution of cholesterol-rich gallstones and in the treatment of chronic liver diseases associated with cholestasis. UDCA is more hydrophilic and less toxic than another therapeutically valuable bile acid, chenodeoxycholic acid (CDCA), the 7α-epimer of UDCA. Procedures for sample preparation and HPLC determination of UDCA in blood serum were developed and validated. A higher homologue of UDCA containing an additional methylene group in the side chain was synthetized and used as an internal standard (IS). Serum samples with IS were diluted with a buffer (pH = 7). The bile acids and IS were captured using solid phase extraction (C18 cartridges). The carboxylic group of the analytes was derivatized using 2-bromo-2′-acetonaphthone (a detection-oriented derivatization), and reaction mixtures were analyzed (HPLC with UV 245 nm detection; a 125–4 mm column containing Lichrospher 100 C18, 5 μm; mobile phase: acetonitrile–water, 6:4 (v/v)). Following validation, this method was used for pharmacokinetic studies of UDCA in humans. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Ursodeoxycholic acid; Higher homologue of ursodeoxycholic acid; Solid phase extraction of serum; Detection-oriented derivatization; Reversed phase HPLC; Pharmacokinetics in humans

1. Introduction

Ursodeoxycholic acid (3α, 7β-dihydroxy-5β-cholanoic acid, UDCA, see Fig. 1) was discovered as the principal bile acid in the polar bear by Hammersten in 1902 [1]. In humans, UDCA is formed only in minor amounts from its 7α-epimer (3α, 7α-dihydroxy-5β-cholanoic acid, CDCA). This occurs via intestinal bacterial oxidation of CDCA to 7-ketolithocholic acid followed by cata-

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PII: S0731-7085(00)00563-X

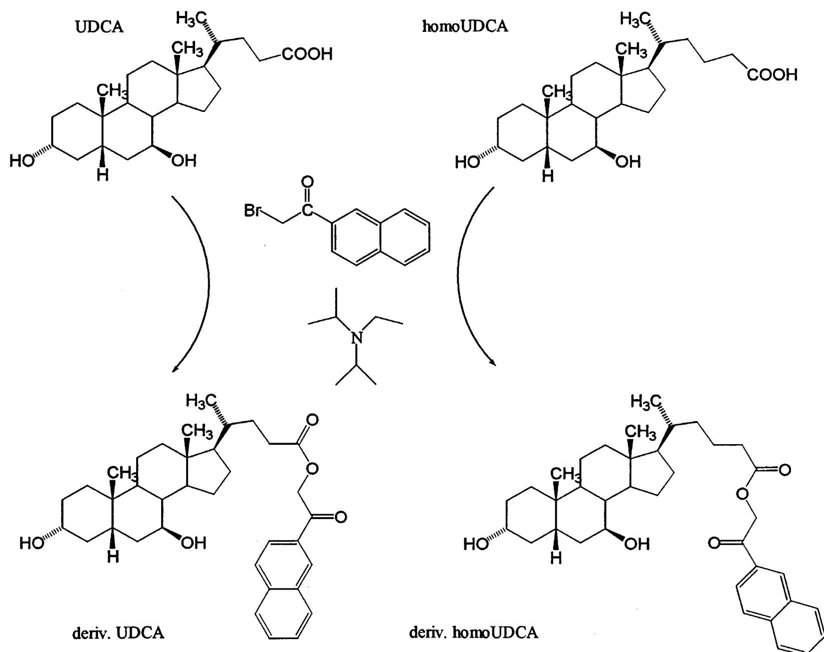

Fig. 1. Chemical structures of ursodeoxycholic acid (UDCA), higher homologue of UDCA (homoUDCA, used as an internal standard) and their 2-naphthacyl esters after the derivatization with 2-bromo-2′acetonaphthone.

lytic reduction to UDCA by both intestinal and hepatic enzymes [2].

UDCA supresses biliary secretion of cholesterol and inhibits its intestinal absorption in humans [3,4]. It is used for the dissolution of cholesterol-rich gallstones in patients with functioning gall-bladers [5,6], and in the treatment of primary biliary cirrhosis. Furthermore, it has been tried in the treatment of some chronic liver diseases associated with cholestasis such as liver disease in cystic fibrosis, cholestasis associated with pregnancy, sclerosing cholangitis, chronic active hepatitis and viral hepatitis. UDCA has also shown some promise in the treatment of non-alcoholic steatohepatitis and refractory graft-versus-host disease of the liver in transplant patients [7,8].

The clinical pharmacokinetics of UDCA is well described [9]. Thus, therapeutically administered UDCA is absorbed, transported to the liver and efficiently biotransformed, predominatly by conjugation with glycine and taurine. Other conjugation products (C-3 sulphate and 7β-glycosylated metabolites) as well as products of Phase I — biotransformation (ring and side-chain hydroxylated products) are formed.

UDCA is efficiently conjugated with taurine during its enterohepatic recycling in rats. An interesting metabolite of UDCA, Δ^{22}-UDCA (3α,7β-dihydroxy-5β-chol-22-en-24-oic acid), was identified in plasma, bile, intestinal contents and liver tissue of Sprague–Dawley rats [10].

Different analytical approaches to the determination of unconjugated and conjugated bile acids in biological material using TLC [11], GC [12–15], HPLC [16–20] and CE [21–23] have been described.

Solid phase extraction (SPE) methods have been routinely used for sample preparation from various body fluids containing bile acids [14]. Supercritical fluid extraction (SFE) has likewise shown to be suitable for extraction of bile acids from solid samples (faeces) [24].

Bile acids do not show significant UV absorption. Neither do they have fluorescent or electrochemical properties suitable for their sensitive and selective detection. UV detection at 200 nm is

non-specific due to biological matrix interference. Pre-column labelling of bile acids with either a chromophore or fluorophore is usually necessary [25].

Derivatization procedures involving the carboxylic group in the side chain appear to be the method of choice. 2-Bromoacetyl-6-methoxynaphthalene [26,27], 1-bromo-acetylpyrene [28] or 4-bromomethyl-7-methoxycoumarine [29] have been described for the fluorometric derivatization. Carboxylic acids can be derivatized directly in an aqueous matrix by using a micellar system [30].

1-Anthroyl nitrile [31] has also been used for the fluorescence labelling; in this case, bile acids were derivatized through the hydroxyl group at C-3 (which is important for the detection of bile acid glycine and taurine conjugates).

HPLC determination of bile acids via a post-column enzymatic reaction and fluorescence detection has also been reported [32].

This paper describes a sample preparation method involving solid phase extraction of UDCA and its higher homologue (as an internal standard) from serum samples, pre-column detection-oriented derivatization of analytes to their UV-absorbing naphthacyl derivatives and subsequent HPLC analysis. The validated bioanalytical method was used in pharmacokinetic studies of pharmaceutical preparations containing ursodeoxycholic acid.

2. Experimental

2.1. *Chemicals, preparations and materials*

Ursodeoxycholic acid (UDCA, 3α, 7β-dihydroxy-5β-cholanoic acid, $C_{24}H_{40}O_4$, F.W. = 392.58 g mol^{-1}) and URSOSAN® (ursodeoxycholic acid 250 mg capsules) were obtained from PRO.MED. CS Praha a.s. (Czech Republic). URSOFALK® (ursodeoxycholic acid 250 mg capsules, Dr. Falk Pharma GmbH, Freiburg, Germany) was tested as a reference preparation. Authentic bile acids (chenodeoxycholic acid, cholic acid, lithocholic acid) were obtained from Sigma-Aldrich, Praha and Dr. Falk Pharma GmbH, Freiburg, Germany. Internal standard, 3α,7β-dihydroxy-24-homo-5β-cholanoic acid (higher homologue of ursodeoxycholic acid, homoUDCA, $C_{25}H_{42}O_4$, F.W. = 40 661 g mol^{-1}) was synthetized and its structure confirmed in the laboratories of the Faculty of Pharmacy (Section 2.2). 2-Bromo-2′-acetonaphthone and *N*,*N*-diisopropylethylamine (both 99%, Sigma–Aldrich) were used in the derivatization procedure. Acetonitrile (gradient grade, LiChrosolv^R, Merck), methanol (p.a. Penta Chrudim, Czech Republic), sodium hydrogenphosphate dodecahydrate, potassium dihydrogenphosphate (both p.a. LACHEMA, Neratovice, Czech Republic) were used for the mobile phase and sample preparation and for solid phase extraction. Methoxymethyl chloride, dichlormethane, potassium cyanide, triphenylphosphine, imidazole and Dowex 50 (for the synthesis of higher UDCA homologue) were purchased from Sigma-Aldrich. Deuterated methanol (CD_3OD, 99.8% D) was purchased from Armar AG, Döttingen (Switzerland). Ultra-high-quality (UHQ) water was prepared using Elgastat UHQ PS apparatus (Elga Ltd., Bucks, England). A phosphate buffer (pH 7) was prepared by mixing of 400 ml of 0.067 M KH_2PO_4 (4.54 g in 500 ml UHQ water) and 600 ml of 0.067 M $Na_2HPO_4 \cdot 12\ H_2O$ (23.88 g in 1000 ml UHQ water). Visiprep Solid Phase Extraction Vacuum Manifold (12-port, Supelco) with SPE columns (Supelclean LC-18, 1 ml tubes, Supelco, Bellefonte, USA) were used for the solid phase extraction. S-Monovette syringe with coagulant activator (aluminium silicate) for the blood collection and serum preparation (Sarstedt, Nümbrecht, Germany) were used.

2.2. *Synthesis of 3α,7β-dihydroxy-24-homo-5β-cholanoic acid (internal standard, see Fig. 2)*

2.2.1. *Homologation of the side chain*

Methoxymethyl chloride (4 ml, 52.7 mmol) and diisopropylethylamine (9.2 ml, 52.8 mmol) were added to a solution of UDCA (5 g, 12.7 mmol) in CH_2Cl_2 (30 ml), which was allowed to stand at ambient temperature for 24 h. The solvent was then evaporated and the residue dissolved in ethyl acetate (EtOAc) and washed with water (2 ×).

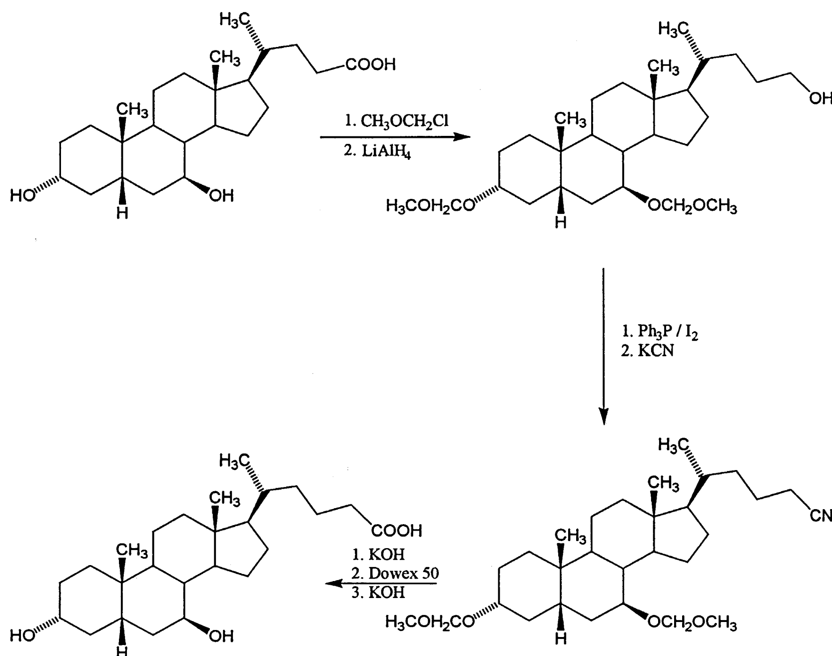

Fig. 2. Synthesis of 3α,7β-dihydroxy-24-homo-5β-cholanoic acid (internal standard to UDCA).

The organic phase was dried over anhydrous Na_2SO_4 and the solvent removed under reduced pressure to yield UDCA with the hydroxy groups protected as MOM ethers and the carboxyl masked as the MOM ester (6.66 g, quant. yield). The crude product (6.66 g, 12.7 mmol) was dissolved in diethyl ether (50 ml), the solution cooled to 0°C, and $LiAlH_4$ (250 mg, 6.6 mmol) then added in several portions. After 2 h at 0°C, the mixture was warmed to ambient temperature, and stirred for a further 2 h. EtOAc (10 ml) and H_2O (2 ml) were cautiously added to the reaction mixture, and the resultant slurry was vigorously stirred for 30 min. The mixture was poured into EtOAc/H_2O, and thoroughly washed. The organic phase was separated, dried over anhydrous Na_2SO_4, and the solvent removed in vacuo to furnish the crude 24-hydroxy derivative (5.2 g, 88%).

The crude hydroxy compound (5.2 g, 11.2 mmol) was dissolved in CH_2Cl_2 (40 ml). PPh_3 (3.8 g, 14.5 mmol) was added, followed by imidazole (990 mg, 14.5 mmol) to the solution. After 5 min, a solution of I_2 (3.68 g, 14.5 mmol) in toluene (40 ml) was added dropwise over 1 h, and the reaction mixture was stirred for further 3 h. The mixture was then concentrated, poured into EtOAc, and successively washed with 5% aqueous $Na_2S_2O_3$, 5% aqueous HCl (3 ×), H_2O (3 ×), and 5% aqueous $NaHCO_3$ (3 ×). The organic phase was concentrated, and chromatographed on silica gel (petrolether/ether 95:5) to afford the primary iodide (5.2 g, 81%).

The iodide (5.2 g, 9 mmol) was dissolved in DMSO (10 ml) and KCN (880 mg, 13.5 mmol) added. The resultant mixture was stirred at ambient temperature for 12 h. The solution was than poured into H_2O, and the resultant mixture extracted with EtOAc (3 ×). The combined EtOAc extracts were dried over Na_2SO_4, concentrated, and the residue chromatographed on silica gel (petrolether/ether 90:10) to furnish the corresponding nitrile (3.86 g, 90%).

2.2.2. *Hydrolysis and deprotection*

The nitrile (3.86 g, 8.1 mmol) was dissolved in butanol/H_2O (3:1, 20 ml), KOH (4.5 g, 80 mmol) was added, and the resultant mixture was heated

at reflux for 48 h. The reaction mixture was acidified to pH = 1 and diluted with EtOAc. The solution was washed with water (3 ×), dried over Na_2SO_4, and the solvent removed. The crude acid (3.3 g, 6.7 mmol) was redissolved in $MeOH/H_2O$ (3:1, 20 ml), Dowex 50 (3.3 g) in the H^+ cycle was added and the heterogeneous mixture heated at reflux for 24 h. The catalyst was then filtered off, and the solvents evaporated. Since ^1H NMR analysis revealed that the carboxyl was converted to a methyl ester under these conditions (singlet at 3.63 ppm corresponding to 3 protons), the residue was again dissolved in $MeOH/H_2O$ (3:1, 20 ml), NaOH (800 mg, 20 mmol) added and the mixture stirred at ambient temperature for 12 h, and then at reflux for 10 h. The solution was acidified to pH = 1, diluted with EtOAc, washed with H_2O (1 ×), dried over Na_2SO_4, concentrated, and chromatographed on silica gel to give the desired 3α,7β-dihydroxy-24-homo-5β-cholanoic acid as an amorphous powder (2.06 g, 76%). A Varian Mercury-Vx BB 300 NMR spectrometer was used for the NMR analyses. The NMR spectra were recorded at 300 MHz for ^1H, and 75 MHz for ^{13}C. Chemical shifts are given as δ values in ppm, the coupling constants are given in Hz.

^1H NMR (CD_3OD) δ: 0.71 (3H, s, 18-CH_3), 0.96 (3H, s overlapped, 19-CH_3), 0.96 (3H, d overlapped, $J = 6.4$ Hz, 21-CH_3), 1.00–2.00 (25H, m), 2.00–2.10 (1H, dm), 2.15–2.35 (2H, m), 3.40–3.60 (2H, m, H3β and H7α). ^{13}C NMR (CD_3OD) δ: 12.7, 19.3, 22.4, 22.7, 24.0, 27.9, 29.-4, 31.0, 35.2, 35.4, 36.1, 36.6, 36.9, 38.0, 38.6, 40.7, 41.6, 44.0, 44.5, 44.7, 56.6, 57.5, 71.9, 72.1, 177.7.

2.3. Volunteers, study design and biological material

Four healthy volunteers (two males and two females), all 22–30 years old and 63–105 kg of body weight (mean 84 kg) entered the pilot study. All the subjects were healthy according to their medical history, physical examination, haematology, clinical chemistry and urinalysis. The study was approved by the state authority and the institutional ethics committee.

Two ursodeoxycholic acid preparations (both containing 250 mg of UDCA in one capsule) were compared in the study: a test preparation of Ursosan and a reference preparation of Ursofalk.

The study was of open, single dose, randomized, two-way, cross-over design with a 2 week washout period between treatments. Smoking, medication and alcohol, methylxanthine and chinine containing beverages were restricted 5 days before and during the study periods.

Blood samples of 5 ml were collected 24 h before (pre-dose period, 12 samples) and 72 h after (post-dose period, 14 samples) the oral administration of UDCA preparations (dose 1 g, i.e. four capsules). Samples were taken from the cubital vein in non-heparinized Sarstedt syringes. Thirty minutes after withdrawal, the blood was centrifuged ($3000 \times g$, 10 min), the serum removed and stored in plain polypropylene tubes at − 65°C until analysis.

2.4. Sample preparation and solid phase extraction (SPE)

Homo UDCA 600 μl 10^{-4} M, i.e. 60 nmol of internal standard) in methanol was transferred into a 9 ml tube and evaporated gently (45°C, stream of nitrogen) to dryness. A serum sample (1 ml) containing UDCA was then added and the tube content mixed and diluted with 5 ml of the phosphate buffer (pH 7).

SPE columns (Supelclean LC-18) were activated on the SPE vacuum manifold with 2 ml of methanol followed by 3 ml of UHQ water (flow rate 3 ml min^{-1}). The serum spiked with the internal standard and diluted with the phosphate buffer was passed through SPE column with a flow rate of 1 ml min^{-1}. After washing the SPE column with 2 ml of UHQ water and 2 ml of 20% methanol (flow rate of 3 ml min^{-1}), the captured analytes (UDCA and homoUDCA) eluted from the column with 3 ml of methanol (flow rate 1 ml min^{-1}). The methanolic extract was evaporated (45°C, stream of nitrogen) to dryness.

2.5. Derivatization procedure

2-bromo-2-acetonaphthone (130 μl of 10^{-3} M) and *N*,*N*-diisopropylethylamine (260 μl of 10^{-3}

M, both in acetonitrile solutions) were added into the tube with the dried serum eluate. The mixture was allowed to react at 60°C for 20 min. Following reaction, the remaining acetonitrile was evaporated (45°C, stream of nitrogen) and the dry residue (a mixture of UV-absorbing naphthacyl esters of bile acids, the derivatizing agent and the amine) was reconstituted in 200 μl of the mobile phase and transferred into a vial containing a glass insert (230 μl volume). One hundred microlitre of the sample was then injected into the chromatographic column.

2.6. Chromatography

Chromatographic analyses were performed using a *Thermo Separation Products* (formerly Spectra Physics) chromatograph. The chromatographic system consisted of a SCM400 solvent degasser, a P4000 quaternary gradient pump, AS 3500 autosampler with 100 μl sample loop, SpectraFOCUS high-speed scanning UV–VIS detector, SN4000 system controller and a data station (Intel-Pentium 166 MMX, RAM 64 MB, HDD 2GB) with analytical software ChromQuest 2.1 (ThermoQuest, Inc., San Jose, CA, USA) working under a Windows NT Workstation 4.0 operating system (Microsoft Corporation). The flow rate was 1 ml min^{-1}. A LiChroCART® 125–4 mm analytical column packed with a LiChrospher® 100 RP-C18, 5 μm and a LiChroCART® 4–4 mm precolumn with the same stationary phase (Merck, Darmstadt, Germany) were used for the analyses. The mobile phase was a simple mixture of acetonitrile-UHQ water (6:4, v/v). UV detection was either performed in single wavelength mode (245 nm) or high-speed scanning mode for the collection of the UV spectra. The range in the latter case being 195–365 nm with a 1 nm distance.

2.7. Calibration

Standard stock solutions (39.26 mg of UDCA in 100 ml and 40.66 mg of homoUDCA in 100 ml, both 10^{-3} M methanolic solutions) were prepared. Lower concentrations (10^{-4} M) of each compound were obtained by dilution with methanol. A calibration series of UDCA/homoUDCA(IS) mixtures were prepared: to 600 μl 10^{-4} M homoUDCA in 9 ml tubes were added 10, 30, 50, 100, 200, 300 μl of 10^{-4} M UDCA (6 samples for each concentration). Methanol in the tubes was evaporated (45°C, stream of nitrogen) to dryness. Drug-free human serum (1 ml) was then added and following the sample preparation, the solid phase extraction and derivatization procedure was performed as described in Section 2.4 and Section 2.5.

A water solution of human albumine (50 mg ml^{-1}) was tested for the determination of the limit of quantification (LOQ).

2.8. Testing and statistical evaluation of the analytical procedure

Six-level calibration series with six analyses at each concentration level were measured. The on-line statistical processing of the calibration analyses by the least-squares method was performed automatically using the ChromQuest software. The linearity of the calibration curve was tested and evaluated ($y = kx + q$, where x is the concentration ratio of UDCA to homoUDCA(IS) and y is the corresponding peak-area ratio UDCA/homoUDCA (IS). The correlation coefficient (r) was also expressed. Accuracy was calculated as the percentage found on the standard curve, and the precision of the method, expressed as the relative standard deviation (R.S.D. = 100 S.D./mean), was also evaluated. Both statistical parameters were calculated for every concentration level. The limit of quantification (LOQ) was determined as the lowest concentration on the standard calibration curve which was measured with a precision of 20% and accuracy of 80 or 120% [33]. The limit of detection (LOD; at a UDCA signal-to-noise ratio of 3, average from 6 chromatograms) was then calculated.

3. Results and discussion

3.1. Internal standard

In the development of a new bioanalytical method, one of the principal issues to be addressed

is the choice of the internal standard. A number of different fatty acids [24,26,28] or bile acid derivatives [31,32] have previously been tested as possible internal standards. In our study, the retention times of the naphthacyl esters of some available bile acids were measured (Table 1) in order to find a suitable internal standard. Unfortunately, some of these derivatives were eluted with impurities (cholic acid), while others (chenodeoxycholic acid and lithocholic acid) exhibited very long retention times and were obviously unsuitable for analyses of large series of samples.

The compound used as an internal standard should be of a similar polarity (to have the retention time sufficiently close to the compound determined and exhibit similar behaviour during solid phase extraction). Additionally, the reactivity of bile acid(s) and the internal standard during derivatization should be similar so that reproducible yields of UV absorbing derivatives are obtained. With a view to these requirements, the higher homologue of UDCA (incorporating an additional methylene group in the side chain) was synthetized.

The synthesis of the internal standard (Fig. 2) was quite straightforward. Following the protection of both the hydroxyl and carboxylic groups in UDCA with methoxymethyl chloride, the carboxylic group (now masked as a MOM ester) was reduced to the primary alcohol with lithium aluminium hydride. The primary OH function was converted to the iodide, then reacted with cyanide to afford the corresponding nitrile. Finally, the nitrile was converted to a carboxyl via base-catalyzed hydrolysis. Treatment of the resulting homologue with Dowex 50 in methanol/water resulted in deprotection of methoxymethyl groups. The resultant 3α,7β-dihydroxy-24-homo-5β-cholanoic acid was analyzed by ^{1}H and ^{13}C NMR spectroscopy. Further structural proof was provided via a DEPT experiment which confirmed the presence of eleven methylene groups in the molecule.

Table 1
Retention times and capacity factors of the naphthacyl esters of cholic acid (CA), ursodeoxycholic acid (UDCA), higher homologue of ursodeoxycholic acid (homoUDCA), chenodeoxycholic acid (CDCA) and lithocholic acid (LCA)[a]

Naphthacyl ester of	Retention time (min)	Capacity factor
CA	12.4	10.8
UDCA	16.8	15.0
HomoUDCA	24.4	22.2
CDCA	37.3	34.5
LCA	>80	151.8

[a] Measurements were carried out under chromatographic conditions described in Section 2.6.

3.2. *Sample preparation and derivatization procedure*

According to the literary sources [10,14,20, 26,28] and in our experience, the most effective way to remove bile acids from blood samples is solid phase extraction (SPE) of a diluted serum. Typically, recovery of bile acids from haemolytic, chylous or anticoagulant (heparine) containing samples is low. Experiments which involved processing biological samples (plasma, serum) using liquid–liquid extraction turned out to be unsuccessful as well. Detailed and optimalized SPE process was described in Section 2.4.

For the enhancing of the detector response and the specificity of the bile acid determination, a pre-column derivatization procedure based on the esterification of the carboxylic group with 2-bromo-2′-acetonaphthone (UV-absorbing chromophore, [34]) was developed (Fig. 1). The derivatization procedure was performed in acetonitrile in the presence of diisopropylethylamine (a scavenger of protons). The influence of temperature and time on the yields of the naphthacyl esters was also studied. Optimal conditions are described in Section 2.5. A UV spectrum of the naphthacyl esters of UDCA and homoUDCA (with λ_{max} of 245 nm) is shown in Fig. 3.

A calibration series of acetonitrile solutions with the same concentrations of UDCA/homoUDCA as described in Section 2.7 was derivatized (no solid phase extraction step was performed in this case) and analyzed in order to estimate if the amount of 2-bromo-2′-acetonaphthone and diisopropylethylamine added to the

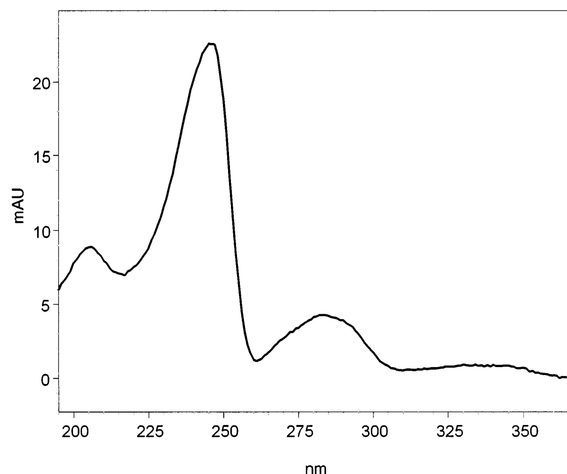

Fig. 3. UV spectrum of naphtacyl ester of UDCA (spectrum of derivatized homoUDCA is the same). Spectra were obtained using SpectraFOCUS detector during the HPLC analysis.

sample was sufficient to achieve reproducible results. The calibration curve was found to be linear with $r = 0.9998$. Evaluation of the accuracy (average value 99.71%, range 96.12–102.02%), precision (average value 1.59%, range 0.256–7.02%) confirmed that good reproducibility of the derivatization procedure in the range of concentrations of 1–40 nmol ml^{-1} of serum could be obtained.

4. Chromatography

After the solid phase extraction and derivatization, the UV-absorbing naphthacyl esters of the bile acids were analyzed under conditions described in Section 2.6. Typical HPLC analyses are shown in Fig. 4. The upper chromatogram corresponds to the analysis of the derivatized extract from the human serum sample taken in the post-dose period (after the oral administration of 4 capsules containing UDCA, Section 2.3). The lower chromatogram shows the analysis of the derivatized extract from a 'drug free' serum, where only very small amount of endogenous UDCA in the form of its naphthacyl derivative was detected.

The analytical procedure involving the solid phase extraction, derivatization and HPLC analysis of the derivatized UDCA and homoUDCA (internal standard) is accurate and precise. The calibration curve was found to be linear ($y = 3.9273x - 0.0144$, $r = 0.9995$). The accuracy and precision of UDCA determination (using homoUDCA as the internal standard) in spiked human serum samples were found to be 101.89% (range of 94.79–117.25%) and 7.6% (range of 5.71–8.62%), respectively. The mean recovery of UDCA (based on the determination of 6 spiked serum samples) was calculated at 94% (SD = ± 5.2%). The limit of quantification (LOQ) was calculated at 0.5 nmol UDCA/ml of serum (196.3 ng UDCA/ml of serum). The LOD was calculated at 25 ng UDCA/ml of serum.

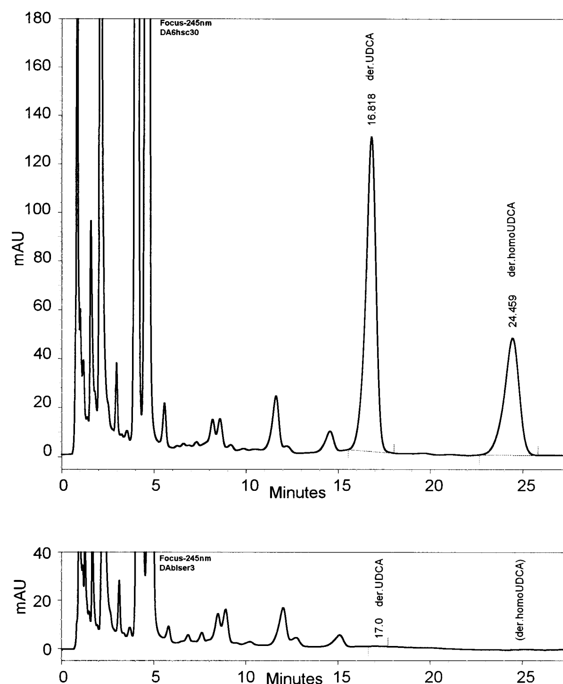

Fig. 4. Typical chromatograms of the derivatized extract from the human serum. The upper chromatogram contains the peak of the naphtacyl ester of UDCA ($t_R = 16.8$ min; corresponds to 28 nmol i.e. 11 μg of UDCA) and the peak of the naphthacyl ester of homoUDCA ($t_R = 24.5$ min, internal standard). The lower chromatogram demonstrates a blank serum after the derivatization procedure with a very small amount of endogenous UDCA (in the form of its naphthacyl ester).

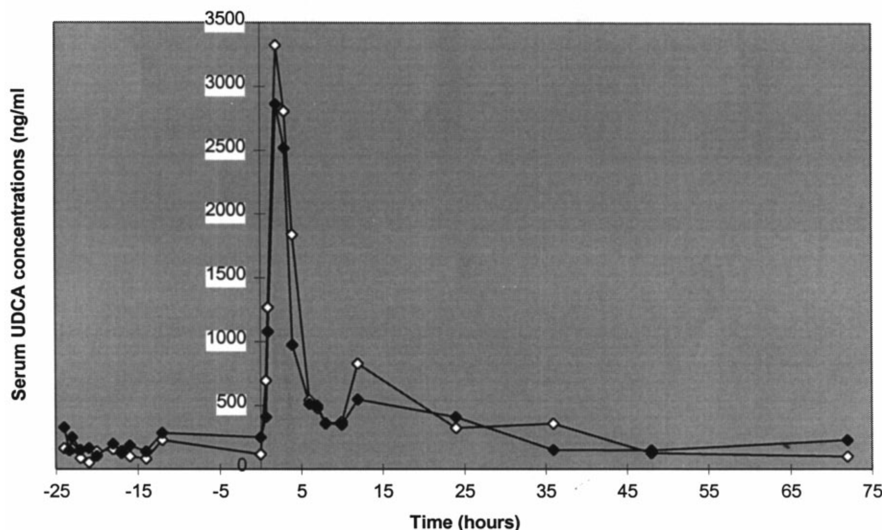

Fig. 5. Serum concentrations of UDCA versus time profiles in the first healthy volunteer (male, 105 kg) 24 h before and 72 h after the oral administration of 1 g of UDCA (◆ Ursosan, ◇ Ursofalk).

4.1. Pharmacokinetics

The applicability of the present method was demonstrated in the pilot comparative pharmacokinetic study with 4 healthy volunteers. Fig. 5 shows serum levels of UDCA 24 h before and 72 h after a single oral dose of 1 g of both UDCA preparations.

Serum concentrations of endogenous UDCA (measured in the pre-dose period) ranged mostly between the LOQ and LOD values but some were higher then the LOQ concentration (usually corresponding with a meal intake). In the post-dose period, the maximum concentrations of UDCA were found between 1 and 3 h after the Ursosan or Ursofalk administration. In the period between t_{max} and 10 h, the UDCA concentrations fell strongly. After 10 h, they decreased only slightly revealing one or two secondary peaks. Serum UDCA concentrations reached approximately the value of the LOQ 72 h after administration of UDCA.

The mean derived pharmacokinetic parameters are summarized in Table 2. AUC_{0-24} and c_{max} were calculated as the net parameters, i.e. after the baseline subtraction (difference between the pre-dose AUC_{-24-0} and the post-dose AUC_{0-24}, difference between the post-dose c_{max} and the corresponding pre-dose concentration) [35,36].

The presence of secondary peaks in the serum concentration-time profile (typical for drugs with enterohepatic recirculation) and endogenous UDCA levels made calculating parameters of $t_{1/2}$ and k_e impossible.

The c_{max} and t_{max} obtained in this study are consistent with those from the previous reports [9,5].

Table 2
UDCA pharmacokinetic parameters following single oral administration of 1 g of UDCA[b]

Pharmacokinetic parameter[a]	Ursosan	Ursofalk
Net AUC_{0-24} [μg h ml^{-1}]	18.7 ± 6.8	22.1 ± 5.7
Net c_{max} [μg ml^{-1}]	5.14 ± 2.74	7.16 ± 4.04
t_{max} [h]	2.0 ± 0.8	2.0 ± 0.8

[a] Net AUC_{0-24}, net area under the serum UDCA concentration-time curve from time 0 to 24 h; net c_{max}, net peak serum UDCA concentration; t_{max}, time to reach the peak drug concentration.

[b] Results are given as mean ± S.D. ($n = 4$).

5. Conclusions

The HPLC method presented for determination of ursodeoxycholic acid in blood serum is applicable not only to human pharmacokinetic studies as shown here, but also to preclinical pharmacokinetic and biodistribution investigations. Results from the latter studies will be published in due course.

Acknowledgements

This work was partially supported by the Ministry of Education of the Czech Republic — project No. VS 97124.

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