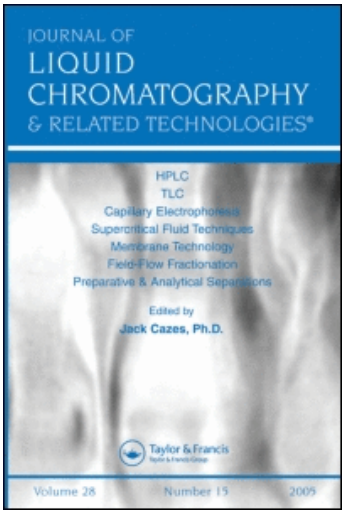

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ASSAY OF URSODEOXYCHOLIC ACID AND RELATED IMPURITIES IN PHARMACEUTICAL PREPARATIONS BY HPLC WITH EVAPORATIVE LIGHT SCATTERING DETECTOR

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ABSTRACT

An HPLC method was developed for assay and purity control of ursodeoxycholic acid, which is separated from all related compounds. Using an evaporative light scattering mass detector (ELSD), equipped with a micronebulizer and a narrow bore column, the bile acids (ursodeoxycholic acid, ursocholic acid, cholic acid, chenodeoxycholic acid, deoxycholic acid and litocholic acid) were efficiently separated on an Hypersil ODS-RP-18 column (100 x 2.1 mm) with methanol - acetonitrile - water (60 : 22: 18), adjusted at pH 4.00 with acetic acid, as mobile phase. Linearity response, accuracy, and precision of the ELSD over the range of sample amounts of interest were established for all compounds. The method demonstrates a suitability of the detector for the assay of ursodeoxycholic and related impurities and was applied for the analysis of ten pharmaceutical preparations.

INTRODUCTION

The bile acids (BA) present in humans are cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA) which occur primarily as glicine or taurine conjugates.[1] Administration of exogenous bile acids in treatment of asymptomatic cholesterol gallstones can reduce biliary cholesterol concentrations and has been tried in an attempt to dissolve the cholesterol component of gallstones.[2] It has also been reported to slow disease progression in primary biliary cirrhosis,[3] cystic fibrosis,[4] sclerosing cholangitis,[5] chronic active hepatitis and viral hepatitis.[6,7,8] Chenodeoxycholic acid was the first agent to be tried but has largely been replaced by UDCA, which is more effective and is associated with fewer adverse effects.[9,10]

UDCA is produced using bile as a starting material by many companies and is available commercially under several names and formulations. Related compounds usually present in commercial samples of UDCA for medicinal use are, in addition to chenodeoxycholic acid (CDCA) itself, deoxycholic (DCA), ursocholic (UCA), cholic (CA), and litocholic (LCA) acids. Since the pharmaceutical formulations of UDCA could contain impurities that have been reported to be toxic, like litocholic acid (LCA),[11] or poorly tolerated CDCA, even if only present in small quantities, an accurate, precise, and simple technique for quality control of UDCA in pharmaceutical dosage forms is required. The maximum acceptable limits of these impurities are reported in some Pharmacopoeias.[12,13,14]

High performance liquid chromatography (HPLC) has been used for determination of UDCA in pharmaceutical dosage forms. However, the conventional UV detection system has a limited sensitivity for the unconiugated bile acids.[15,16,17] UV detection of BA at 200 - 210 nm is limited by the poor absorptivity of BA at that wavelength; solvents, impurities and other matrix constituents with high absorptivity at 200 nm greatly affect the identification of the eluted BA. Therefore, a pre-derivatization procedure is required to increase sensitivity,[18,19,20] and the main disadvantage of this method is the time consuming and difficult controllable additional steps. The detection limits for UDCA and related impurities is shown to be much less using sensible and sensitive HPLC direct analysis with electrochemical detection (HPLC - ED)[21] and HPLC - ED after pre - column derivatization.[22] An alternative solution, suitable for the detection of the bile acids is the evaporative light scattering mass detector (ELSD) and RI detector.[23]

A current literature is reviewed, which demonstrates the applicability of the ELSD for use with phospholipids, fats and fatty esters, carbohydrates, synthetic polymers, steroids, inorganic counter ions, and pharmaceutical compounds.[24]

The use of this detector is also reported in the determination of bile acids in humans[25] and in the assay of UDCA and minor related impurities in pharmaceutical preparations in a competitive evaluation of four different detectors.[23] The last report describes the ELSD lack of sensitivity at low concentrations.

The aim of this work was to improve the sensitivity of ELSD by miniaturisation of the chromatographic system using a narrow bore column and a micronebulizer [26] and test its potentiality for quantitative analysis of trace bile acids present as impurities in formulations of UDCA.

EXPERIMENTAL

Chemicals and Reagents

All the chemicals were of analytical grade, and the solvents and reagents for HPLC analysis were of HPLC grade. They were purchased from Farmitalia Carlo Erba (Milan, Italy). Bile acids standards, i.e. ursodeoxycholic acid (UDCA), ursocholic acid (UCA), cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and litocholic acid (LCA) were purchased by Sigma (St. Louis, MO, USA). Pharmaceutical dosage forms containing UDCA were commercial samples.

Instrumental and Conditions

The chromatographic system consisted of LC pump Series 410 (Perkin-Elmer, Norwalk, CT, USA) and ELSD SEDEX 55, equipped with HPLC Micro Nebulizer (S.E.D.E.R.E., Alfortville, France). Signals from the detector were integrated by a computer system, Nelson model 1020, (Perkin - Elmer). The compressed air used to spray the column effluent was obtained with Minor Jun Air Compressor (Jun Air, Norresundby, Denmark). Separations were performed on a 3 μm particle size Hypersil ODS-RP-18 column (100 x 2.1 mm), and eluted isocratically with methanol - acetonitrile - water (60 : 22 : 18 v/v) adjusted to pH 4.00 with glacial acetic acid. The mobile phase was filtered through filters 0.22μm (Gelman Sciences, Michigan, USA).

Chromatography was performed at ambient column temperature, at a solvent flow rate of 0.08 mL/min, air carrier gas flow of 2.1 bar and drift tube temperature 70°C. The peaks were first identified by comparison of their retention times with those of BA standards. Spiking the samples with standards provided additional reference points. Peak areas were quantified by the external standardisation method.

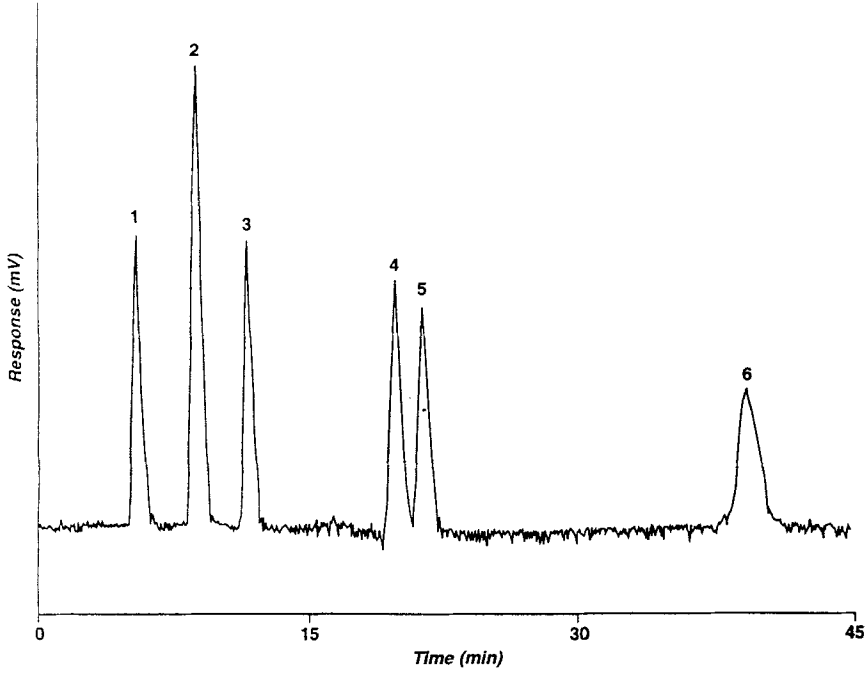

Figure 1. Chromatogram of standard mixture peak 1: UCA (0.28 μg), peak 2: UDCA (0.42 μg), peak 3: CA (0.28 μg), peak 4: CDCA (0.28 μg), peak 5: DCA (0.28 μg) and peak 6: LCA (0.28 μg); Column: Hypersil ODS-RP-18, 100 x 2.1 mm ID, 3μm; Mobile phase: methanol - acetonitrile - water (60:22:18), adjusted at pH=4 with acetic acid; isocratic flow 0.08 mL/min.

Preparation of Samples

Standard solutions of UDCA, UCA, CA, CDCA, DCA, and LCA in the examined concentration range were prepared dissolving known amounts of each bile acid reference material in methanol. Capsules and tablets were extracted with methanol, mixing thoroughly with shaking and sonication. Solutions were then filtered through a 0.22 μm nylon syringe filter. A volume of 5 μL was injected into the chromatographic column.

RESULTS AND DISCUSSION

The micro nebulizer tube used was constructed in a way that permits the entire effluent to be carried down directly into the drift tube where the eluent is

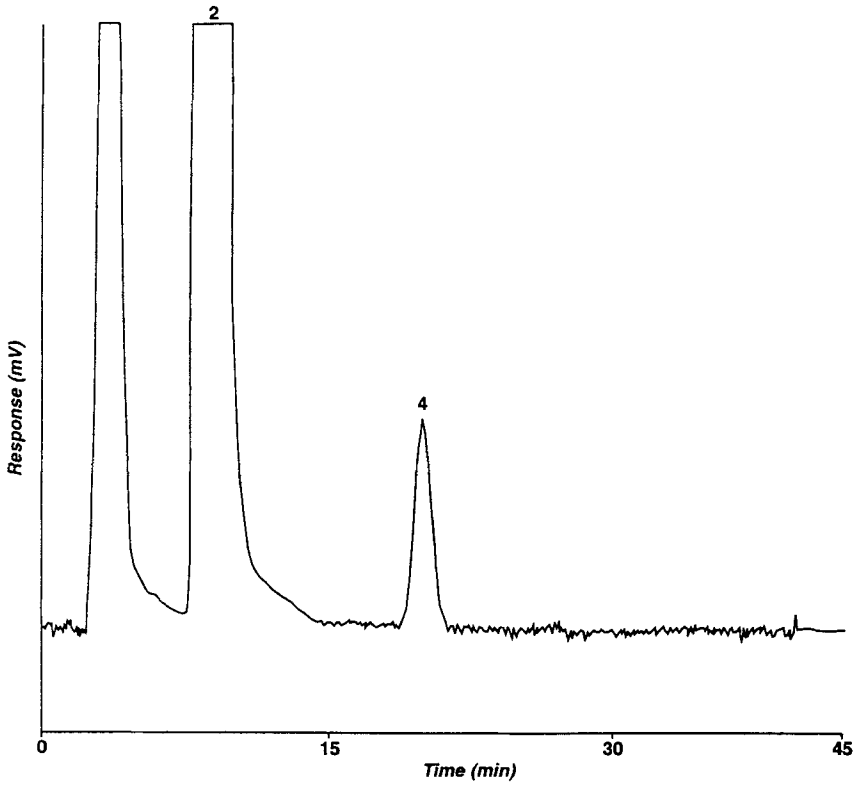

Figure 2. Chromatogram of UDCA tablet sample containing CDCA as impurity (0.23%). Column and chromatographic conditions as described in Fig 1.

evaporated. The connection between the column and ELSD was reduced to offer the smallest void volume. We have chosen 2.1 bar of nebulise gas pressure and 70°C of drift tube temperature in order to obtain the optimum vaporisation of the eluent and good operation condition for work.

With the mobile phase described above at a flow rate 0.08 mL/min or lower, the background current and noise were not significantly different from the values observed without solvent flow. When the solvent flow rate increased, both background current and noise increased because of incomplete vaporisation of the solvents. The developed method allowed separation within 40 min of all bile acids present as potential impurities in pharmaceutical preparations of UDCA. Figure 1 shows the separation of a standard mixture of the six bile acids tested. Figure 2 shows the chromatogram of a tablet formulation of UDCA with

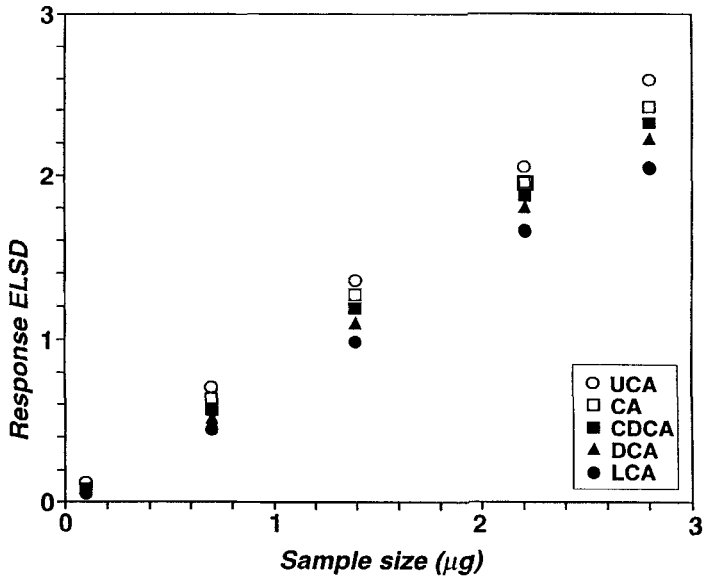

Figure 3. Detector response of the studied bile acids as a function of the amount injected.

Table 1

Statistical Data for Calibration Graphs for UDCA and Related Impurities

Compound	Range Tested (μg)	Correlation Coefficient	Slope	Intercept
UDCA	0.20 - 5.60	0.9997	1.1326	0.0365
UCA	0.10 - 2.80	0.9993	1.0490	0.0251
CA	0.10 - 2.80	0.9987	1.1423	0.0376
CDCA	0.10 - 2.80	0.9994	1.1992	0.0521
DCA	0.10 - 2.80	0.9986	1.2223	0.0541
LCA	0.10 - 2.80	0.9992	1.3240	0.0709

CDCA as impurity. No interference were noted from excipients present in the formulation tested at the retention times of UDCA and related impurities. The detection limit of described method was dependent on the conditions of the detector. Under those experimental conditions the detection limit expressed in terms of ng of the component injected were 30 ng for UCA, 35 ng for UDCA, and CA, 40 ng for CDCA and DCA and 60 ng for LCA as highly retained BA.

Table 2

Resproducibility of Within-Assay and Between-Assay of UDCA

Concentration (μg)	% RSD				
	Day 1[a]	Day 2[a]	Day 3[a]	Day 4[a]	Overall[b]
0.2	1.60	3.25	3.32	2.56	2.86
1	1.72	2.49	1.44	1.43	2.12
2	0.99	1.98	2.03	2.37	1.96

[a] Each value represents the mean of five determinations.
[b] Mean of 20 determinations.

Calibration curves were obtained by injecting UDCA in a concentration range of 40 - 1120 μg/mL and UCA, CA, CDCA, DCA, and LCA in a concentration range of 20 - 560 μg/mL. The areas of the chromatographic peaks were measured. The values of slope and intercept at the calibration straight - line and the regression coefficients are given in Table 1. In order to evaluate <0.05% level of the impurities it required concentrated UDCA solution, with the peak of UDCA off the scale. For quantification of UDCA itself, a more dilute solution was injected. The relationship between the sample size and detector response is shown in Figure 3 for all examined impurity compounds. The slopes obtained from the plot of area against amount injected gave the deviation of the linearity and the difference on detector response for each component.

Both accuracy and within - and between - assay reproducibility of the described method were tested. Accuracy was evaluated by calculating the percent difference between the amount found and the corresponding nominal amount at four concentrations of UDCA and related impurities. The deviation from theoretical values were < 5 %. The within - assay and between - assay reproducibility was studied at three concentration levels of UDCA. The results are shown in Table 2, from which one can see that the coefficient of variations ranged from 0.99 to 3.32 %.

Ten different formulations of UDCA, which are commercially available in Italy, were assayed using the proposed method (Table 3). The amount of active ingredient found experimentally is within 3% of the amount declared. The percentage of the related impurities, only in the few cases examined, is found to be a bit over the limits of 1% for CDCA allowed in Italian pharmacopoeia; it is, nevertheless, allowed in American and Japanese pharmacopoeias (1.5%). Minor impurities such as LCA and UCA were present only in some samples.

Table 3

Assay of UDCA and Related Impurity Levels in Commercial Dosage Forms

Sample	Label Claim	Found ± RSD[a]	Impurity Concentration (%)				
			UCA	CA	CDCA	DCA	LCA
A	150 mg	99.5±1.2	---	---	0.65	---	---
B	150 mg	97.8±2.1	0.11	---	1.14	---	---
C	125 mg	100.7±2.0	---	---	0.62	---	---
D	150 mg	101.6±1.12	---	---	0.17	---	---
E	150 mg	98.6±2.4	---	---	0.23	---	---
F	300 mg	102.0±2.1	---	---	0.11	---	---
G	150 mg	100.8±1.8	---	---	0.41	---	<0.05
H	150 mg	100.1±1.7	---	---	0.95	---	---
I	50mg	98.4±2.4	0.06	---	1.06	---	<0.05
J	300 mg	99.5±2.7	---	---	0.06	---	---

[a] Each value represents the mean (± relative standard deviation of six determinations.

CONCLUSIONS

For unconjugated BA with poor UV molar absorptivity, the ELSD has been demonstrated to be a more universal and accurate method of detection. Using a narrow bore column and micronebulizer good performance and separation and lower sensitivity levels have been obtained. Although it is not yet competitive with prederivatization procedures it is much simpler and less expensive. The system can be successfully applied, according to good reproducibility, sensitivity, and precision for direct and simple assay of the impurities present in UDCA formulations.

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