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HPLC study of the impurities present in different ursodeoxycholic acid preparations: comparative evaluation of four detectors

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Abstract: The use of HPLC with different detectors has been investigated for the analysis of bile acid impurities present in four different commercially available ursodeoxycholic acid preparations. The bile acids were efficiently separated by C_{18} reversed-phase HPLC using methanol–water (3:2, v/v) as the mobile phase. The detectors used for bile acid detection were: UV at 200 nm refractive index (RI) and an evaporative light scattering mass detector (ELSD II). A prederivatization method with the formation of a fluorescent naphthacyl ester has also been used. GC–MS analysis of Me–TMS bile acid derivatives was included as a reference method.

The four ursodeoxycholic acid samples were 98–99% pure. The main impurities present in the samples were chenodeoxycholic acid and to a lesser extent lithocholic acid. Only one sample was found to be almost 100% pure using all the detectors. Significant agreement of the data was found between RI, ELSD II detectors and the fluorescent method; the UV detector was unsuitable for use in this method. The analytical performances of the four detectors for bile acid analysis are reported and discussed. When the four-detector data were compared with the GC–MS method, reasonable agreement resulted. Discordant results were found in the quantitation of trace impurities like lithocholic acid and/or other minor bile acids present in amounts less than 0.1%.

Keywords: *Bile acids; ursodeoxycholic acid; HPLC analysis; pharmaceutical analysis; impurities.*

Introduction

Ursodeoxycholic acid (UDCA) is a well established drug for dissolution of cholesterol gallstones and more recently has been found to be useful for the treatment of mild cholestatic liver diseases [1–8]. This drug has been shown to be safe, well tolerated and without major side-effects for patients with cholesterol gallstones or with a variety of liver diseases [9].

UDCA is the 7β epimer of chenodeoxycholic acid (CDCA) which is also available commercially and used for the treatment of cholesterol gallstone diseases [10–11]. Unfortunately, numerous clinical and animal studies have indicated that chronic oral CDCA administration causes many side-effects including diarrhoea and mild liver disease, with a transient increase in some biochemical liver function tests such as transaminases [12]. Therefore CDCA, although still on the market, has been practically abandoned for clinical use. In addition, a most important consideration is that unlike UDCA which is useful for cholestatic liver disease even in pregnant women, CDCA cannot be taken during pregnancy.

UDCA is produced using ox bile as a starting material; the cholic acid is then isolated and, following different syntheses and chemical reactions, is converted to UDCA [13–14]. The method includes the removal of an hydroxy group in the 12 position and the epimerization of the 7-hydroxyl from the α- to the β-orientation. Finally, UDCA must be isolated and properly crystallized.

UDCA is produced by many companies around the world and available commercially under several brand names and formulations. Since potential impurities present in UDCA have been reported to be toxic, like lithocholic acid (LCA) [15–16], or poorly tolerated as CDCA [9], it is important to establish the impurities present in UDCA available from different companies. Even if only present in small quantities (i.e. less than 1%), in view of the metabolism of their long term accumulation in a given target organ, a detailed analytical study is warranted.

Therefore, the objectives of the present work were to evaluate the impurities present in four different UDCA samples obtained from different companies and to evaluate critically the results obtained using an HPLC method

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with four different detector systems including UV, refraction index (RI), a evaporative light scattering mass detector and a prederivatization fluorescence method. Four different detectors were used because whereas high-performance liquid chromatography (HPLC) is the preferred method for routine assessment of drug purity owing to its simplicity, for bile acid (BA) impurities detection needs more accurate techniques [17].

The conventional UV detector lacks sensitivity for compounds with low molar absorptivity, such as unconjugated bile acids. The refractive index detector, on the other hand, requires highly standardized chromatographic conditions but does offer the advantage of being universal. A recently developed detector has also been included [18]. This is an evaporative light scattering mass detector whose response is related to the mass of eluted BA and can be considered universal. In order to improve the sensitivity of the system, pre-column derivatization with the formation of highly fluorescent naphthacyl esters was also used [19].

The detection limits, accuracy and precision of the various detectors have been compared and the results obtained with the four UDCA preparations evaluated and discussed. In addition, results obtained by GC–mass spectrometric analysis were included as a reference method for the quantitative and qualitative detection of impurities.

Materials and Methods

Chemicals

All the chemicals used were of analytical grade, and the solvents and reagents for HPLC analysis were of HPLC grade. They were purchased from Merck (Darmstadt, Germany) and Mallinkrodt (MO, USA).

Pelargonic (nonanoic) acid and tetrakis-(decyl)ammonium bromide (TDeABr) were obtained from Fluka (Switzerland). Lauric (dodecanoic) acid was from Farmitalia Carlo Erba (Milan, Italy). The reagent 2-bromoacetyl-6-methoxynaphthalene (Br-AMN) and the 2-naphthacyl ester of pelargonic acid (used as an internal standard) were prepared as previously described [19].

Bile acids (BA) standards, i.e. cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), lithocholic acid (LCA) were purchased by Sigma (St Louis, MO, USA), ursocholic acid (UCA) was supplied by Giuliani SpA (Milan, Italy). UDCA samples under study were supplied by: Tokio Tanabe (Tokyo, Japan) (sample A); Diamalt GmbH (München, Germany) (sample B); Erregierre Industria Chimica SpA (Bergamo, Italy) (sample C); PCA SpA (Italy) (sample D). The powders were vacuum-dried before use in the analytical procedures.

Synthesis of the naphthacyl esters

The carboxylic acid (3 mmoles) in about 15 ml of acetonitrile was treated with 1 mmole of the reagent 2-bromoacetyl-6-methoxynaphthalene in the presence of 1 ml of triethylamine for 30 min at 70°C. After cooling the reaction mixture was treated as described below.

6-methoxynaphthacyl ester of UDCA. The reaction mixture was evaporated to dryness under reduced pressure and the oily residue was chromatographed on a silica gel column (flash chromatography) using ethyl acetate–methanol (98/2, v/v) with UV detection at 254 and 366 nm. This produced a white product with m.p. 94–96°C; calculated for $C_{37}H_{50}O_6$, C 75.22, H 8.53%; found C 74.9, H 8.61. IR (cm^{-1}): 1740 (CO ester), 1690, 1625, 1270, 1155, 1025, 850. UV (ethanol): λ_{max} = 312 nm ($\epsilon = 1534 \times 10^4$).

6-methoxynaphthacyl ester of dodecanoic acid. The reaction mixture was diluted with 30 ml of water and extracted with ethyl ether (3 × 10 ml). The collected extracts were washed with 10 ml of 5% sodium bicarbonate solution and 3 × 10 ml of water in succession; the ethereal layer was dried over anhydrous sodium sulphate and then the solvent was removed under vacuum. The residue obtained was purified by crystallization from methanol–water to give a white compound which was homogeneous by TLC (using ethyl acetate–petroleum ether (7:3, v/v)) with UV detection at 254 and 366 nm. m.p. 83–85°C calculated for $C_{25}H_{34}O_4$% C 75.34, H 8.6; found C 76.01, H 9.00. IR (cm^{-1}): 1735 (CO ester), 1690, 1620, 1285, 1160, 1025, 905, 855, 815. UV (ethanol): λ_{max} = 313 nm ($\epsilon = 1.350 \times 10^4$).

Instrumentation

A Waters 600-MS high-performance multi-solvent delivery system liquid chromatograph (Waters, Milford, MA, USA) was used. The

chromatograph was equipped with an autosampler Model 717, a column Thermostat TCM, a recorder Data Module Model 746 (Waters) and the following detectors: UV–vis, Waters Model 484 at 200 nm; evaporative light scattering mass detector ELSD II (Varex Corporation, Burtonsville, MD, USA) (nitrogen carrier gas flow, 40 PSI; drift tube temperature, 130°C; exhaust gas temperature, 82°C); refractive index (RI), differential refractometer Waters Model R-401; fluorimeter, Varian 2070 Model with excitation wavelength at 300 nm and emission at 460 nm. The solvents were degassed on line with a degasser ERC-3312 Erma (Tokyo, Japan).

A preliminary direct inspection of the drugs was performed by TLC using both conventional silica gel G plates 0.25-μm thick and C_{18} silica gel plates (Merck) in reversed-phase mode. The mobile phases were: acetic acid–carbon tetrachloride–isopropyl ether–isoamyl acetate–*n*-propanol–benzene (1:4:6:8:20:2, v/v/v/v/v/v); and methanol–water (3:2, v/v).

For BA detection, the plates were sprayed with a mixture of sulphuric acid–acetic anhydride–ethanol (1:1:3, v/v/v), heated at 120°C for 5 min and then exposed to UV light.

IR spectra were recorded using a Nujol mull on a Perkin–Elmer 298 IR spectrophotometer. UV spectra were recorded on a Jasco Uvidec 610 double-beam spectrophotometer.

Chromatographic conditions

Direct HPLC method. When the analysis was carried out directly on UDCA samples, the separation of UDCA from other bile acids was achieved using a 4-μm Nova-Pak C_{18} steel column (300 × 3.9 mm i.d.) (Waters). The column temperature was 37 ± 0.2°C. The mobile phase was methanol–water (65:35, v/v).

The water phase was buffered with 2 mM sodium phosphate (pH 5.4) when either the UV or the refractive index detectors were used. For the light scattering mass detector, a more volatile buffer was required; this was 2 mM ammonium acetate in methanol–water (65:35, v/v) (pH 5.4). The analysis was carried out in the isocratic mode at a flow rate of 0.9 ml min^{-1}.

Fluorescent derivatization method. The HPLC separation of the naphthacyl esters was performed on a 5-μm Ultracarb ODS-30 column (250 × 4.6 mm i.d.) using ternary mixtures of acetonitrile, methanol and water. The analysis of UDCA was carried out under isocratic conditions using the mixture A–B (55:45, v/v), where A = water and B = acetonitrile–methanol (60:40, v/v), at a flow rate of 1.2 ml min^{-1}.

The HPLC analysis of the potential UDCA impurities was performed under gradient elution conditions. The gradient profile adopted was: t = 0, 20% B; t = 15 min, 20% B; t = 25 min, 60% B; t = 45 min, 60% B; t = 50 min, 20% B; t = 60 min, 20% B at a flow rate of 1.2 ml min^{-1}. The column temperature was 35 ± 0.2°C.

Direct HPLC analysis

Preparation of the solution for analysis. 10 mM solutions of each sample of UDCA were prepared by dissolving the sample in the HPLC mobile phase and in water. Formation of the sodium salt in aqueous solution was achieved by adding equimolar amounts of sodium bicarbonate directly to the weighed UDCA and dissolving the salt in the appropriate amount of water under ultrasonication. The titre of these solutions was also assessed by an enzymatic colorimetric method using a 3α-hydroxysteroid dehydrogenase [20].

Analysis of UDCA samples. The content of UDCA in the four preparations was determined using calibration graphs derived from experiments in which known amounts of each BA standard had been analysed. From the graphs of peak area versus BA concentration the UDCA content was calculated. An internal standard, nordeoxycholic acid, was used.

UDCA impurities. Quantitation of the impurities, identified from their retention times, was achieved by external standardization. A calibration plot using BA standards for known concentrations was constructed for each BA and for each detector. The concentration of each unknown was read off the graph and expressed as a percentage of the original UDCA preparation.

Moreover, in order to minimize the method variability, the impurities were also quantified by internal standardization where increased known amounts of pure standards were added to the original UDCA solutions. The amount of a given impurity was calculated by a plot of peak area (y) versus BA concentration (x) and extrapolated at the intercept with the x-axis.

The direct ratio between the corrected peak area of UDCA and impurities in a single chromatographic run was evaluated using only the RI detector. For each analysis, six runs were performed and the results were expressed as mean ± standard deviation.

HPLC analysis with derivatization

Preparation of the solutions for analysis. The reagent (Br-AMN) solution (4.2 mg ml^{-1}) used for pre-chromatographic derivatization was prepared in acetone. The solution was found to be stable for about 2 weeks at 4°C. Tetrakis(decyl)ammonium bromide (TDeABr) solution (10 mM) was prepared in aqueous 5 mM phosphate buffer (pH 7.0). Stock solutions of the BA (2.5 mg ml^{-1}) were prepared in methanol and subsequently diluted with water to give the required final analytical solutions (concentration under Calibration Graphs) in 18% (v/v) methanol.

Derivatization procedure. To 0.2 ml of BA solution, 0.15 ml of 10 mM tetrakis(decyl)-ammonium bromide (TDeABr) in 5 mM phosphate buffer (pH 7.0) and 0.1 ml of the reagent solution (4.2 mg ml^{-1}) were added. The derivatization reaction was allowed to proceed for 10 min at 40°C, under ultrasonication. Then, 0.15 ml of the appropriate internal standard solution was added; the reaction mixture was ultrasonicated at room temperature for 1 min and a 50-μl aliquot of the resulting clear solution was injected into the chromatograph.

Calibration graph. Standard solutions of UDCA (0.025–0.1 mg ml^{-1}) in 18% (v/v) aqueous methanol were subjected to the derivatization procedure, using pelargonic acid naphthacyl ester (45.4 μg ml^{-1}) as the internal standard. The peak-height ratio of bile acid to internal standard was plotted against the corresponding acid concentration to obtain the calibration graph.

Analysis of UDCA samples

For UDCA. A sample was dissolved in methanol and diluted with water to provide a final analytical solution containing about 50 μg ml^{-1} of the drug in 18% (v/v) aqueous methanol. A 0.2 ml aliquot of this solution was then subjected to the derivatization and HPLC procedures and the drug content in each sample was determined by comparison with an appropriate standard solution.

For UDCA impurities. A sample solution of UDCA (100 μg ml^{-1}) was prepared in 18% (v/v) aqueous methanol as described above. To 0.2 ml of this solution 50-μl aliquots of standard solutions of CDCA and LCA (10–25.5 μg ml^{-1}) in 18% (v/v) methanol were added and the resulting solutions were subjected to the derivatization and HPLC procedures, using the naphthacyl ester of dodecanoic acid as the internal standard (10 μg ml^{-1}).

The peak-height ratios of analyte (CDCA and LCA) to internal standard were plotted against the corresponding amounts added to obtain for each analyte a standard addition calibration graph. The *x*-intercept was then used for calculating the content of CDCA and LCA in the UDCA sample analysed.

Gas-chromatography–mass spectrometry

GC–MS analyses were kindly performed by Dr K. Setchell, Clinical Mass Spectrometry Laboratories, Department of Pediatric Gastroenterology and Nutrition, Children's Hospital Medical Center, Cincinnati, OH, USA.

GC–MS analyses of the four UDCA samples were performed on the methyl ester trimethylsilyl ether derivative. The Me-TMS derivatives were separated using a 30 m × 0.4 i.d. capillary column DB-1 (J&W Scientific, Fisons, Folsom, CA, USA) using temperature program operation as previously described [21]. MS analysis was carried out using a Finnigan 4635 quadrupole instrument with electron impact ionization mode.

The purity of each sample was determined from the peak height response obtained for UDCA expressed as a percentage of the total peak height response for all peaks in the chromatogram excluding the internal standard, nordeoxycholic acid.

Results

TLC analysis

A preliminary direct TLC qualitative examination of the four UDCA samples showed the presence of detectable amounts of bile acid impurities in some samples. Both TLC systems confirmed the presence mostly of CDCA and lithocholic acid (LCA) together with another minor unknown BA.

Sample C was extremely pure and no other major spots were found. The presence of CDCA and LCA was observed in samples A, B and D, as indicated by the comparison with the R_f values of reference standards both in normal- and reversed-phase mode.

BA separation by HPLC

Bile acid can be efficiently and selectively separated on a C_{18} column in the isocratic mode according to structure and lipophilicity [22] as shown in Fig. 1 for a mixture of BA including UCA, UDCA, DCA, CDCA, LCA and CA. Slight differences in retention times were found when the phosphate buffer was replaced with ammonium acetate, but only when the same concentration, ionic strength and apparent pH.

Derivatized bile acids were separated under reversed-phase conditions (C_{18}) using gradient elution (Fig. 2).

BA detection

UV detector. For unconjugated bile acids, the UV detector lacks sensitivity since these bile acids do not have functional groups with adequate molar absorptivity. The detection limit of the UV detector is 100 nmole for a 10-μl volume injected (Table 1), requiring the injection of a highly concentrated BA solution. In order to estimate a 0.1% impurity, a 1 M solution of UDCA was required and although UDCA as a sodium salt is extremely soluble in water or methanol, preparation of a 1 M solution presents problems of solubility. Moreover, the BA loading required greatly compromises the analytical performance of the column in terms of peak resolution and sharpness.

The impurities present in the samples under analysis could not be accurately evaluated with this detector and data were not included in the present work. Only data on UDCA are reported in Table 2.

Light scattering mass detector. In the light scattering mass detector, the signal is a function of the mass of the eluted BA. The eluent from the column enters a nebulizer and is converted to a fine mist by a stream of nebulizing carrier gas (nitrogen). The fine droplets are carried through a temperature-controlled tube which causes evaporation of the mobile phase (volatile ammonium acetate buffer) and the non-volatile BA passes through a laser beam causing light scattering which is detected by a photodiode. The measured light is related to the amount of sample in the light scattering chamber, and the signal is indicative of molecular size and shape but not of the chemical identity of the BA passing through the beam [18].

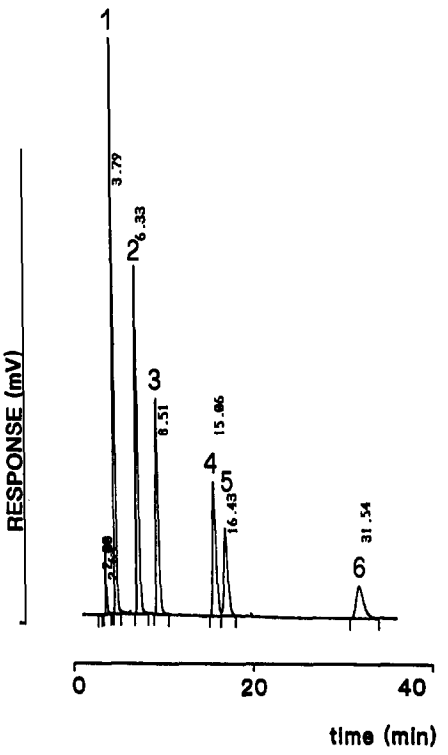

Figure 1
Chromatographic separation of the six major unconjugated bile acids using a 2 mM ammonium acetate in methanol–water (65:35, v/v) (pH* 5.4) as mobile phase (isocratic elution). The eluted BA were detected with the evaporative light scattering mass detector ELSD II. The amount injected was 10 nmoles for each BA. (1) Ursocholic acid; (2) ursodeoxycholic acid; (3) cholic acid; (4) chenodeoxycholic acid; (5) deoxycholic acid; (6) lithocholic acid.

The detection limit of the ELSD II detector is 0.5 nmol for 10 μl injected; this limit is much lower (at least 200 times) than that for the conventional UV detector (Table 1).

The detector response is accurate from 0.5 to 500 nmoles injected and the logarithm of the peak area is linearly related to the logarithm of the BA concentration in the sample. Since the detector response is also a function of the width of the peak, i.e. the retention time, accurate standardization is required. The detector response is higher for bile acids poorly retained by the column, such as UDCA, and is indirectly related to the retention time. The detection limit of LCA, a highly retained BA, is twice than that of UDCA (Fig. 1).

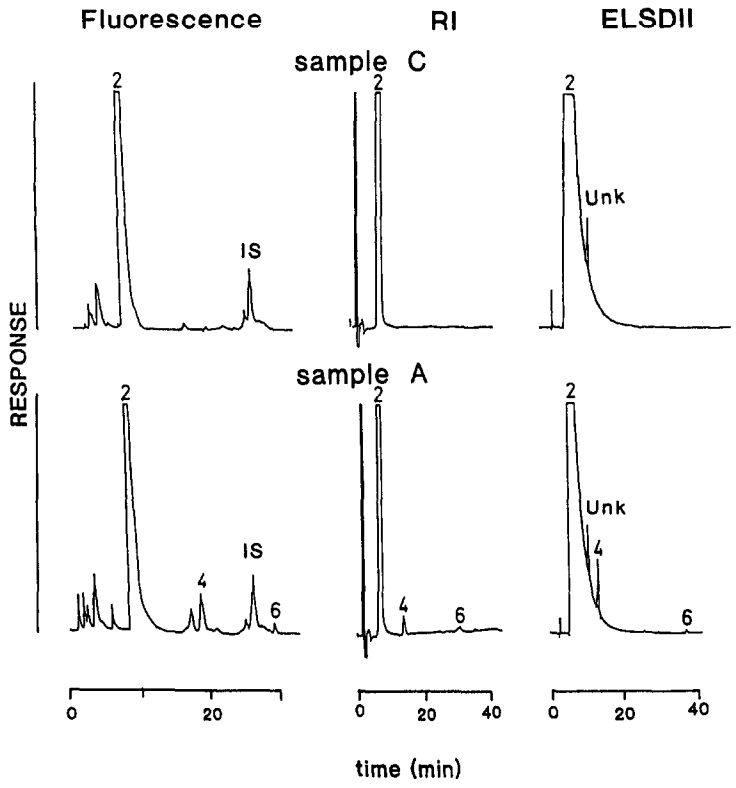

Figure 2
Typical chromatograms of samples A (Tokio Tanabe, Japan) and C (Erregierre SpA, Italy) obtained using the prederivatization procedure, the RI and ELSD II detectors. IS: internal standard; Unk: unknown compound.

Table 1
Analytical performance of the four methods used for the analysis of UDCA impurities

	Detector*			
	UV 200 nm	RI	ELSD II	Fluorescence
Detection limits (nmoles/injection)	100	0.1	0.5	0.002
Dynamic range (nmoles/injection)	100–10,000	0.1–500	0.5–500	0.002–25
Precision* (RSD%)				
Low concentration 5 nmoles	—	2	2.5	3.5†
Moderate concentration 50 nmoles	—	1.5	2	2
High concentration 500 nmoles	2	2	1.5	1.5

*Data refer to six repeated injections using the autosampler 717. The RSD of the injection system is always below 0.2%.

†The precision of the fluorescence detector was evaluated and studied at pmolar levels (5–50–500 pmoles/injection).

Figure 3 shows the relationship between the log of the peak area and the log of BA concentration; according to the slope of the curve, the ELSD II lacks sensitivity at low concentration. In order to evaluate a 0.1% level of impurity, a 100 mM UDCA solution is required. A 20-μl injection resulted in a very high UDCA peak, often off the scale. Under such analytical conditions it was possible to quantify the impurities but not UDCA itself. A subsequent injection of a more dilute solution allowed proper quantitation of UDCA.

Refractive index detector. The use of differential refractometry requires well standardized and constant mobile phase flow and composition and under the reported analytical conditions it was possible to detect less than 0.1% of impurities in the original solution. This detector shows a wide dynamic range with

Table 2
Results for the determination of ursodeoxycholic acid and its impurities in four samples* using different HPLC systems and GC–MS. Results are expressed as the mean value of six replicates ±SD

	UDCA				CDCA				LCA			
	A	B	C	D	A	B	C	D	A	B	C	D
UV	99.5	99.2	99.9	99.1	†	†	†	†	†	†	†	†
	0.2	0.4	0.1	0.7								
RI	99.1	99.1	99.9	99.2	0.80	0.68	—	0.80	<0.02	0.04	—	0.05
	0.4	0.6	0.4	0.6	0.05	0.06	—	0.04	—	0.001	—	0.01
ELSD	99.4	99.2	100.2	99.4	0.66	0.59	—	0.79	<0.02	0.07	—	0.12
	0.7	0.4	0.8	0.4	0.04	0.02	—	0.02	—	0.001	—	0.01
F	99.5	99.2	100.1	99.2	0.58	0.56	—	0.89	—	0.15	—	0.20
	0.5	0.4	0.4	0.5	0.02	0.02	—	0.05	—	0.005	—	0.01
GC–MS	98.6	96.8	99.9	98.6	0.70	0.60		0.92	—	0.02	—	0.10

*A, Tokio Tanabe (Japan); B, Diamalt (Germany); C, Erregierre (Italy); D, PCA (Italy).
†The UV detector cannot be used for the determination of the impurities CDCA and LCA.

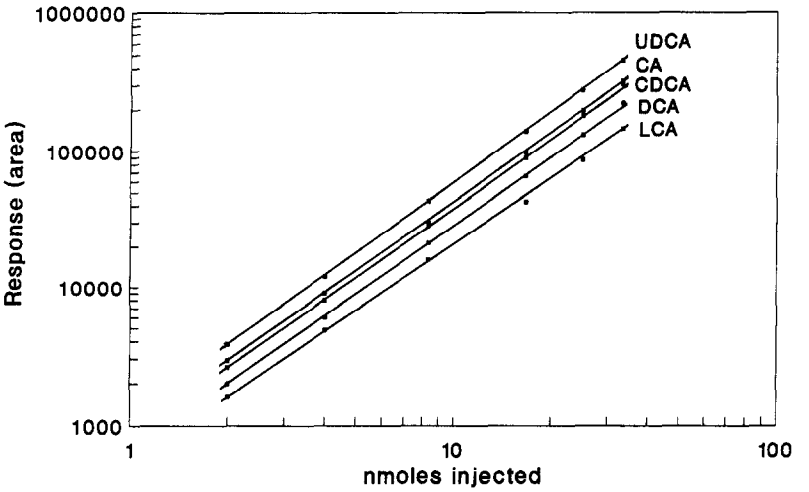

Figure 3
Detector response of the studied bile acids as a function of the amount injected using the ELSD II detector.

the possibility of simultaneously detecting UDCA and its impurities in a single chromatographic run.

Fluorogenic derivatization method. The developed derivatization method with the formation of fluorescent BA naphthacyl esters is much more sensitive than all the other direct methods and has a detection limit of 2 pmole per injection (Table 1).

The derivatization procedure, in aqueous medium in the presence of tetrakis(decyl)-ammonium bromide, is rapid under mild reaction conditions (10 min at 40°C) and proved to be essentially quantitative by comparison with an authentic specimen of UDCA naphthacyl ester. The overall precision (RSD) of the method, including the derivatization and chromatographic steps, was 1.5–3.5%, over the BA concentration range examined.

Two separate chromatographic runs were performed for the determination of UDCA and its impurities. Moreover, in order to minimize the method variability (fluorimetric detection) the impurities were also quantified by internal standardization in which increased known amounts of pure standards were added to the original UDCA solutions (standard addition method). The amount of a given impurity was calculated by using the x-intercept of a plot of peak-height ratio (analyte to internal standard) (y) versus BA added (x). A typical regression line obtained for the analysis of CDCA in sample B was: $y = 3.70\,x - 0.35$; $r = 0.9995$ ($n = 5$) (Fig. 4).

Detector comparison

The analytical performance of the various detectors in terms of detection limit, linearity and reproducibility are reported in Table 1.

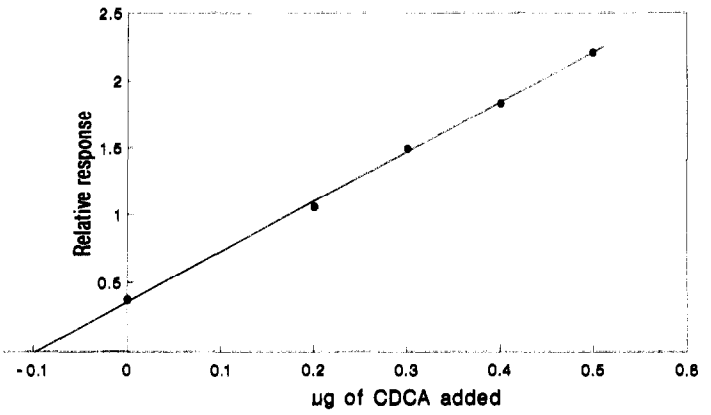

Figure 4
Plot of peak-height ratio (CDCA to internal standard) versus amount of CDCA added to the sample B solution. The peak-height ratio, determined by extrapolating the calibration plot to zero amount added, agreed with that obtained by direct analysis of the sample solution.

The detection limit is similar for the ELSD II and RI whereas it is much higher for the UV detector.

The derivatization method with the formation of 24-naphthacyl esters and their fluorescent detection is highly sensitive and about 2 pmoles can be evaluated.

The use of a new Waters 717 autosampler reduces the variability in the injection step, and the precision between runs of the detectors can be evaluated. When known samples at low, moderate and high concentrations were injected six times, the RSD of the injection system calculated from the mean values and their standard deviation was always below 0.2%. The overall RSD of the different methods was 1.5–3.5% (Table 1).

Analysis of UDCA samples

The results obtained (mean values ±SD of six replicates) are reported in Table 2 for the four commercial UDCA samples analysed using the four different HPLC systems.

All UDCA samples were more than 99% pure for all the detector methods; results for sample C showed that it was almost 100% pure. Differences in the amounts of BA impurities which were less than 1% of the total BA content were observed between the four methods used. Typical chromatograms recorded on samples C and A using the four detectors are shown in Fig. 2.

The main impurity identified was CDCA ranging from 0.5 to 0.9% in the different samples but absent (i.e. less than 0.01%) in sample C. The second impurity present in the same samples was LCA which is present only in samples B and D. Other minor BA impurities were present in some samples, but account for only less than 0.05% of total BA.

GC–MS analysis

The results obtained by the GC–MS analysis of the samples are reported in Table 2. The results indicated that only sample C was more than 99.9% pure whereas samples A, B, D contained BA impurities, mainly CDCA and to a lesser extent LCA, as identified by the mass spectra.

Discussion

The results obtained in the present work demonstrate the critical role of the detector in HPLC analysis. This is particularly evident in BA analysis and for the detection of impurities present in small amounts, i.e. less than 0.5–1%. In order to correctly quantify the amount of unknown impurities, it must be ascertained that the detector response for each impurity is similar to that for the major constituent, in this case UDCA.

For UV detectors the results clearly show a lack of sensitivity for unconjugated BA. Moreover, for molecules like BA that poorly absorb, the accuracy could be markedly affected by the presence of functional groups in the impurities with stronger molar absorptivity than those present in UDCA. Sources of error can be encountered when, for example, a mixture of amidated bile acid (glycine or taurine conjugates) with free BA is analysed. In this case, the molar absorptivity of the amidated BA is 50–100 times higher than the

free acid with a consequent underestimation of the free fractions [17]. UV detection can, therefore, be excluded as not being sufficiently accurate for impurity detection.

More accurate detection can be achieved with the RI and light scattering mass detectors since their analytical signal is mainly related to the bulk molecular size and not to the presence of a particular functional group and both detectors can thus be considered universal.

Despite similar detection limits, the ELSD II detector offers the advantage of easier running conditions since detection does not require major standardization; the only limitation is the use of a volatile buffer. Moreover, the detection response is not affected by variations in mobile phase flow or composition as in the case of the refractive index detector. This offers the advantage of possible programmable gradient studies which can be of extreme importance in increasing selectivity and reducing analysis time. This is the case in the detection of LCA which is highly retained by the C_{18} column due to its high lipophilicity [18].

On the other hand, the refractive index detector is preferable to the ELSD II in the evaluation of impurities at 0.1–0.5% levels. The detector response in the refractive index model is linearly related to BA concentration with a dynamic range which allows simultaneous evaluation both of UDCA and the impurities. With the ELSD II detector the main problem is that the detector response is affected by the width of the eluted peak, which is a function of the retention time, if the run is carried out in the isocratic mode. As a consequence, the calibration must be done for all bile acids under analysis.

Moreover, the log of the signal is linearly related to the log of the weight of BA injected. As a consequence, the detector response of BA standards is reduced at low concentration; according to the slope, for a reduction of 1/100 of the concentration, the area of the peak falls more than 2000-fold thus compromising the peak area evaluation. However, with two separate runs it is possible to properly quantify the impurities.

The derivatization method with fluorimetric detection is at least 1000 times more sensitive than both the RI and ELSD detectors but a pre-analytical step is required.

The reported results on the accuracy and precision of the overall procedure including the derivatization step, suggest the validity of this method for the evaluation of UDCA impurities. However, the method involves great use of the derivatization reagent and separate determinations of UDCA and its impurities. Moreover, the actual sensitivity achieved is determined by the reduced UDCA solubility in the reaction medium and, according to the derivatization reaction, only the acidic impurities can be detected. A possible modification of the impurity profile could also result from different or uncontrolled yield in the derivatization procedure.

When the results obtained with the four methods were compared, a reasonable agreement was found; despite a slight variability in the percentage of CDCA and LCA in the different samples, all the four methods, for example, excluded the presence of impurities up to 0.1% in sample C.

The variability in the unknown impurity is method-dependent and further detailed studies are required to identify those impurities which could also be non-BA material.

When the data obtained using HPLC with the different detectors were compared with those obtained by GC–MS, agreement was found. For example, sample C was almost 100% pure and the amounts of impurities like CDCA in the other samples were of the same order of magnitude.

More recently an HPLC–MS method for BA analysis based on the use of a thermospray interface has been developed [23]. The direct assay of BA, without the need of a precolumn derivatization as in GC–MS, associated with MS detection could possibly be the method of choice. However, both LC–MS interface technology and standardization procedure require further studies.

In conclusion, the results show that the UV detector cannot be used for BA quality control studies; both IR and ELSD II can be used but similar results can only be achieved under well standardized conditions. To further increase detectability, the proposed derivatization method with fluorimetric detection can be applied because of its good analytical performance in terms of reproducibility and accuracy.

Many commercially available UDCA samples contain detectable amounts of chenodeoxycholic, the α-epimer of UDCA, and only in one sample was CDCA absent; minor impurities like LCA were present only

in some samples. Sample C was almost 100% pure and could be proposed as a reference UDCA material for analytical purposes.

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References

[1] W.H. Bacharach and A.F. Hofmann, *Dig. Dis. Sci.* **27**, 737–761 (1982).
[2] S. Erlinger, A. Le Go, J.M. Husson and J. Fevery, *Hepatology* **4**, 308–314 (1984).
[3] H. Fromm, J.W. Roat, V. Gonzalez, R.P. Sarva and S. Farivar, *Gastroenterology* **85**, 1257–1264 (1983).
[4] E. Roda, F. Bazzoli, A.M. Morselli-Labate, G. Mazzella, A. Roda, C. Sama, D. Festi, R. Aldini and L. Barbara, *Hepatology* **2**, 804–810 (1982).
[5] F. Ichida, *Diagn. Treat.* **36**, 388 (1961).
[6] K. Miyai, *Jpn. J. Clin. Expl. Med.* **53**, 1395 (1976).
[7] U. Leuschner, J. Leuschner, J. Sieratzki and W. Kurtz, *Dig. Dis. Sci.* **30**, 642–649 (1985).
[8] R. Poupon, Y. Chretien, R.E. Poupon, Y. Calmus, F. Ballet and F. Darnis, *Lancet* **1**, 834–836 (1987).
[9] A. Ward, R.M. Brogden, R.C. Heal, T.M. Speight and G.S. Avery, *Drugs* **27**, 95–131 (1984).
[10] R.G. Danzinger, A.F. Hofmann, L.J. Schoenfield and J.L. Thistle, *N. Engl. J. Med.* **286**, 1–8 (1972).
[11] J.L. Thistle and A.F. Hofmann, *N. Engl. J. Med.* **289**, 655–659 (1973).
[12] J.W. Marks, S.O. Sue, B.J. Pearlman, G.G. Banorris, P. Varady, J.M. Lactin and J.L. Schoenfield, *J. Clin. Invest.* **68**, 1190–1197 (1981).
[13] T. Kanazawa, A. Shimazaki, T. Sato and T. Hoshino, *Nippon Kaguku Zasshi* **76**, 863 (1981).
[14] B. Samuelsson, *Acta Chem. Scand.* **14**, 17 (1960).
[15] J. Scholmerich, M.S. Becher and K. Schmidt, *Hepatology* **4**, 661–666 (1984).
[16] T.R. Gadacz, R.N. Allan, E. Mack and A.F. Hofmann, *Gastroenterology* **70**, 1125–1192 (1976).
[17] S.S. Rossi, J.L. Converse and A.F. Hofmann, *J. Lipid Res.* **28**, 589–595 (1987).
[18] A. Roda, C. Cerrè, P. Simoni, C. Polimeni, S. Vaccari and A. Pistillo, *J. Lipid Res.* **33**, 1393–1402 (1992).
[19] R. Gatti, V. Cavrini and P. Roveri, *Chromatographia* **33**, 13–18 (1992).
[20] O. Fausa and B.A. Skalhegg, *J. Gastroenterol.* **9**, 249–254 (1974).
[21] K.D.R. Setchell, R. Dumaswala, C. Colombo and M. Ronchi, *J. Biol. Chem.* **263**, 16637–16644 (1988).
[22] A. Roda, A. Minutello, A. Angellotti and A. Fini, *J. Lipid Res.* **31**, 1433–1443 (1990).
[23] K.D.R. Setchell and C.H. Vestal, *J. Lipid Res.* **30**, 1459–1469 (1989).

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