

Determination of intraluminal individual bile acids by HPLC with charged aerosol detection

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Abstract An isocratic HPLC charged aerosol detector (CAD) method was developed, validated, and applied for the determination of individual bile acids in human gastric and duodenal aspirates. The method requires a low volume of aspirates (50–100 μ l) and minimal sample pretreatment. A Hypersil BDS RP-C₁₈ column (250 \times 4.6 mm, 5 μ m particle size) was equilibrated with a mobile phase composed of methanol-[ammonium formate 20 mM, formic acid 0.5%, triethylamine 0.2% (pH 3)] 67:33 v/v. Its flow rate was 1 ml/min. The elution times for taurocholate, glycocholate, taurochenodeoxycholate, ursodeoxycholate, glycochenodeoxycholate, cholate, and glycodeoxycholate were approximately 9.9, 16.2, 18.2, 21.3, 31.6, 34.5, and 38.5 min, respectively. Calibration curves in the mobile phase were constructed in the concentration range of 0.5–500 μ M. Limits of detection and quantification were in the range of 0.07–0.60 μ M and 0.20–1.80 μ M, respectively. This method was applied first, in gastric aspirates collected in the fasted state, in which bile acid presence is minimal and, second, in duodenal aspirates collected in the fed state, in which a large number of potentially interfering compounds exists. Intra-day relative standard deviation in fasted gastric aspirates and in fed duodenal aspirates was less than 2.2% and 6.0%, respectively.—Vertzoni, M., H. Archontaki, and C. Reppas. Determination of intraluminal individual bile acids by HPLC with charged aerosol detection. *J. Lipid Res.* 2008. 49: 2690–2695.

Supplementary key words charged aerosol detector • gastric aspirates • duodenal aspirates • high-performance liquid chromatography

Intraluminal bile acids promote lipid absorption. They are essential for adequate absorption of fat-soluble vitamins and are likely to enhance the absorption of multivalent metal ions such as iron and calcium from the small intestine (1). In addition, bile acids may affect oral absorption of drugs administered in solid dosage forms with two mechanisms: first, by decreasing the intraluminal surface tension and, thus, facilitating the wetting of solid particles; and second, by acting as potent solubilizing

agents of lipophilic drug molecules in the small intestine. Lipophilic drug molecules have increased steadily in recent years (2), whereas the solubilizing capacity of bile acids varies with the identity of bile acid (3).

Over the last three decades, various methods have been proposed for the determination of intraluminal bile acid levels. As early as 1974, Fausa (4) enzymatically determined bile acids in the fed duodenum, after almost complete separation of individual bile acids with TLC. Many years later, a similar enzymatic determination of total 3 α -hydroxy bile acids in duodenal human aspirates was applied for the determination of intraluminal bile acid levels using a commercially available kit (5, 6). However, only concentrations higher than about 200 μ M could be quantified, and therefore, this method has limitations in the determination of bile acids in the fasted stomach, especially after administration of a glass of water, which is typically administered in oral drug absorption studies (6).

Determination of individual bile acid levels in aspirates collected from the upper gastrointestinal lumen has also been possible by applying liquid chromatography. Initially, HPLC coupled with refractive index detector was proposed, but its low sensitivity limited its application (7). HPLC coupled with ultraviolet (UV) detection was also proposed (8, 9), but the similar chemical structure of bile acids and their low UV light absorbance resulted in limited selectivity and sensitivity, respectively. Recently, it has been shown that by using evaporative light-scattering detection (ELSD), both selectivity and sensitivity are improved (10, 11). ELSD is advantageous particularly when a compound has low volatility and lacks a chromophore, as is the case with bile acids. However, compared with newer semi-universal detectors, such as the charged aerosol detector (CAD), ELSD has limitations in sensitivity, precision, and

Abbreviations: C, cholate; CAD, charged aerosol detector; ELSD, evaporative light-scattering detection; GC, glycocholate; GCD, glycochenodeoxycholate; GDC, glycodeoxycholate; LC-MS, liquid chromatography-mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; TC, taurocholate; TCDC, taurochenodeoxycholate; UDC, ursodeoxycholate; UV, ultraviolet.

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dynamic range (12, 13) that may create problems in cases in which intraluminal bile acid concentrations are low, e.g., in stomach and in colon.

In the CAD, the aerosol particles are charged with an ionized gas (typically nitrogen). After the removal of high-mobility particles (mainly excess N_2 ions), the aerosol particles are electrically measured. CAD has been demonstrated to provide a uniform response to nonvolatile ana-

lytes independently of their nature. CAD is claimed to be more sensitive than ELSD, has a broad and useful dynamic range (four orders of magnitude) and requires little or no human intervention (12, 13).

In this manuscript, the usefulness of CAD in the determination of bile acid levels in the upper gastrointestinal lumen was evaluated. Specifically, a simple and fast isocratic HPLC-CAD method was developed, validated, and applied

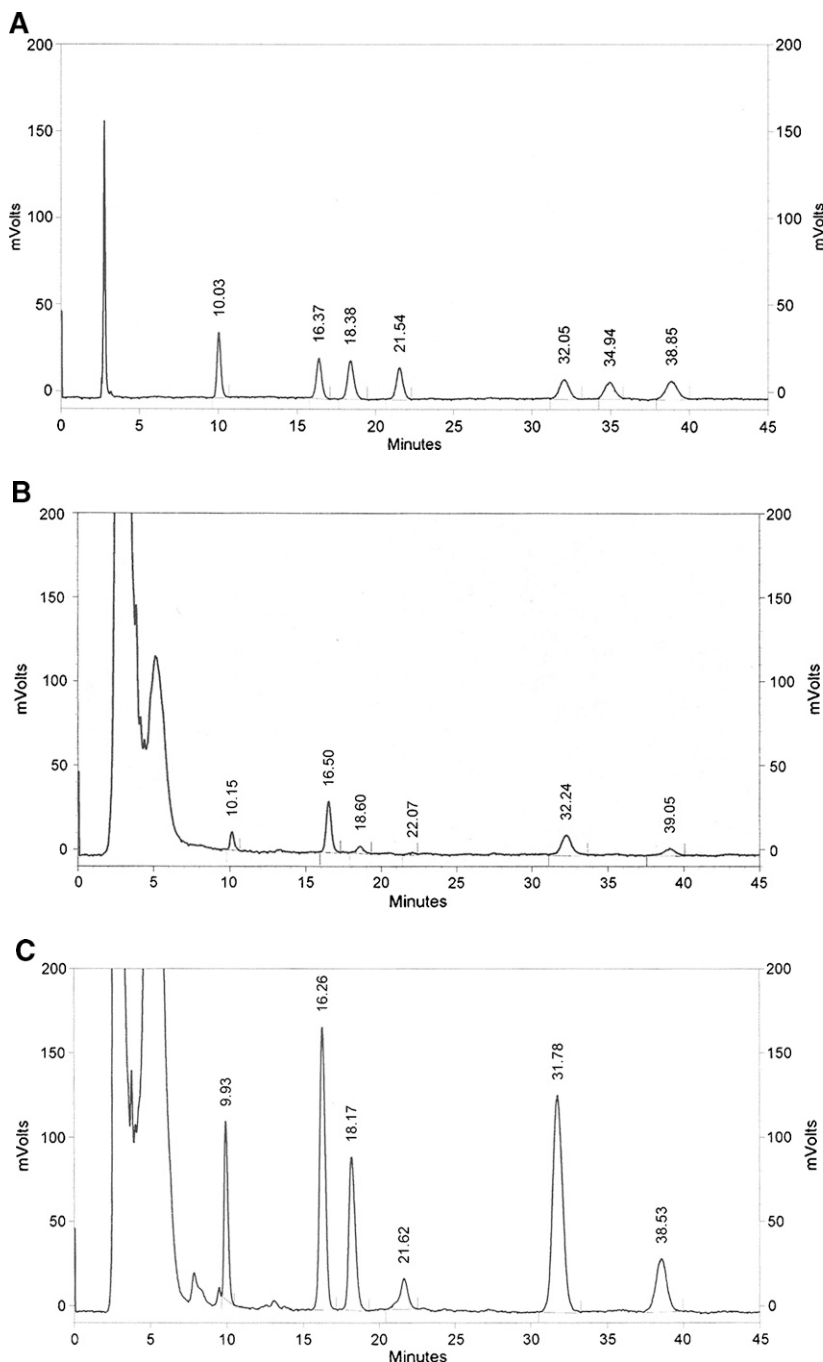


Fig. 1. A: A typical chromatogram of a standard solution of bile acids in mobile phase, in which concentration of each bile acid was 10 μ M. B: Chromatogram of HGF^{60}_{fasted} diluted with mobile phase (1:1; v/v). C: Chromatogram of HIF^{90}_{fed} previously treated as described in the text. From left to right, the numbers indicate the retention times of taurocholate, glycocholate, taurochenodeoxycholate, ursodeoxycholate, glychenodeoxycholate, cholate, and glycodeoxycholate. No cholate was detected in HGF^{60}_{fasted} and HIF^{90}_{fed} (peak was expected at ~ 34.9 min).

for the determination of individual bile acids under conditions in which minimal presence of intraluminal bile acids is expected, i.e., in gastric aspirates collected in the fasted state, and under conditions in which determination can be problematic, owing to the large number of potentially interfering compounds, i.e., in duodenal aspirates collected in the fed state.

MATERIALS AND METHODS

Instrumentation

The chromatographic system consisted of a Spectra System P1000 pump, a CAD (Corona CAD; ESA Inc., Chelmsford, MA), and an autosampler AS 3000. Nitrogen gas, regulated at 35 psi, was introduced into the detector, and the resultant gas flow rate was regulated automatically and monitored by CAD device. The only parameter that required user input was the response range, which was set to 100 pA full scale. The above system was controlled by a Spectra System Controller SN 4000 and a software package (Chromquest; Thermoquest, Inc., San Jose, CA). A Universal 32R centrifuge (Hettich, Tuttlingen, Germany) was utilized to centrifuge samples.

Chemicals and reagents

Acetonitrile of HPLC grade and methanol of HPLC and liquid chromatography-mass spectrometry (LC-MS) grade were purchased from E. Merck (Darmstadt, Germany). Sodium salts of taurocholate (TC), glycocholate (GC), taurochenodeoxycholate (TCDC), ursodeoxycholate (UDC), glycochenodeoxycholate (GCDC), cholate (C), and glycodeoxycholate (GDC) were purchased from Sigma Co. (St. Louis, MO). Water purified with Labconco water pro ps system (Kansas City, MO) was used in all procedures. All other chemicals were of analytical grade.

Human aspirates

Human gastric fluids were aspirated 60 min after the administration of 250 ml of water to fasted healthy adults (6). HGF_{fasted}^{60} was created by pooling aspirates (2 ml each) from 5 individuals. Human intestinal fluids were aspirated 90 min after the administration of 500 ml Ensure Plus® to fasted healthy adults (6). HIF_{fed}^{90} was created by pooling aspirates (1 ml each) from 14 individuals. All samples were kept at -70°C until used. The study was held in the Red Cross Hospital of Athens after receiving approval by the Scientific and the Executive Committee of the Hospital. The study followed the tenets of the Declarations of Helsinki promulgated in 1964. All volunteers had given informed consent prior to their participation in this study.

Chromatographic conditions

A reversed-phase Hypersil BDS- C_{18} column (250×4.6 mm, $5 \mu\text{m}$ particle size) equipped with a precolumn Hypersil BDS- C_{18} (10×4 mm, $5 \mu\text{m}$ particle size) was used. The mobile phase was composed of methanol-[ammonium formate 20 mM, formic acid 0.5%, triethylamine 0.2% (pH 3)] 67:33 v/v, and its flow rate was 1 ml/min. Injection volume was 50 μl . Experiments were performed at ambient temperature. The elution times for TC, GC, TCDC, UDC, GCDC, C, and GDC were approximately 9.9, 16.2, 18.2, 21.3, 31.6, 34.5, and 38.5 min, respectively.

Solution preparation

Stock solutions. Ten millimolar stock solutions of each bile acid (TC, GC, TCDC, UDC, GCDC, C, and GDC) were prepared by

dissolving the appropriate amount of each compound in 10 ml methanol. These solutions were stored at 4°C and were stable for at least 6 months.

Standard solutions. Bile acids working standard solutions were prepared in the concentration range of 0.5–500 μM for construction of calibration curves and estimation of limit of detection (LOD) and limit of quantification (LOQ). All dilutions were performed with mobile phase.

Sample treatment

Gastric samples. One-hundred microliters of sample (HGF_{fasted}^{60}) was transferred to a microcentrifuge tube, and 100 μl of mobile phase was added. After vortexing for 30 s, the sample was centrifuged for 10 min (10,500 g, 10°C). The clear supernatant was injected into the HPLC system.

Duodenal samples. Fifty microliters of sample (HIF_{fed}^{90}) was transferred to a microcentrifuge tube, and 100 μl of acetonitrile was added. After vortexing for 30 s, the sample was centrifuged for 10 min (10,500 g, 10°C). The clear supernatant was diluted with the appropriate volume of mobile phase and then injected into the HPLC system.

Method validation

Calibration curves. Calibration curves for bile acids in mobile phase were constructed in the concentration range of 0.5–500 μM ($n = 10$) by measuring the peak area of each individual bile acid as a function of bile acid concentration. In addition, calibration curves of bile acids standards obtained in very low concentration region (0.5–10 μM) were constructed for the estimation of LOD and LOQ. In this case, regression equations were obtained through unweighted least-squares linear regression analysis and applied to the peak area of each bile acid as a function of bile acid concentration.

Recovery. Recovery data were collected by applying the standard addition method in gastric and duodenal aspirates. Each sample was first prepared as described in Sample treatment. Then an appropriate amount of bile acids was added, and the sample was treated and analyzed for bile acid content again. From the difference between the two concentrations (added vs. endogenous), recovery values were obtained. For each bile acid, the added concentration ranged between 20 and 400 μM (gastric aspirates) and 120 and 1,800 μM (duodenal aspirates).

Precision. Intra- and inter-day relative standard deviations were calculated using 2, 50, 100, 300, and 500 μM bile acid concentrations in mobile phase. In addition, precision was evaluated by estimating intra-day relative standard deviation of bile acid concentrations in HGF_{fasted}^{60} and HIF_{fed}^{90} . Each sample was prepared and measured five times.

TABLE 1. Limit of detection and limit of quantification of individual bile acids when using the HPLC-CAD method developed in this study

	TC	GC	TCDC	UDC	GCDC	C	GDC
	μM						
LOD	0.41	0.23	0.07	0.36	0.51	0.24	0.60
LOQ	1.26	0.71	0.20	1.08	1.55	0.71	1.80

CAD, charged aerosol detector; TC, taurocholate; GC, glycocholate; TCDC, taurochenodeoxycholate; UDC, ursodeoxycholate; GCDC, glycochenodeoxycholate; C, cholate; GDC, glycodeoxycholate; LOD, limit of detection; LOQ, limit of quantification.

TABLE 2. Intra-day precision for the determination of individual bile acids in mobile phase, in HGF_{fasted}^{60} and in HIF_{fed}^{90} with the HPLC-CAD method developed in this study

Medium	Concentration (μM)	Intra-day Relative Standard Deviation						
		TC	GC	TCDC	UDC	GCDC	C	GDC
Mobile Phase	2	2.8	2.6	3.4	3.6	3.9	0.2	3.9
	50	0.8	2.4	2.5	2.7	1.7	1.1	1.8
	100	1.0	2.5	3.0	2.8	1.5	1.2	2.9
	300	1.7	1.6	1.1	0.7	2.3	0.9	0.2
	500	0.9	2.6	2.7	0.8	2.8	0.8	1.4
HGF_{fasted}^{60}	<i>a</i>	1.5	0.7	2.1	2.2	0.8	<i>b</i>	2.2
HIF_{fed}^{90}	<i>a</i>	1.2	1.1	0.5	6.0	0.9	<i>b</i>	2.4

a Concentrations of individual bile acids are presented in Table 4.

b Relative standard deviation could not be estimated, because no C was detected in HGF_{fasted}^{60} or HIF_{fed}^{90} .

RESULTS

A typical chromatogram of a standard solution of bile acids in mobile phase, the chromatogram of HGF_{fasted}^{60} and the chromatogram of HIF_{fed}^{90} , are shown in **Fig. 1**. Resolution was greater than 2 for all bile acid peaks in all chromatograms. The variation in the retention times of the bile acids was less than 1.5% (Fig. 1). Because samples were treated with a simple dilution, no internal standard was used for the quantification of bile acids with the developed method. However, the use of an internal standard could correct the small variations in the retention times in different runs. The plot of analyte concentration versus area response of CAD showed that in agreement with theory (12, 13), CAD generated a nonlinear response for an injected concentration range of 0.5–500 μM . Based on the standardized residuals plots, in all cases, the quadratic model ($y = y_0 + ax + bx^2$) was fitted better than the linear or the logarithmic model. Coefficients of determination ranged between 0.993 and 0.9999. For the estimation of LOD and LOQ, linear calibration curves were obtained in a very low concentrations region (0.5–10 μM). LOD and LOQ were calculated by using the standard error of estimate (i.e., the square root of the variance of deviations from the regression line), $s_{y/x}$, and the slope, b , of the regression line according to the following equations (14)

$$LOD = \frac{3.3s_{y/x}}{b} \quad \text{and} \quad LOQ = \frac{10s_{y/x}}{b}$$

The calculated LOD and LOQ values for each bile acid are presented in **Table 1**. The highest LOD and LOQ values were estimated for GDC (0.60 and 1.80 μM , respectively).

The lowest intra-day precision of standard samples in mobile phase (concentration range 1–500 μM) was 3.9%

(**Table 2**). Intra-day precision in HGF_{fasted}^{60} and in HIF_{fed}^{90} was 2.2% and 6%, respectively (Table 2). The lowest inter-day precision of standard samples in mobile phase (concentration range 10–300 μM) was at 10 μM (11.9%). At higher concentrations, the lowest inter-day precision was 7.8%.

Table 3 shows the recovery for each bile acid from HGF_{fasted}^{60} and HIF_{fed}^{90} . Depending on the bile acid, mean recovery ranged from 98.2% to 113.0%. Based on these data, and because gastric and duodenal aspirates always contain bile acids, quantification was based on calibration curves constructed in the mobile phase.

Table 4 shows the individual and total bile acid levels in HGF_{fasted}^{60} and HIF_{fed}^{90} . The predominant bile acids in HGF_{fasted}^{60} are GCDC and GC, whereas, based on the absence of C, it may be claimed that unconjugated bile acids are probably absent from the healthy fasted stomach. These data are in agreement with the data reported by Scalia et al. (9), in which individual bile acids had been determined in patients with reflux gastritis. Total bile acid concentration in HGF_{fasted}^{60} was 81.09 μM . This value is in agreement with a mean \pm SD value of $80 \pm 30 \mu M$ reported by Rhodes et al. (15), where the assay method involved radiolabeling of the bile acid pool. In other studies in which enzymatic methods were applied, either intragastric total bile acid levels could not be estimated reliably, owing to the high LOQ (6, 16), or the measured concentration range was 0.0–2.5 mM (median 0.1 mM), without reporting the LOQ (5). It should be noted that to date, only total bile acid content has been reported for the fasted stomach of healthy adults, probably owing to the very low individual bile acid levels.

The major bile acids in HIF_{fed}^{90} were found to be GDC, GCDC, and GC (Table 4). This is in agreement with a recent study (17). Also, very recently, by using a gas chromatography method, it has been reported that cholate and chenodeoxycholate constitute most of the total bile acid pool in the fed duodenum (18). However, the relevant method does not allow distinguishing between conjugated and unconjugated bile acids. Total bile acid concentration in HIF_{fed}^{90} was 9.2 mM (Table 4). Using enzymatic methods, Armand et al. (19) reported mean values of 6.7–13.4 mM up to 4 h after the administration of a meal, whereas Kalantzi et al (6) reported values in which the medians dropped from 11.2 mM at 30 min to 5.2 mM at 180 min post meal administration. Using an HPLC-ELSD method (17) the mean \pm SD total bile acid level in the fed jejunum was estimated to be 8 ± 0.1 mM. Very recently, Clarysse et al. (18), using a gas chromatography method, reported that within 30 min after meal administration, individual intraduodenal concentrations of total bile acids ranged

TABLE 3. Percent recovery of each bile acid from HGF_{fasted}^{60} and HIF_{fed}^{90} using the HPLC-CAD method developed in this study

Medium	Percent Recovery						
	TC	GC	TCDC	UDC	GCDC	C	GDC
HGF_{fasted}^{60}	102.6 \pm 5.3	101.7 \pm 3.5	102.5 \pm 5.4	101.6 \pm 3.4	101.0 \pm 4.7	99.5 \pm 1.1	100.6 \pm 1.8
HIF_{fed}^{90}	101.3 \pm 5.3	113.0 \pm 25.5	99.8 \pm 1.9	98.2 \pm 3.5	107.6 \pm 12.6	98.6 \pm 5.5	103.3 \pm 6.4

Mean \pm SD values of the five different standard additions.

TABLE 4. Individual and total bile acid concentrations in HGF_{fasted}^{60} and in HIF_{fed}^{90} measured in this study

Sample	Bile Acid Concentration							
	TC	GC	TCDC	UDC	GCDC	C	GDC	Total
	μM							
HGF_{fasted}^{60}	4.62	29.01	3.62	1.35	31.82	<LOD	10.67	81.09
HIF_{fed}^{90}	562.9	1,348.5	754.5	216.1	1,881.0	<LOD	4,448.9	9,211.9

from 6.9–9.3 mM. It is worth mentioning that in all the works cited above, the administered meals contained all major nutrients in a balanced way.

DISCUSSION

During the development of the present method, both HPLC and LC-MS grade methanol were tested. Because the signal-to-noise ratio remained unaltered, the validation procedure was applied by using HPLC-grade methanol. In addition, several concentrations of ammonium formate were tested. A concentration of 20 mM of ammonium formate (adjusted to pH 3) and a mixture of methanol-buffer (67:33; v/v) constituted the most appropriate mobile phase for the clear separation of all bile acids without the need for gradient elution.

The method developed in the present study for determining individual bile acids in gastric and duodenal aspirates involved simple sample preparation procedures. Specifically, for gastric aspirates collected in the fasted state, a dilution of 1:1 (v/v) with mobile phase was adequate for obtaining a clear solution to inject directly into the HPLC system. For duodenal aspirates collected in the fed state, the procedure involved simply the addition of acetonitrile (for protein precipitation), centrifugation, and injection into the HPLC system. Thus, with the proposed method, liquid-liquid extraction or other tedious preparation procedures required by previous methods such as sample evaporation (7) or solid-phase extraction (9, 11, 20, 21) were avoided. However, the LOD of the developed method was lower than that in previously proposed HPLC-UV (LOD = 15 μM) (8) or HPLC-ELSD (LOD = 12 μM) (11) methods. In addition, the sample volume required for the analysis was kept low (100 μl for gastric samples and 50 μl for duodenal samples). In previously proposed methods, sample volumes were always higher than 500 μl , due to the low sensitivity of the method (7) and/or owing to the need for solid-phase extraction prior to injection into the HPLC (8, 17).

Gas chromatography has recently been applied for quantifying bile acids in the duodenum (18). Issues with gas chromatography include the need for silylation (i.e., the need for appropriate sample preparation procedures) and also the fact that conjugated bile acids cannot be distinguished from nonconjugated bile acids (18). Finally, an LC-tandem mass spectrometry method, originally developed for assaying individual bile acids in human serum (22), has been applied for the determination of intra-duodenal bile acid levels (20, 21). No data on analytical

characteristics of this method in aspirates from the gastrointestinal lumen have been provided to date.

In summary, compared with previously proposed HPLC-UV or HPLC-ELSD methods for the determination of intraluminal individual bile acid levels, the HPLC-CAD method developed and validated in the present investigation has a number of advantages, including simple sample preparation procedure (important especially when aspirates are collected in the fed state), low intra-day precision (<6%), high recovery (>98.2%), small sample volume ($\leq 100 \mu l$), and low (<0.60 μM) LOD (important especially when aspirates are collected from the fasted stomach or from the colon) **■**

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