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Journal of Pharmaceutical and Biomedical Analysis
32 (2003) 949–956
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Determination of ursodeoxycholic acid in pharmaceutical preparations by capillary electrophoresis with indirect UV detection

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Received 24 April 2002; received in revised form 18 December 2002; accepted 20 December 2002

Abstract

A simple and rapid capillary electrophoretic method, with indirect UV detection, for the quantification of ursodeoxycholic acid (UDCA) in pharmaceutical preparations was developed in this study. Sodium *p*-hydroxy benzoate was used as background electrolyte (BGE) (5 mM, pH 8.0) and visualization agent. Separation was carried out on a fused-silica capillary (50 μm × 72 cm) at a potential of 25 kV under ambient temperature and detected at 250 nm. Glycocholic acid was used as internal standard for quantification. Both run-to-run repeatability and day-to-day reproducibility of migration time were below 0.1% relative standard deviation (R.S.D.). Repeatability and reproducibility of relative peak height were 3.3 and 3.8% R.S.D., respectively. Accuracy was tested by spiking two pharmaceutical tablets with standards and the recoveries were 101.9 ± 9.87 and 99.6 ± 9.60% ($n = 3$), respectively. Linearity of relative peak height was tested in the range 20–100 μg/ml. Limit of detection (LOD) was 3 μg/ml. The method could be used to assay UDCA raw materials and formulation products.

Keywords: Ursodeoxycholic acid; Capillary electrophoresis; Indirect UV detection

1. Introduction

Ursodeoxycholic acid (UDCA, Fig. 1) was first isolated in 1902 from the polar bears by Hammarsten, Sweden [1]. It is effective in dissolving cholesterol gallstones. Liver diseases, including primary biliary cirrhosis, primary sclerosing cholangitis, hepatic allograft rejection, nonalchoholic steatohepatitis and cystic fibrosis are treated with UDCA. Mechanisms for the favorable effects of UDCA can be classified into three categories: hepatoprotective effect, effects on endogeneous bile acids and bile flow and immunomodulation [2,3].

Many HPLC methods with various detections have been proposed for the analysis of UDCA. Direct UV detection usually detected UDCA at the wavelength around 200 nm [4,5]. However, UDCA does not show significant UV absorption and the sensitivity is, therefore, limited. USP 24

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doi:10.1016/S0731-7085(03)00196-1

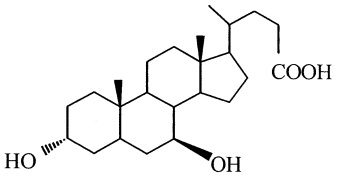

Fig. 1. Structure of ursodeoxyxholic acid (UDCA).

prescribes HPLC coupling with refractive index detector to assay UDCA in pharmaceutical preparations [6]. Electrochemical and evaporative light scattering detectors have also been employed to assay UDCA [7,8]. Other methods involved sample derivatization, followed by fluorescence or UV detection, to determine UDCA in pharmaceutical preparations and biological fluid [9–11]. Although sensitivity and specificity could be increased with these approaches, the procedure is laborious. Pulsed amperometry and tandem mass spectrometry were also used for LC detection to determine UDCA and other bile acids in biological fluids; however, the tested species are the glycine, taurine and glucuronide conjugates of the acids [12,13].

In capillary electrophoresis (CE), micellar electrokinetic chromatography had been used for the determination of UDCA in pharmaceuticals [14]. The wavelength was set at 185 nm to maintain a suitable detection with UV.

Indirect detection can be applied to detect compounds that do not show good UV absorbance. HPLC using indirect UV detection is mainly applied for qualitative analysis. CE using indirect detection shows high accuracy in quantitative analysis. It works by adding an UV absorbing agent (called visualization agent) with the same charge as the analyte to the background electrolyte (BGE). When an analyte displaces the visualization agent, it will decrease the absorbance and cause a negative peak.

Quaglia et al. applied CE with indirect UV detection method to separate UDCA and its related compounds [15]. However, quantitative analysis of UDCA was not included in their work. The aim of this study was to develop an effective capillary electrophoretic method with indirect UV detection to determine the amount of UDCA in pharmaceutical preparations.

2. Experimental

2.1. Chemicals and materials

All bile acids, namely, UDCA, chenodeoxycholic acid, glycochenodeoxycholic acid, glycocholic acid, glycodeoxycholic acid, hyodeoxycholic acid, lithocholic acid, taurochenodeoxycholic acid and taurodeoxycholic acid, were purchased from Sigma (St Louis, MO, USA).

Benzoic acid and 4-hydroxy benzoic acid were purchased from Acros (Geel, Belgium). Potassium phthalic acid was purchased from Wako (Osaka, Japan). Sodium 2-naphthalenesulfonate was purchased from TCI (Tokyo, Japan). Sodium salicylate was purchased from Riedel-de Haën (Seelze, Germany). Terephthalic acid was purchased from Sigma. Phosphoric acid, sodium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Merck (Darmstadt, Germany). Sodium hydroxide was purchased from Fluka (Buchs, Switzerland). Methanol (MeOH) was purchased from Mallinckrodt (Paris, KY, USA). All the reagents and solvents used were of analytical and chromatographic grade.

2.2. Stock and standard preparations

UDCA stock solution (1000 μg/ml) was prepared by dissolving 10 mg UDCA in 10 ml MeOH. Stock solution of glycocholic acid, the internal standard, was prepared by the same way. Adequate amounts of UDCA and glycocholic acid stock solutions were mixed at different ratios to prepare standard solutions for respective experiments.

2.3. Sample preparation

Commercial tablets containing 100 mg of UDCA per tablet manufactured from two different pharmaceutical companies were obtained from the market. The tablets were ground to fine powders. A certain amount of powder from each sample was weighed and added to a volumetric flask. MeOH was added to make the volume. After sonication, the mixtures were filtered through 0.45 μm filters. The solutions contained

UDCA 1000 μg/ml. Each of these solutions was mixed with an appropriate volume of the glycocholic acid stock solution described above in a volumetric flask. MeOH was added to make the volume. Final sample solutions contained UDCA 50 μg/ml and glycocholic acid 60 μg/ml.

2.4. Apparatus and conditions

MEKC separations were carried out on a CE system consisting of a Lauer Lab's (Emmen, The Netherlands) Prince programmable injector and a 30 kV high voltage supply, connected to a Dynamax (Rainin, Emeryville, CA, USA) UV–C absorbance detector. A fused-silica capillary (72 cm × 50 μm i.d., 60 cm effective length) from Polymicro Technologies (Phoenix, AZ, USA) was used. Electropherograms were recorded using an EZChrom (Scientific Software, San Ramon, CA, USA) chromatographic data system.

The new fused-silica capillary was conditioned by flushing consecutively with 1.0 N sodium hydroxide for 10 min, 0.2 N sodium hydroxide for 10 min, deionized water for 5 min, and the running buffer for 10 min. In the beginning of each day, the capillary was washed by 0.2 N sodium hydroxide for 5 min and then the running buffer for 5 min.

The operating conditions were as following: voltage, 25 kV; injection time, 6.0 s (hydrodynamic, 50 mbar, injection volume about 6.0 nl); detection wavelength, 250 nm; temperature, ambient (23 ± 1 °C).

The BGE solution was composed of 5 mM sodium *p*-hydroxy benzoic acid, pH 8.0. The pH of BGE solution was adjusted to 8.0 with 0.2 N sodium hydroxide. All the solutions were filtered through 0.45-μm filters (Millipore, Bedford, MA, USA) prior to use.

3. Results and discussion

3.1. Method development

3.1.1. Selection of visualization agent

A suitable visualization agent (background-providing chromophore) is essential for obtaining a sensitive detection and an accurate quantification. The minimal detectable concentration, or limit of detection (LOD) is determined by concentration of the visualization agent (C_m), transfer ratio (TR) and dynamic reserve (DR):

$$\mathrm{LOD} = C_m/(\mathrm{TR} \cdot \mathrm{DR}) \quad (1)$$

DR is the ratio of the intensity of the background signal to the noise. It is defined as:

$$\mathrm{DR} = (\varepsilon L C_m)/\mathrm{AN} \quad (2)$$

where ε is the molar absorptivity; L is light path and AN is the absorbance noise.

Eq. (2) describes that DR is determined by the quality of the detector and the molar absorptivity of the visualization agent. Visualization agent with higher molar absorptivity results in higher DR value when using the same detector [16]. TR is defined as the number of molecules of visualization agent displaced by one molecule of analyte ion and its value depends on their mobilities. One to one displacement (TR = 1) occurs when their mobilities are the same. Minimal peak broadening and largest plate number are obtained when TR = 1 [17].

By substituting the dynamic reserve from Eq. (2) for DR in Eq. (1), we obtain:

$$\mathrm{LOD} = \mathrm{AN}/(\mathrm{TR} \cdot \varepsilon L) \quad (3)$$

It can be seen that the minimum detectable concentration (LOD) is proportional to the absorbance (or background) noise; a larger background noise will lead to a dramatic decrease in sensitivity and in the quantitative reliability [18]. It is generally observed that the magnitude of the non-instrumental noise depends strongly on the CE conditions, including the BGE composition [19].

In theory, a suitable visualization agent should have a mobility that matches the analyte ion. Its molar absorptivity should be high and its absorption wavelength should not overlap with that of the analyte. BGE should be made as simple as possible. The simpler the BGE is, the fewer system zones and disturbances can be expected [17,19]. It is a common practice in indirect photometric detection that the visualization agent makes up the principal component of the BGE. Better

sensitivity can be obtained with a BGE (also acting as visualization agent) having same charge as that of the analyte ion [20].

Literature reported that benzoic acid ($\varepsilon_{max} = 10\,580$ at $\lambda_{max} = 220$ nm), salicylic acid ($\varepsilon_{max} = 14\,650$ at $\lambda_{max} = 214$ nm), *p*-hydroxy benzoic acid ($\varepsilon_{max} = 13\,490$ at $\lambda_{max} = 250$ nm), phthalic acid ($\varepsilon_{max} = 40\,000$ at $\lambda_{max} = 205$ nm), terephthalic acid ($\varepsilon_{max} = 15\,540$ at $\lambda_{max} = 240$ nm), and naphthalene 2-sulfonic acid ($\varepsilon_{max} = 11\,730$ at $\lambda_{max} = 220$ nm) are often used as visualization agents in indirect UV detections. They were selected as potential candidates in this study. Mobilities of these compounds determined with the following conditions: 50 mM KH_2PO_4, pH 8.0, 25 kV and dimethyl sulfoxide as EOF marker, are listed in Table 1 (accompanied with their pK_a). For the convenience sake of comparison, relative mobilities of these potential visualization agents with respect to UDCA are shown in Fig. 2. Phthalic acid and terephthalic acid were not listed because they had far greater mobilities (due to carrying two negative charges at pH 8.0) than could be detected with positive electrode polarity. Napthalene 2-sulfonic acid had mobility closest to UDCA; however, it showed a high background noise. Molar aborptivities of benzoic acid, salicylic acid and *p*-hydroxy benzoic acid were similar but the mobility of *p*-hydroxy benzoic acid matched that of UDCA the best. In fact, when these three compounds were tested as visualization agent, *p*-hydroxy benzoic acid gave highest peak height response compared with the others. *p*-Hydroxy benzoic acid was, therefore, chosen as visualization agent, which was also acting as background electrolyte BEG, in this study.

Table 1
Electrophoretic mobilities and pK_a values of the potential visualization agents, UDCA and glycocholic acid

	Mobility[a] (10^{-2} cm^2/V per s)	pK_a
Benzoic acid	3.06	4.2
Salicylic acid	3.17	2.9
p-OH benzoic acid	2.82	4.5, 9.3
Phthalic acid	–[b]	2.9, 5.5
Terephthalic acid	–[b]	3.5, 4.8
Naphthalene 2-sulfonic acid	2.78	Strong acid
UDCA	1.45	6.4
Glycocholic acid	1.39	4.4

[a] Determined by CZE in 50 mM phosphate buffer, pH 8.0.
[b] Undetectable with positive polarity condition.

3.1.2. Effect of pH

The pH of the BGE solution is an important parameter which influences the sensitivity in indirect UV detection. The pK_a value of UDCA is 6.4. For such weak electrolyte to be analyzed by indirect detection, the pH chosen must be high enough to guarantee a substantial amount of analyte in the ionized form. In this way TR is increased and will be less dependent upon the pH [21]. Moreover, resolution between analyte and internal standard (for quantitative use) is also affected by the pH. It optimum value must be sought.

A pH range of 7.0–11.0 was tested for the BGE (*p*-hydroxy benzoic acid) solution at its optimum concentration (5 mM, see below). Peak height response and resolution between UDCA and glycocholic acid (internal standard) were monitored. Results are shown in Fig. 3. At pH 8.0 both peak height response and resolution were at their maximum. Beyond pH 8.0 peak height declined rapidly. This could be due to the strong influence of the competitive migration of the OH^- ions [22].

3.1.3. Concentration of visualization agent

To enhance the sensitivity of indirect detection, it is suggested the concentration of BGE (also acting as visualization agent) be as low as possible so that the background noise is decreased [21]. However, decreasing the concentration of BGE decreases the linear dynamic range, also the peak shape becoming asymmetric and broad [23]. Concentrations of 2–10 mM of BGE are typically used. This concentration should be at least 100-times that of the analyte ions. *p*-Hydroxy benzoic acid concentration between 1 and 15 mM at pH 8.0 was, therefore, investigated. The result is shown in Fig. 4. Highest Peak height response was found at 5 mM of *p*-hydroxy benzoic acid, where the resolution between UDCA and glycocholic acid was also at its maximum.

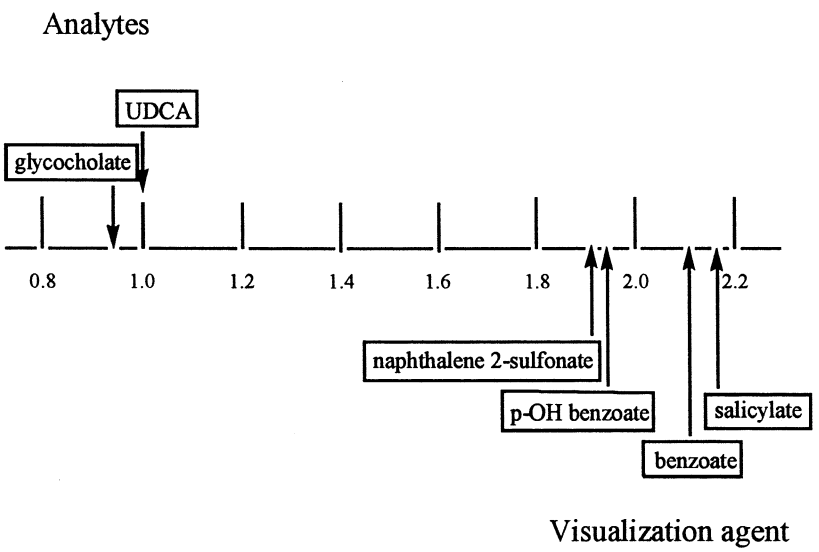

Fig. 2. Relative electrophoretic mobilities (with respect to UDCA) for four potential visualization agents and glycocholic acid.

3.1.4. Selection of internal standard

In the beginning of this experiment, many amino acids were tested as internal standard. However, a suitable amino acid could not be found, probably due to their large difference in structures from UDCA. A number of bile acids, including chenodeoxycholic acid, glycochenodeoxycholic acid, glycocholic acid, glycodeoxycholic acid, hyodeoxycholic acid, lithocholic acid, taurochenodeoxycholic acid and taurodeoxycholic acid, which all have similar structures to UDCA, were tested afterwards. It was found that glycocholic acid was migrating just ahead of UDCA but was baseline separated from the latter (Fig. 5). Its peak shape and response factor were also similar to UDCA.

In summary, an optimum condition for the analysis of UDCA has been developed and the parameters are listed as follows: BGE, 5 mM *p*-hydroxy benzoic acid, pH 8.0; voltage, 25 kV; temperature, ambient (23 ± 1 °C); detection wavelength, 250 nm; fused-silica capillary, 50 μm i.d., 350 μm o.d., total length of 70 cm, detection length of 60 cm; injection, 50 mbar, 6.0 s. With this condition, UDCA and its internal standard glycocholic acid were baseline separated within 5.5 min.

3.2. Method validation

3.2.1. Precision

Run-to-run repeatability ($n = 10$) and day-to-day reproducibility ($n = 3$) of migration time, peak height, and peak area ratios were tested. Glycocholic acid of 60 μg/ml was added to the standard solution of UDCA as internal standard. Both repeatability and reproducibility in terms of migration time ratio were within 0.1% relative standard deviation (R.S.D.). Precision of peak height and peak area ratios was tested at 20 and 100 μg/ml of UDCA (the lower and higher levels of the linearity, see below). The corrected peak area ratios were obtained by correcting peak areas with respect to migration times. Repeatability and reproducibility of peak area ratios at 20 μg/ml level were 3.84 and 4.02% R.S.D., respectively; those of peak height ratios were 2.99 and 3.56% R.S.D., respectively. Repeatability and reproducibility of corrected peak area ratios did not change better after time correction of the peak areas (3.85 and 4.01% R.S.D., respectively). Precessions at 100 μg/ml level were about the same as those at 20 μg/ml level. R.S.D.s of peak height ratios were the smallest among the three categories. Peak height ratio was, therefore, adopted for use in the following validation terms.

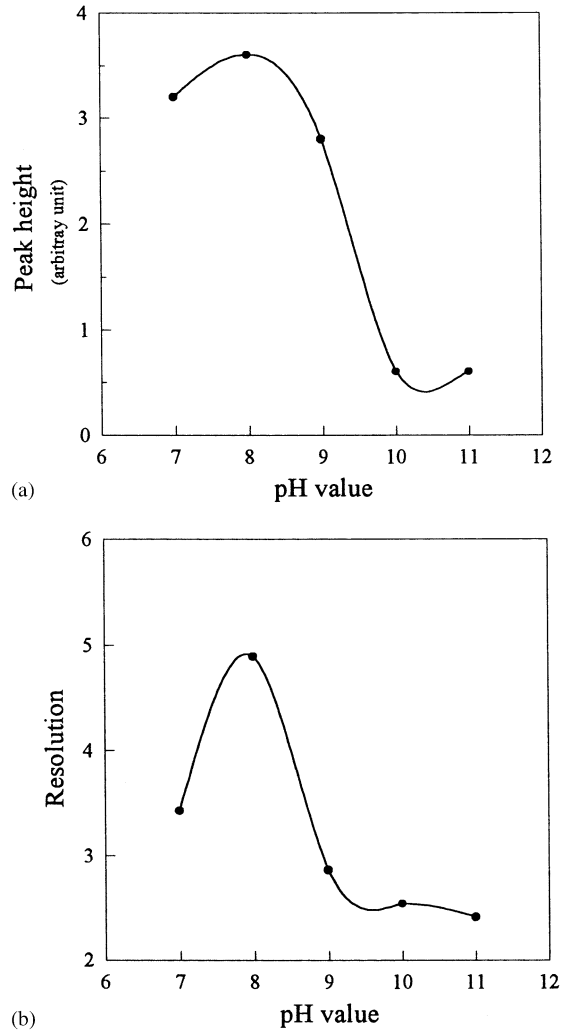

Fig. 3. Effect of pH of BGE solution on (a) peak height of UDCA and (b) resolution between UDCA and glycocholic acid (internal standard).

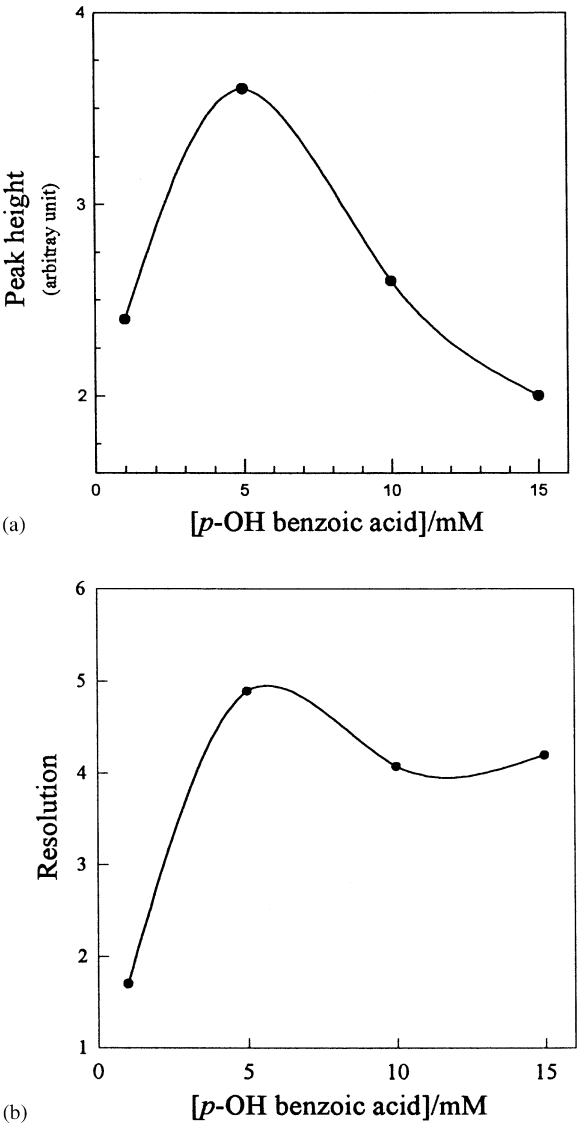

Fig. 4. Effect of concentration of BGE on (a) peak height of UDCA and (b) resolution between UDCA and glycocholic acid (internal standard).

3.2.2. Linearity and accuracy

Linearity of the method was tested by preparing five standard solutions from 20 to 100 μg/ml of UDCA and measuring their responses (peak heights) relative to internal standard (60 μg/ml of glycocholic acid). Each solution was tested for three-times. Regression line was $y = 0.01475x + 0.3549$ with correlation coefficient (r) being equal to 0.995.

Accuracy of the method was assessed with recovery. Standard solutions of 30 μg/ml of UDCA were added to the solutions prepared from the commercial tablets with a target concentration of 50 μg/ml UDCA. Two commercial brands of tablets were tested. The concentrations found were calculated against the concentrations

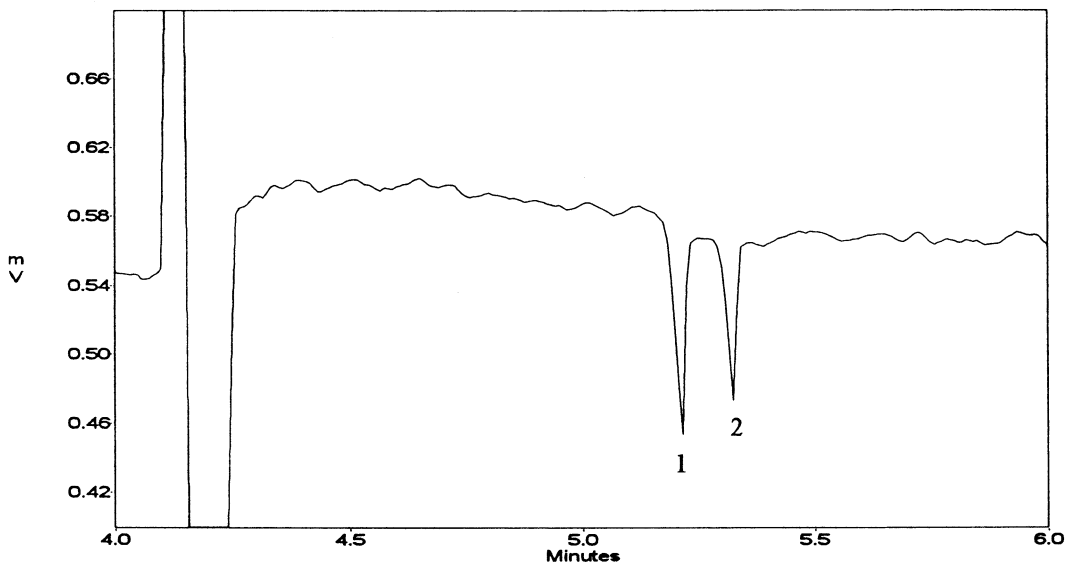

Fig. 5. Electropherogram of UDCA (peak 2), 50 μg/ml in MeOH, with glycocholic acid (peak 1) as internal standard, 60 μg/ml in MeOH. Condition: 5 mM sodium *p*-hydroxy benzoic acid, pH 8.0; fused silica capillary, 50 μm I.D., total length, 72 cm, detection length, 60 cm; injection, 50 mbar, 6 s; voltage, 25 kV; temperature, ambient; detect ion, 250 nm.

added. The recoveries were 101.9 ± 9.87 and 99.6 ± 9.60% ($n = 3$) for the two tablets, respectively.

3.2.3. Limit of detection (LOD) and limit of quantification (LOQ)

LOD of the method was defined as the concentration of UDCA being detected at S/N = 3. It was found to be 3 μg/ml.

LOQ was tested with a concentration of UDCA at S/N = 10. Its value was set to be 10 μg/ml. Repeatability and reproducibility of peak height ratio (with respect to glycocholic acid, 60 μg/ml) were 4.38 and 5.01% R.S.D., respectively.

3.3. Assay of tablets

The contents of UDCA in two commercial tablets manufactured from two different pharmaceutical companies were determined by the developed method. Their label claims of UDCA are 100 mg/tablet. Electropherogram obtained from tablets of one company is shown in Fig. 6 (the other being similar). The calculated UDCA contents were 99.50 and 100.54% of the label amount of the two tablet formulations, respectively.

4. Conclusion

A simple, fast, and sensitive (compared with direct detection) method to quantitate UDCA in pharmaceutical preparations was developed in this study. Noise was the major cause of imprecision in this experiment. Better precision could be obtained by using a more efficient air-cooling or liquid-coolant system to reduce the effect of Joule heat.

Because of its merits such as high efficiency, high speed and extraordinary low mobile phase consumption, CE has become a popular analytical tool. Comparing to UV direct detection, indirect detection of CE provides better sensitivity in analyzing UDCA (results not shown). The method developed in this study could constitute an alternative to USP prescribed method for the quality control of UDCA bulk substance and pharmaceutical preparations.

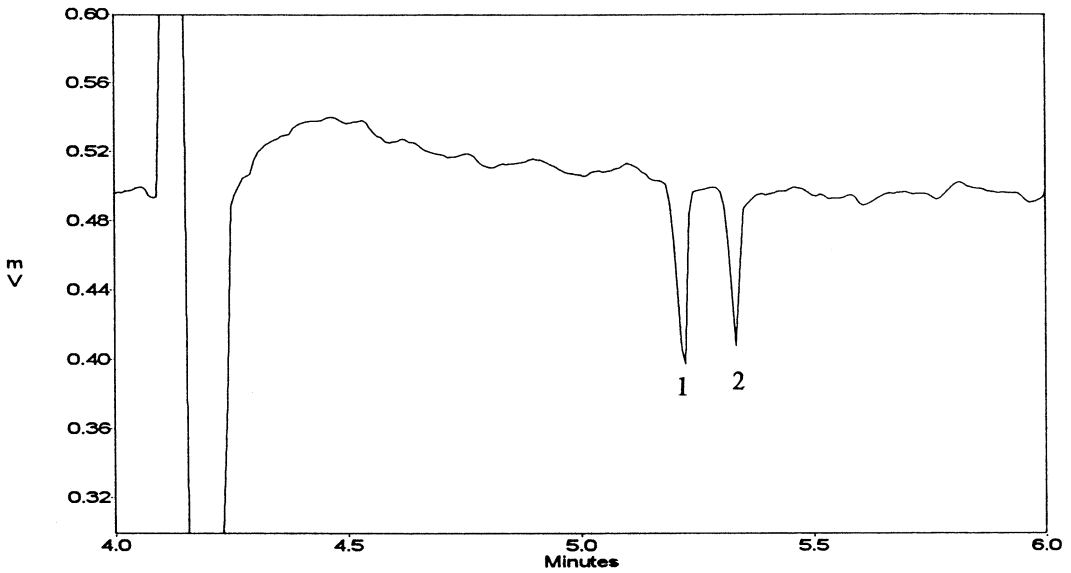

Fig. 6. Electropherogram of UDCA extract from tablets of a pharmaceutical company. UDCA (peak 2): 50 μg/ml, glycocholic acid (internal standard, peak 1): 60 μg/ml. Conditions are as described in Fig. 5.

Acknowledgements

Financial support from the National Science Council, Republic of China (NSC 89-2320-B-002-224), is gratefully acknowledged.

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