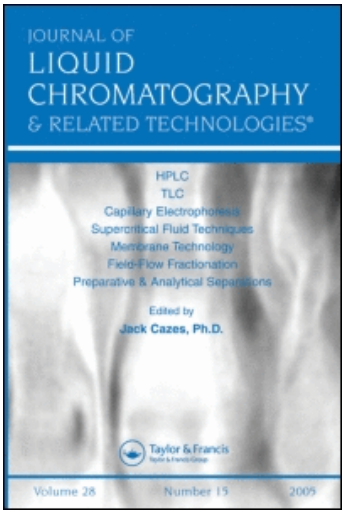

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Bousquet, E. , Santagati, N. A. and Tirendi, S.(1997) 'Determination of Chenodeoxycholic Acid in Pharmaceutical Preparations of Ursodeoxycholic Acid by High Performance Liquid Chromatography with Coulometric Electrochemical Detection', Journal of Liquid Chromatography & Related Technologies, 20: 5, 757 — 770
To link to this Article: DOI: 10.1080/10826079708014140
URL: http://dx.doi.org/10.1080/10826079708014140

J. LIQ. CHROM. & REL. TECHNOL., 20(5), 757–770 (1997)

DETERMINATION OF CHENODEOXYCHOLIC ACID IN PHARMACEUTICAL PREPARATIONS OF URSODEOXYCHOLIC ACID BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH COULOMETRIC ELECTROCHEMICAL DETECTION

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ABSTRACT

1-(2,5-Dihydroxyphenyl)-2-bromoethanone (2,5-DBE) has been used as an electroactive labelling reagent for high performance liquid chromatographic (HPLC) analysis of chenodeoxycholic acid impurity in pharmaceutical formulations of ursodeoxycholic acid.

The preparation of this reagent has been performed in a single step by bromination of 2,5-dihydroxyacetophenone (2,5-DAP), with remarkable improvement as regards the synthesis previously reported. Several experiments have been performed in order to give maximum conversion of the bile acids to their electroactive esters.

The determination of the derivatized compounds has been carried out on an Adsorbosphere column with a methanol-acetonitrile-acetate buffer eluent and detected by a porous graphite electrode set at an oxidation potential of + 0.6 V. The high sensitivity and specificity

shown by this novel method allows the determination of low concentrations of the cited impurity in capsule and tablet formulations containing the above therapeutic agent.

INTRODUCTION

Cholesterol is the precursor of bile acids and is normally present in the bile in high concentrations. The alteration of the bile composition, that is due to an excessive quantity of cholesterol with respect to the bile acids and to the phospholipids, causes the formation of gall-stones.

For the treatment of cholesterol gall-stones disease, two of bile acids present in human, chenodeoxycholic acid (CDCA) and the ursodeoxycholic acid (UDCA) are used.[1,2] However, because of the side-effects associated with CDCA therapy, UDCA is the most commonly administered compound.[3] Since the pharmaceutical preparations of UDCA could contain impurities of CDCA because the above drug is produced from bovine bile, a novel method of analysis of both the compounds has been investigated.

Recently, different procedures for the determination of bile acids in pharmaceutical and biological matrices have been reviewed.[4] These include especially chromatographic techniques, but also electrochemical, enzymatic and immunological methods.

The HPLC technique is the method of choice due to its precision and simplicity. But, the conventional UV detection system has a limited sensitivity for the unconjugated bile acids because they have weak molar absorptivities and short UV wavelengths must be used.[5-7] This results in an increase of the interferences from matrix components.

In order to improve the sensitivity for the HPLC determination of free bile acids, more convenient revelation methods have been used. Alternating voltage polarographic detection,[8] pulsed amperometric detection,[9] direct analysis by electrochemical detection (ED)[10] and direct combination with a mass spectrometer[11,12] represent some significant examples that have appeared in literature. A chemical label should permit measurement at low concentrations and minimize the effect of interfering substances present in complex matrices. In fact, precolumn labeling of bile acids allows sensitive UV and also fluorescence and electrochemical detection.[13-17] Particularly, HPLC coupled with ED represents a very sensitive method providing enhanced selectivity as a result of the limited number of substances which can undergo redox reactions under certain conditions.[18]

This paper describes a highly sensitive and selective method for the determination of impurity of CDCA in tablets and capsules of UDCA by HPLC-ED after pre-column derivatization. To this purpose we established a novel reaction of derivatization of these two bile acids with 2,5-DBE to form electroactive esters measurable by HPLC with ED. Moreover, a conventional on-line UV detector was present. The electrochemical probe 2,5-DBE was previously synthesized from 1,4-dimethoxybenzene,[19] or from 2,5-diacetoxy-α-diazoacetophenone[20] with different steps of reaction and low yields.

We have synthesized the 2,5-DBE with more satisfying results and in a single step of reaction from 2,5-DAP by direct bromination with phenyltrimethyammonium bromide tribromide ($PTMABr_3$). The brominating agent we have used has the advantage to reduce the forming of byproducts and to exploit a less toxic reagent than the ones generally used.[21]

EXPERIMENTAL

Apparatus

The HPLC apparatus comprises two Model 510 pumps, a Model 712 WISP auto-injector and a Model 490E absorbance detector (Waters Assoc., Milford, MA, USA) set at 257 nm and 0.05 absorbance units full scale. The UV detector was connected in series with the electrochemical detector (Model 5100A Coulochem; ESA, Bedford, MA, USA) which consisted of a control module and an analytical cell (Model 5010) containing two on-line porous graphite coulometric electrodes.

The analysis was performed in the oxidative mode. The ED sensitivity range and response time were set at 100 nA and 10 s, respectively. Signals from the detectors were converted to chromatographic traces and integrated by an APC IV computer system (NEC, Boxborough, MA, USA) using Maxima 820 software (Waters Assoc., Milford, MA, USA).

Mass spectra were obtained on a model Kratos MS 25 RF. IR spectra were recorded on a Perkin-Elmer 1600 Fourier transformed spectrometer as KBr disks. The ^{1}H-NMR spectra were recorded at 80 MHz on a Bruker WP instrument for $CDCl_3$ solutions with tetramethylsilane (TMS) as internal standard. Chemical shifts are expressed in ppm (δ). Elemental analysis for C,

H, N were obtained on a Carlo Erba 1106 analyzer (Milan, Italy) and agreed with theoretical values to within ± 0.4%. UV absorption spectra were recorded on a Uvikon 860 (Kontron, Zurich, Switzerland) spectrometer in CH_3CN/MeOH 9:1 solution. Analytical thin layer chromatography (TLC) was performed on Merck 60 F_{254} silica gel plates.

Chemicals

2,5-Dihydroxyacetophenone, phenyltrimethylammonium bromide tribromide, ursodeoxycholic acid, and chenodeoxycholic acid were obtained from Fluka (Buchs, Switzerland). HPLC-grade methanol, acetonitrile and water were from Carlo-Erba (Milan, Italy). Other chemicals used were of reagent grade or better.

Chromatographic Conditions

Separations were performed on a 3 μm Adsorbosphere column (100x4.6 mm i.d.; Alltech, Deerfield, IL, USA) fitted with a guard column (Hypersyl ODS RP-18, 5 μm particles, 4x4 mm i.d.; Policonsult, Rome, Italy) and eluted, isocratically, with methanol:acetonitrile:sodium acetate buffer 0.1 M (60:20:20, v/v) at pH 6.5. The mobile phase was filtered through GS-type filters (0.22 μm, Millipore, Bedford, MA, USA) and on-line degassed with a Model ERC-3311 solvent degasser (Erma, Tokyo, Japan). Chromatography was performed at room temperature (21°C) and a flow-rate of 1.0 mL/min.

Synthesis

1-(2,5-Dihydroxyphenyl)-2-bromoethanone(2,5-DBE)

Phenyltrimethylammonium bromide tribromide ($PTMABr_3$), (2.5 g, 6.6 mmol) was slowly added to a solution of 2,5-dihydroxyacetophenone (2,5-DAP) (1g, 6.6 mmol) in 20 mL of dry THF. The mixture was kept under stirring overnight at room temperature (21°C) and checked by TLC with eluent cyclohexane:ethyl acetate (7:3 v/v). The precipitate formed was removed by suction filtration, and then rotaryevaporated to dryness. The residue was purified by flash chromatography (cyclohexane:ethyl acetate 7/3 v/v) giving

960 mg of 2,5-DBE (yield 63%). UV: λmax 255 nm, ε = 10,356 M^{-1} cm^{-1} I.R. (KBr cm^{-1}) λ_{max}: 3335 (OH), 1620 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 11.4 (s, 1H, OH); 11.1(s, 1H, OH); 7.3-6.7(m, 3H, ArH); 4.4(s, 2H, CH_2). MS (m/z): 232 (M^+ +2), 230, 150, 136, 108.

1-(2,5-Dihydroxyphenyl)-2-ethanone-2-chenodeoxycholate (2,5-DE-UDC)

The solution of UDCA (196 mg, 0.5 mmol) in 5 mL of dry CH_3CN was added to 2,5-DBE (120 g, 0.5 mol), 100 μL of triethylamine (0.71 mmol), and heated at 70°C for 2 h. The mixture was diluted with 20 mL of H_2O and was extracted three times with diethyl ether. Then the organic layer was washed with saturated $NaHCO_3$ and water, dried (Na_2SO_4), evaporated and purified by flash chromatography (hexane:ethyl acetate 7:3 v/v) giving 185 mg of product (yield 70%). UV: λmax 257nm, ε = 15,890 M^{-1} cm^{-1}; IR (KBr, cm^{-1}) νmax: 3335 (OH), 2931 (CH_2), 1729 (CO), 1660 (Ph-CO); MS (m/z): 542 (M+), 507, 415, 373, 341, 150, 137, 109.

1-(2,5-Dihydroxyphenyl)-2-ethanone-2-ursodeoxycholate (2,5-DE-CDC)

This compound was obtained by the same procedure described for the 2,5-DE-UDC.

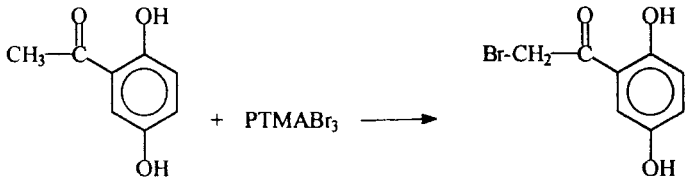
UV: λmax 257 nm, ε=15,503 M^{-1} cm^{-1}; I.R. (KBr, cm^{-1}) νmax: 3445(OH), 1732(CO), 1657(Ph-CO); MS (m/z): 542(M^+), 507, 357, 150, 137, 109.

RESULTS AND DISCUSSION

Derivatization

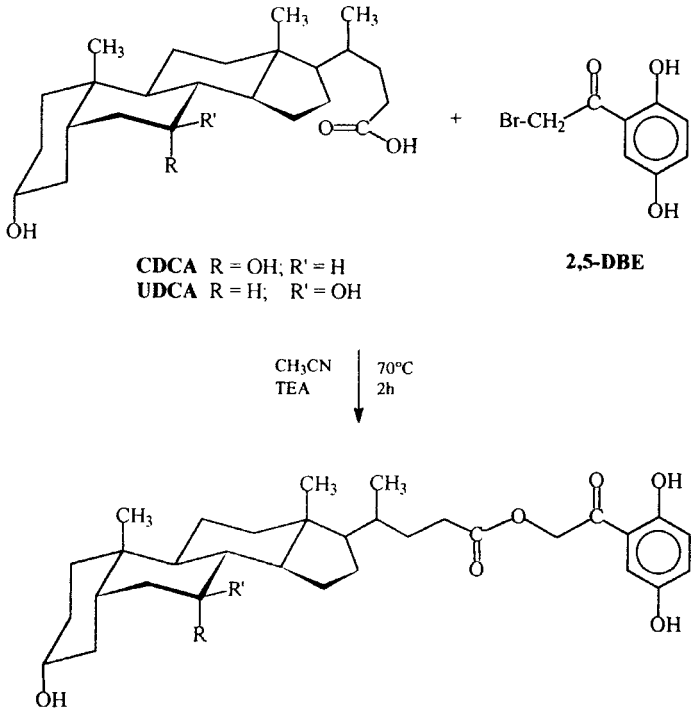
Scheme 1 illustrates the novel synthesis of 2,5-DBE. The preparation of this labeling reagent has been performed in one step. In fact, the treatment of 2,5-DAP with $PTMABr_3$ has given the brominated product immediately.

Scheme 2 represents the CDCA and UDCA reaction of esterification with 2,5-DBE to give the derivatized compounds 2,5-DE-CDC and 2,5-DE-UDC by nucleophylic substitution. The experiments have been performed to determine optimum derivatization time in order to give maximum conversion of the bile acids to their electroactive derivatives.



2,5-DAP 2,5-DBE

Scheme 1: Reaction of 2,5-DAP with $PTMABr_3$ to give labelling reagent 2,5-DBE.



CDCA R = OH; R' = H
UDCA R = H; R' = OH

2,5-DBE

2,5-DE-CDC R = OH; R' = H
2,5-DE-UDC R = H; R' = OH

Scheme 2: Derivatization reaction of CDCA and UDCA with 2,5-DBE to give the electroactive esters 2,5-DE-CDC and 2,5-DE-UDC.

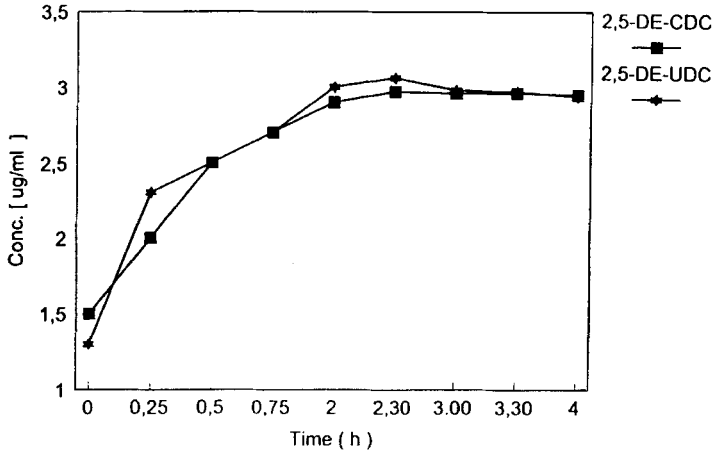


Figure 1. Concentration of derivatized esters 2,5-DE-CDC and 2,5-DE-UDC *versus* time curve.

In fact, figure 1 shows the trend of the derivatization procedure which is complete after 2 h with 70% yield. The synthesized esters show stability in the reaction mixture up to 12 h after the optimum.

Optimization of Detection

Several parameters has been examined in order to optimize the electrochemical detection of the above compounds. Under the chromatographic conditions, which are soon after described, the ester derivatives responded at the ED oxidation potentials higher than +0.2 V. The enhanced signals were obtained as the working electrode potential has been increased from + 0.2 V to +1.0 V. With the additional applied potential, no further increases in ester peak heights occurr and a rise in the background current can be observed. Electroactive properties of the compounds 2,5-DE-CDC and 2,5-DE-UDC have been examined by their hydrodynamic voltammograms (figure 2). The figure indicates that the best potential is +0.6 V, because for superior potentials the response would be an amplified one only for the derivatization reagent 2,5-DBE.

The ED performance has been markedly influenced by the ionic strength but not by the pH of the mobile phase. With increasing concentrations of the sodium acetate buffer (from 0.05 to 0.1 M), an increase of the bile

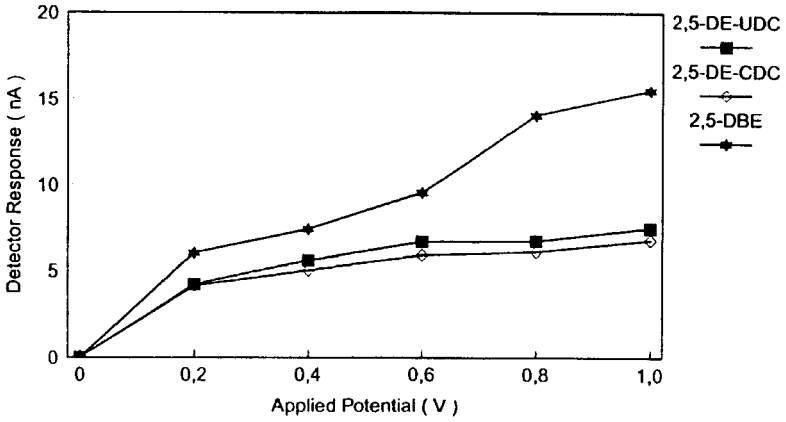


Figure 2. Hydrodynamic voltammograms of the electroactive compounds.

esters electrochemical response has been observed. No significant improvement in the detector response has been achieved by further increasing the sodium acetate molarity, which was consequently fixed at 0.1 M and the pH at 6.5.

Chromatography

Figure 3 shows a representative HPLC chromatogram with ED detection of standards. Chromatographic separations have been carried out under isocratic reversed phase conditions on a 3 μm Adsorbosphere column. The mobil phase consists of the ternary mixture methanol:acetonitrile: sodium acetate 0.1 M (60:20:20, v/v), at flow-rate of 1 mL/min.

The analysis is complete within 9 minutes and the retention times are 4.07 for 2,5-DE-UDC and 8.71 for 2,5-DE-CDC, at concentrations of 4.4 and 3.9 nmol/mL, respectively. The injected volume is 5 μL.

In the described analysis conditions, from the conventional UV detector connected on-line and set at 257 nm there was no signal. When the concentration values of the bile esters are increased by about twenty times, we observe only the relative peak to the derivatized drug. So, the UV detection is also applicable but only for concentrations superior to 80/90 pmol/mL and for the assay of the principal compound.

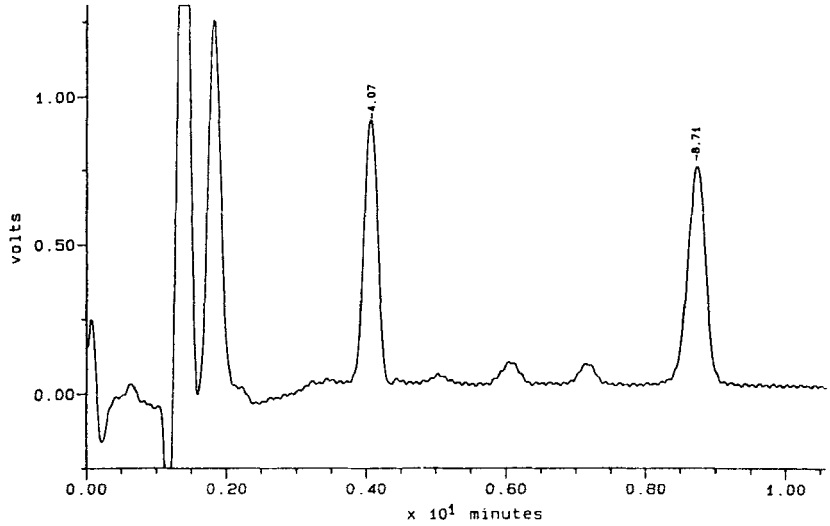


Figure 3. Chromatogram obtained by HPLC with ED detection of 5 µL of the standard solutions of electroactive esters 2,5-DE-UDC (4.4 nmol/mL) and 2,5-DE-CDC (3.9 nmol/mL).

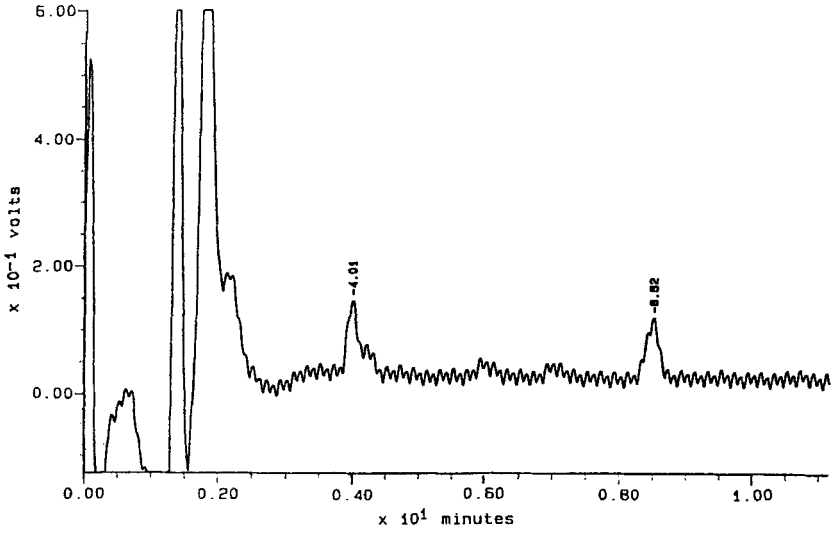


Figure 4. Chromatographic recording of detection limit of electroactive esters 2,5-DE-UDC (0.88 pmol/mL) and 2,5-DE-CDC (0.78 pmol/mL).

Linearity and Detection Limit

The linearity of response has been examined for both esters 2,5-DE-CDC and 2,5-DE-UDC in the range 0.2-2.0 μg/mL. The coefficients of linear regression of the standard curves have been consistently greater than 0.99.

Detection limit (figure 4) is determined by five runs and it is in the range of 0.88 and 0.78 pmol for 2,5-DE-UDC and 2,5-DE-CDC respectively (S/N= 5).

Analysis of Pharmaceutical Formulation

Four different commercial formulations containing UDCA have been subjected to the derivatization and HPLC analysis using the proposed ED system. The high sensitivity achieved by ED monitoring permits an accurate quantification of the trace of the CDCA impurity present in the pharmaceutical preparations. Figure 5 shows a representative HPLC chromatogram of a tablet formulation.

Under the same analysis conditions as the impurity traces there was an excessively high signal due to 2,5-DBE and 2,5-DE-UDC. This has made it impossible to quantify both compounds in the same chromatogram. Then the active principle can be determined by dilution or by an on-line UV detection system.

Figure 6 shows the HPLC chromatogram of the same formulation using the UV detector. Other potential bile acid impurities or other ingredients of the formulations do not interfere with the analysis of both compounds when the detection is carried out with a UV detection system and with ED.

The results, presented in Table 1, are in agreement with the label claim and demonstrate the precision of the method.

CONCLUSION

The derivatization of bile acids CDCA and UDCA with 2,5-DBE yields stable and highly sensitive electroactive esters, which are measurable by HPLC. The derivatization procedure developed in this study allows the detection of CDCA impurities at a level of less than 1 pmol.

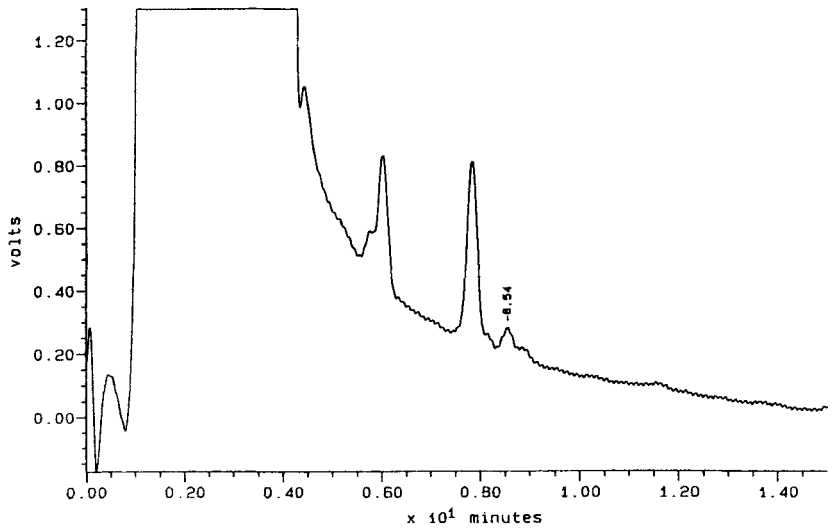


Figure 5. Chromatographic recording of derivatized CDCA impurity present in a tablet preparation.

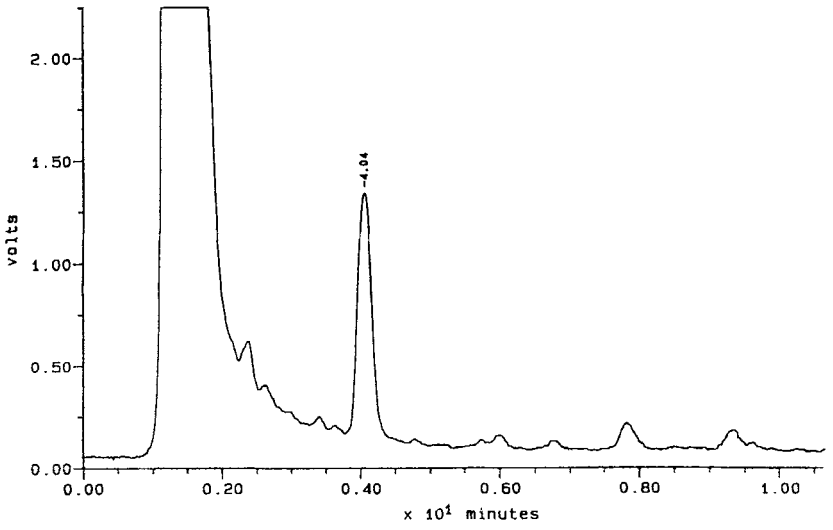


Figure 6. Chromatographic recording of UDCA obtained by HPLC with UV detection.

Table 1

Assay Results for the HPLC Determination of CDCA Impurity in Commercial Pharmaceutical Formulations of UDCA

Formulation	Label Claim (mg)	% Found*	%CDCA*
Tablet 1	UDCA 300	99.7 (0.3)	1.2 (2.5)
Tablet 2	UDCA 150	101.0 (0.8)	1.3 (4.5)
Capsule 1	UDCA 450	101.6 (1.4)	0.6 (3.9)
Capsule 2	UDCA 250	100.2 (1.1)	0.8 (5.4)

* Mean (RSD) of six determinations

The sensitivity obtained is higher than that produced by the electrochemical methods reported in the literature.[8-10] The applied potential permits the selective oxidation of the esters derivatives without interference because of the limited number of substances which can undergo redox reactions under this condition.

Because of the selectivity achieved, no interference from the commercial product matrix was observed by the method described here and consequently complex extraction procedure are not required. Since this method offers a means of enhancing the selectivity and sensitivity, it can be used to determine the trace amounts of the electroactive compound CDCA in quality control assays of commercial pharmaceutical formulations containing the UDCA drug.

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Received May 5, 1996
Accepted August 12, 1996
Manuscript 4180