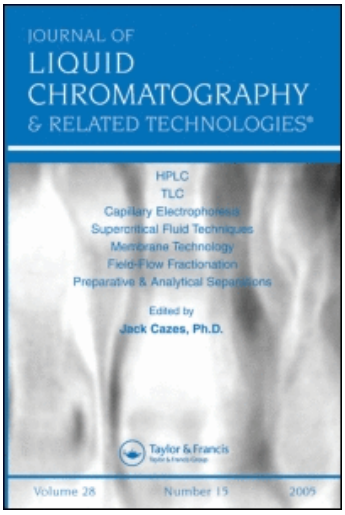

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Roubies, N. and Botsoglou, N. A.(2008) 'Liquid Chromatographic Determination of Individual Taurine-Conjugated Bile Acids in Dog Serum', Journal of Liquid Chromatography & Related Technologies, 31: 10, 1511 — 1522
To link to this Article: DOI: 10.1080/10826070802039572
URL: http://dx.doi.org/10.1080/10826070802039572

Journal of Liquid Chromatography & Related Technologies®, 31: 1511–1522, 2008

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070802039572

Liquid Chromatographic Determination of Individual Taurine-Conjugated Bile Acids in Dog Serum

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Abstract: A liquid chromatographic method is described for the determination of taurine-conjugated cholic, deoxycholic, chenodeoxycholic, and lithocholic acids in dog serum. Samples are mixed with aqueous formic acid, cleaned-up by solid phase extraction, and analyzed by liquid chromatography with detection at 195 nm. Using a mobile phase of acetonitrile/trifluoroacetic acid solution, the analytes are eluted from the column well resolved from serum interferences. The method shows analytical characteristics well within acceptable limits. Recoveries were in the range 96.2–100.2%, precision, expressed as percent coefficient of variation, in the range 1.8–9.5%, and limits of detection in the range 0.04–0.1 μg/mL.

Keywords: Bile acids, Dog, Serum, Taurine-conjugated, Liquid chromatography

INTRODUCTION

Bile acids are cholesterol derivatives that are important for the digestion and absorption of lipophilic nutrients in the gut.[1] The liver synthesizes two primary bile acids, cholic acid and chenodeoxycholic acid, which are further converted by intestinal bacteria to the secondary bile acids, deoxycholic acid and lithocholic acid. These amphipathic molecules are stored in the bile, secreted into the small intestine, efficiently reabsorbed from the ileum-proximal colon and returned to the liver via the portal vein.[2,3] Bile

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acids occur in biological fluids primarily as glycine and taurine conjugates, but there are major species differences in the extent of the specificity of bile acid conjugation. In humans, the glycine/taurine ratio is normally 3:1[4] while, in animals like the dog, bile acids are mainly conjugated with taurine.[5]

It has been reported that hepatobiliary and intestinal dysfunctions are marked by variation in the concentrations and relative proportions of the major bile acids.[3,6,7] This increasing interest in serum bile acid profile, as indicator of metabolic disorders and diseases, has led to development of many analytical methods for their determination, such as gas chromatography,[8,9] liquid chromatography,[10–13] and capillary electrophoresis.[14]

Liquid chromatography (LC) has become the most widely used analytical technique for the analysis of bile acids in biological fluids, as it offers the potential to directly analyze these ionic, highly polar, and hydrophilic compounds without prior deconjugation and subsequent derivatization.[3] However, in combination with UV detection at around 200 nm, it suffers from limited sensitivity and serious matrix interferences due to lack of a strong chromophore in the bile acid molecule.

Several LC derivatization procedures for overcoming the poor UV detection by modifying the bile acid structure have been described, but they are rather complicated and time-consuming.[13,15] Electrochemical detection has been also applied in the analysis of bile acids but its applicability in biological samples has not been yet examined.[16,17] To improve the detectability, some off- or on-line sample purification procedures have been recommended for removing interferences but they are rather complicated and inconvenient.[10,11,13] Recently, a few reports on the sensitivity and selectivity offered by mass spectrometry in the LC analysis of bile acids in biological samples have been described, but the high instrumentation cost and the need for qualified analysts remain limiting steps in this kind of analysis.[18–21] Thus, liquid chromatography with UV detection remains the method of choice for easy and fast routine determination of conjugated bile acids in serum samples.

In the present study, a simple, rapid, and precise LC method for monitoring taurine-conjugated cholic, deoxycholic, chenodeoxycholic, and lithocholic acids in dog serum is described. Samples are mixed with formic acid, cleaned-up by solid phase extraction, and analyzed by liquid chromatography, with detection at 195 nm. Using a mobile phase of acetonitrile/trifluoroacetic acid solution, the analytes are eluted from the column, well resolved from serum interferences.

EXPERIMENTAL

Instrumentation

Liquid chromatography was carried out with a Shimadzu system (Shimadzu Corp. Kyoto, Japan) consisting of a model LC-10AD piston pump, a model

FCV-10AL low pressure gradient controller unit, a model SPD-10AV spectrophotometer set at a wavelength of 195 nm, and a model CTO-6AS oven set at 36°C. An on-line vacuum degasser was also used (Manifold kit, Jour Research, Swenden), whereas injections were made using a Rheodyne, Model 7725, injection valve (Cotati, CA) equipped with a 100-μL sample loop. The chromatographic system was controlled by Class-LC (version 4.3, Shimadzu) software.

A Model G-560E vortex mixer (Scientific Industries, Bohemia, NY), and a Centra-MP4 centrifuge (IEC, Needman Heights, MA), were used for sample treatment. A Model Aquatron A400D compact Ultrapure water system (Bibby Sterilin Ltd, UK) was also used for purification of tap water.

Reagents

LC grade methanol was from Millinckrodt Baker B.V. (Deventer, Holland), while HPLC grade acetronitrile from Carlo Erba Reagent SpA (Rodano, Italy). Analytical grade trifluoroacetic acid and formic acid were from Sigma-Aldrich Chemical (Steinheim, Germany).

Standard taurocholate (TC), taurodeoxycholate (TDC), taurochenodeoxycholate (TCDC) and taurolithocholate (TLC) sodium salts were from Sigma-Aldrich chemical (Steinheim, Germany).

Preparation of Standard Solutions

Individual standard stock solutions of bile acids were prepared in 25 mL volumetric flasks by dissolving ca 25 mg of each reference standard in 7 mL of methanol and diluting to volume with acetonitrile. Intermediate solutions were prepared by appropriately diluting aliquots from stock solutions with acetonitrile. A mixed standard intermediate solution containing all 4 analytes was also prepared by combining appropriate aliquots from each of the stock solutions in a 10-mL volumetric flask and diluting to volume with acetonitrile. Mixed working solutions in the range 2 to 16 μg/mL were prepared by evaporating under N_2 at 40°C aliquots from the mixed intermediate solution and diluting successively with mobile phase. All solutions were kept in a freezer when not in use. Both stock and mixed standard intermediate solutions were stable for at least 3 months in a freezer at −25°C. Mixed standard working solutions were prepared fresh weekly.

Sample Preparation

A 1 mL aliquot of serum sample was transferred into a 10 mL centrifuge tube, and 1 mL of 1 N formic acid was added. The mixture was

vortex-mixed, allowed to stand for 10 min at 37°C, and loaded onto a solid phase extraction (SPE) cartridge containing 100 mg of end-capped ODS (Supelco, Bellefonte, PA, USA) that had been preconditioned by rinsing with 2 mL methanol followed by 2 mL H_2O. After the sample was allowed to percolate through the cartridge, sample washing was carried out by passing through the cartridge 3 mL H_2O. To facilitate the extraction of large numbers of samples, the SPE cartridges were used with the VAC-ELUT (Varian Inc., CA, USA) vacuum apparatus, which would allow 10 cartridges to be run simultaneously. Following cartridge centrifugation at 2,000 g for 5 min, the analytes were eluted with 1 mL methanol followed by 2 mL acetonitrile. The collected effluent was evaporated to dryness under nitrogen and reconstituted with 1 mL mobile phase to be injected into the chromatograph.

Liquid Chromatographic Analysis

Analysis was performed on a Nucleosil 100-5 C_{18}, 250 × 4.6 mm, column protected with a Nucleosil 100-5 C_{18}, 8 × 4 mm, guard column (Nucleosil, Macherey-Nagel, Duren, Germany). The mobile phase consisted of acetonitrile and 0.002 M trifluoroacetic acid, and was passed through 0.2 μm filter before use, degassed using helium, and delivered into the system at a flow rate of 1 mL/min. Elution of the analytes was carried out by programming the acetonitrile-trifluoroacetic acid mobile phase composition (v/v) as follows: 6 min isocratic run at 27:73; 12 min linear gradient run to 38:62; 10 min linear gradient run to 40:60; 10 min purge at 90:10; 10 min equilibration at 27:73. After each day's work, the column was flushed with water until free from acidity and maintained filled with acetonitrile.

Determination

Calibration curves were generated by running mixed standard working solutions, plotting the recorded peak heights y (mV) versus the amounts x (μg) of analytes injected, and computing the slope (b), intercept (a), and least squares fit of the calibration curves according to the equation $y = a + bx$. Calibration curve slopes and intercept data were used to determine the amounts of the analytes in injected extracts. The concentration (μg/mL) of the analytes in serum samples was determined by multiplying the amount of the analytes by the appropriate dilution factors. Serum samples found to contain the analytes at concentrations higher than those of the standard curves were resubmitted to chromatographic analysis after dilution with mobile phase.

RESULTS AND DISCUSSION

Extraction/Cleanup

Since bile acids possess weak chromophores with absorption at around 200 nm, LC with UV detection suffers from limited sensitivity and requires selection of short wavelengths, which result in increased interference from the biological matrix constituents. Even trace amounts of endogenous components with much stronger absorptivities than bile acids would generate large peaks, thus limiting the accuracy of UV detection. In addition, bile acids are present in biological fluids such as serum, at very low concentrations, whereas most are tightly bound to proteins. These characteristics, coupled with the complexity of the serum matrix, necessitate an efficient extraction step in which the bile acids would selectively be isolated from serum components.

The major problem in extracting bile acids and their conjugates from serum arises from their strong binding to serum albumin.[22] The binding of bile acids to albumin can be decreased by increasing pH and temperature.[23] At pH >12, bile acids show minimal binding to protein, whereas heating of the diluted serum appeared to give a higher recovery of the bile acids.[22] For these reasons, common bile acids are usually isolated from serum by dilution with 0.1 M NaOH and incubation at 64°C for 30 min prior to SPE cleanup. However, the use of the alkaline medium for the detachment of bile acids from serum albumin may cause loss of the cleanup efficiency due to protein precipitation and rapid degradation of the SPE packing material.[3] As the chromatographic efficiency would be affected by the alkaline medium, the use of organic solvents with known de-binding ability, such as acetonitrile[20] or methanol,[14] instead of NaOH has been also proposed.

Our initial experiments to extract the taurine-conjugated bile acids from serum of dogs showed that extraction with acetonitrile failed to achieve recovery of the analytes, whereas methanol provided satisfactory recovery of the analytes but high matrix interferences were present. In an attempt to overcome these problems, and considering that, when formic acid is added to serum, analytes that are bound to proteins can be fully released from the sample matrix,[24–27] formic acid was examined instead. Analysis results showed that addition of formic acid in the extraction process prior to SPE cleanup could provide sufficient recovery of the analytes from the serum matrix, whereas interferences were not present in the resulting extracts; this is evident from the chromatograms presented (Figure 1).

Liquid Chromatography

A wide range of eluents has been proposed for the chromatographic analysis of taurine-conjugated bile acids. A literature survey shows that satisfactory

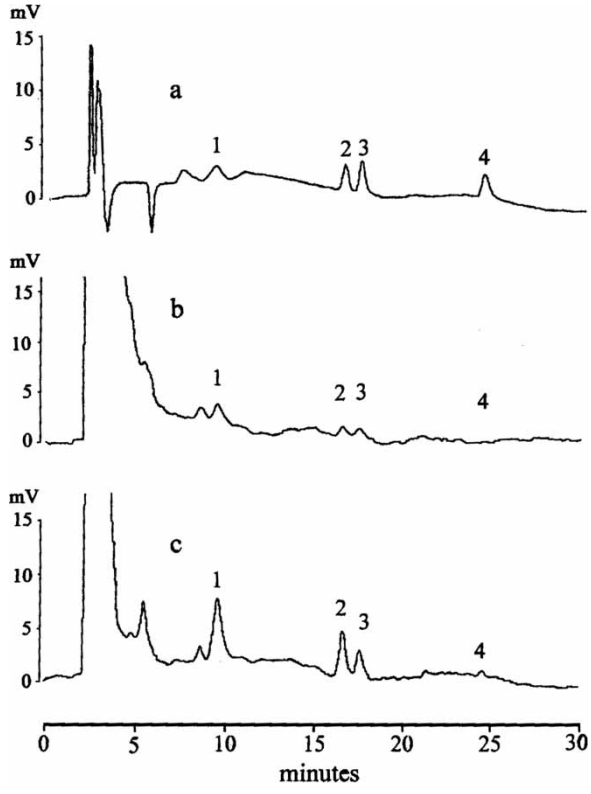

Figure 1. Typical chromatograms of a mixed standard working solution (a), a serum sample from a physiological dog (b), and a serum sample from a dog with hepatic disease (c). Peak identification: taurocholate (1), taurodeoxycholate (2), taurochenodeoxycholate (3) and taurolithocholate (4).

resolution can be obtained with a C_{18} columns using mobile phases containing methanol or acetonitrile as organic modifiers, in the pH range 3–5.[12,28–30] Although methanol exhibits higher solvating power for these hydrophobic bile acids,[12,31] acetonitrile was considered a more appropriate eluent for these compounds because its UV cut-off is lower than that of methanol.[30–33]

Apart from the type of the organic modifier, the chromatographic behaviour of bile acids can be markedly influenced by the pH of the mobile phase.[12,13] Thus, the retention of the unconjugated and glycine-conjugated bile acids, which have pKa values at 4.5 and 6.0, respectively, increases with decreasing pH owing to ionic suppression, which enhances the lipophilic character and the interaction of these compounds with the hydrophobic C_{18} sorbent.[12,30]

On the other hand, the retention of the taurine-conjugated bile acids, which have a pKa values at 1.5 is not significantly influenced by the mobile phase pH.[12] This leveling of the retention behavior of the taurine-conjugated bile acids indicates that at a mobile phase pH where complete ionization of the analytes occurs, retention is controlled mainly by the steroidal nucleus of their molecule, particularly with relation to the hydroxy-substituents in the steroid nucleus.[34,35] Thus, elution is in the order taurine-conjugates < glycine-conjugates < free bile acids, although reversal of this retention sequence occurs with alkaline mobile phases.[28,31] Within each series of bile acids, retention increases with decreasing numbers of hydroxyl groups on the steroid backbone, but modification of this elution order can be caused by the presence of hydroxyl groups which interfere with the interaction of the bile acid's hydrophobic β-surface with the C_{18} packing material.[34–36] In agreement with this, at the mobile phase pH of 3 selected in our experiments, taurocholic acid was eluted at 9.8 min, taurodeoxycholic acid at 16.9 min, taurochenodeoxycholic acid at 17.8 min, and taurolithocholic acid at 24.7 min.

Our preliminary experiments showed that the use of C_{18} columns with acetonitrile-water mobile phases acidified with phosphate buffers was usually accompanied by a rapid deterioration of column performance. This indicated serum build-up on the reversed-phase column, although a 10-min purge with an acetonitrile-water mixture (90:10, v/v) was always carried out at the end of each run. To overcome this problem, various somewhat different cleaning processes were examined, as solvents such as acetonitrile cannot dissolve peptides and proteins and are ineffective for cleaning reversed-phase columns. It was found that trifluoroacetic acid, a cleaning solvent for clogged columns, could efficiently regenerate the contaminated columns after repeated column flushing. Based on this finding, we examined whether the phosphate buffer used for mobile phase acidification could be replaced by trifluoroacetic acid. It was found that total replacement of the phosphate buffer by trifluoroacetic acid could eliminate the frequent column deterioration observed in the past, since no changes in the retention times were noted with continual column use thereafter.

Calibration Curves

Regression analysis of the data obtained by running a series of mixed working solutions showed the response for each analyte to be linear in the range examined ($y = -0.046 + 19.64x$, $R^2 = 0.989$ for taurocholate; $y = 0.035 + 9.40\ x$, $R^2 = 0.996$ for taurodeoxycholate; $y = 0.119 + 7.00\ x$, $R^2 = 0.997$ for taurochenodeoxycholate; $y = 0.041 + 7.75\ x$, $R^2 = 0.997$ for taurolithocholate, where y represents peak heights (mV) and x the quantity (micrograms) of the compound injected.

Accuracy and Precision

Interferences in extracted samples might cause inaccurate determinations, so a standard addition method of analysis was evaluated. In the study, 18 of 24 samples from pooled serum of dogs were spiked at 3 fortification levels with a mixed standard intermediate solution containing all 4 analytes, and all samples were submitted to the developed assay. Least-squares and regression analysis of the data (Table 1) based solely on the 3-level spiking showed that the relationship between "added" and "found" was adequately described by linear regressions. The intercepts of the regression lines (1.77 ± 0.16, 0.18 ± 0.11, 0.11 ± 0.08, and -0.23 ± 0.07 for taurocholate, taurodeoxycholate, taurochenodeoxycholate and taurolithocholate, respectively), which actually represent the values in μg/mL predicted for the unspiked samples, were found by t-tests not significantly different from the

Table 1. Accuracy and precision data for the determination of taurine-conjugated cholic acids in serum

Analytes	Concn added (μg/mL)	Mean concn found[a] (μg/mL)	Coefficient of variation (%)	Recovery (%)
TC	0	1.83 ± 0.121	6.6	—
	2.00	3.63 ± 0.163	4.6	91.7 ± 8.2
	4.00	5.58 ± 0.306	5.8	95.4 ± 7.0
	8.00	9.5 ± 0.358	3.8	95.8 ± 5.7
				Overall
				96.2 ± 3.4
TDC	0	0.18 ± 0.017	9.4	—
	2.00	2.13 ± 0.197	9.5	96.7 ± 9.8
	4.00	4.08 ± 0.160	3.9	97.1 ± 4.0
	8.00	8.00 ± 0.237	3.0	98.5 ± 2.0
				Overall
				97.7 ± 2.4
TCDC	0	0.13 ± 0.012	9.2	—
	2.00	2.03 ± 0.121	6.0	95.2 ± 6.1
	4.00	4.05 ± 0.138	3.4	98.0 ± 3.4
	8.00	7.95 ± 0.152	1.9	97.8 ± 1.9
				Overall
				98.0 ± 1.6
TLC	0	0	—	—
	2.00	1.95 ± 0.105	5.4	100.0 ± 7.1
	4.00	3.98 ± 0.117	3.0	98.3 ± 4.1
	8.00	8.00 ± 0.141	1.8	99.4 ± 2.3
				Overall
				100.2 ± 1.4

[a]Mean of 6 replicates ± SD.

arithmetic means (1.83 $\pm$ 0.12, 0.18 $\pm$ 0.11, 0.13 $\pm$ 0.05, and 0 for taurocholate, taurodeoxycholate, taurochenodeoxycholate, and taurolithocholate, respectively) of the unspiked samples. This finding indicated that interferences were not present in extracted samples.

The lack of interfering peaks permitted accuracy and precision evaluation using the data from both the spiked and the unspiked samples (Table 1). Least-squares and regression analysis of these data showed that linearity was quite acceptable ($R^2 = 0.993$, $R^2 = 0.997$, $R^2 = 0.998$, and $R^2 = 0.999$ for taurocholate, taurodeoxycholate, taurochenodeoxycholate, and taurolithocholate, respectively). Therefore, the slopes of the regression lines (0.962 $\pm$ 1.7, 0.977 $\pm$ 1.2, 0.980 $\pm$ 0.8 and 1.002 $\pm$ 0.7 for taurocholate, taurodeoxycholate, taurochenodeoxycholate, and taurolithocholate, respectively, could be used as estimates of the overall recoveries (96.2, 97.7, 98.0, and 100.2 for taurocholate, taurodeoxycholate, taurochenodeoxycholate, and taurolithocholate, respectively, of the method.

The precision of the method, expressed as percent coefficient of variation (Table 1), was also found quite acceptable, as it was not higher than 9.4% in all samples analyzed.

Limits of Detection

The power of the chromatographic system, coupled with the efficiency of the extraction/cleanup process, allowed reasonable limits of detection to be realized. The limits of detection for taurocholate, taurodeoxycholate, taurochenodeoxycholate, and taurolithocholate in dog serum, defined as the lowest levels that could yield signals clearly distinguished from the signals obtained by blank injections (3/1 ratio of peak height of the analyte to average peak-to-peak amplitude of the blank baseline), were estimated at 0.1 μg/mL, 0.05 μg/mL, 0.04 μg/mL, and 0.05 μg/mL, respectively.

Applicability of the Method

To test the applicability of the method with real samples, serum from 4 dogs with hepatic disease was submitted to analysis for taurine-conjugated bile acids. The concentrations found ranged from 11.1 to 17.8 μg/mL for taurocholate, 6.3 to 9.9 μg/mL for taurodeoxycholate, 2.2 to 4.8 μg/mL for taurochenodeoxycholate, and 1.3 to 2.8 μg/mL for taurolithocholate.

CONCLUSION

The results of the present study suggest that the developed method is a relatively simple, rapid, and inexpensive procedure that presents quite acceptable analytical characteristics with respect to recovery, precision, selectivity, and

sensitivity. Sample preparation and analysis time is minimal, whereas sophisticated and costly equipment is not needed. These advantages make the method valuable for routine clinical applications.

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Received December 12, 2007
Accepted January 16, 2008
Manuscript 6258