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Method Article

A fast and simple ion-pair high performance liquid chromatography method for analysis of primary bile salts in *in vitro* digested bean samples

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A B S T R A C T

Bile salts (BS) play a key role in cholesterol and lipid metabolism as well as in many other key metabolic pathways. High performance liquid chromatography (HPLC) is the most common technique used to analyze BS in diverse type of samples. However, current HPLC analysis methods used to analyze and quantify single BS in *in vitro* digested samples showed poor separation of a complex mixture of BS. In this article, we improved a standard method originally used for quantifying individual BS in food samples subjected to *in vitro* digestion. We also adapted a method previously developed for BS examination in human blood samples to the analysis of these molecules in chyme samples obtained during simulated gastrointestinal digestion. Our method was simple and achieved a fast and successful separation and quantification of four primary BS (sodium salts of taurocholic, glycocholic, taurochenodeoxycholic and glycochenodeoxycholic acids).

- A method used to analyze bile salts in human blood samples has been adapted to separate and quantify four primary bile salts in *in vitro* digested bean samples.
- Addition of an ion-pair reagent led to complete separation of glycine and taurine conjugates of chenodeoxycholic and cholic acids within 10 min, and achieved good peak symmetry.
- The minimum BS concentration that could be measured was as low as 0.03125mM.

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A R T I C L E I N F O

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Method details

Analysis of primary bile salts (BS) (sodium taurocholate, glycocholate, taurochenodeoxycholate, and glycochenodeoxycholate) in simulated chyme was carried out using samples obtained from a previous study evaluating the BS retention/binding-ability of dry beans and bean main components [3]. In this study, dry beans or dry bean fractions (soluble and insoluble dietary fiber, proteins, starch) were digested using the standardized INFOGEST multistage *in vitro* static digestion model that mimicks the human oral, gastric and small intestinal environments [4]. In the intestinal stage, simulated intestinal fluid (SIF) including enzymes and a mixture of primary BS (10mM) was added. A dialysis model consisting of a dialysis membrane of 12–14 kDa cut-off and pore diameter of 25 Å placed into a flask containing SIF, and shaken in an incubator, was used to mimic the small intestinal conditions and to dialyze the free BS non retained by bean materials during 2 h of digestion. The detailed *in vitro* digestion protocol and dialysis method can be found in the original article.

Analysis of permeated (unbound or non-retained) BS collected in the dialysate involved preparation of individual bile salt and bile salt mixture stock solutions; comparison and validation of three HPLC methods (A, B and C) and determination of fundamental chromatographic parameters; use of a dialysis method to avoid additional extraction of BS from chyme; development of standard calibration curves at two concentration ranges to account for the dilution occurred during dialysis; and eventually, HPLC analysis of individual primary BS applying the method C (with an ion-pair reagent -tetrabutylammonium phosphate- in the mobile phase) which showed the fastest and best separation efficiency and peak symmetry among the three methods tested. Namely, method A is a standard HPLC method generally used to analyze and quantify BS in chyme samples generated in *in vitro* digestion studies. Based on method A, we developed the method B by adding 0.1% (v/v) formic acid in the mobile phase, with the goal of improving the separation efficiency. Since methods A and B did not achieved yet a successful separation, peak symmetry and quantification of the four individual BS, we developed method C by adapting a method previously conceived for BS examination in human blood samples in *in vivo* studies to the analysis of BS in simulated chyme samples.

HPLC analysis was carried out using a C18 analytical column with a flow rate of 1mL/min and a UV detector set at a wavelength of 200nm.

Materials

Ultra-pure water
Methanol (HPLC grade)
Phosphate buffer (0.05M, pH6.5)
Formic acid
Acetonitrile (HPLC grade)
Tetrabutylammonium phosphate
Whatman ^{R}filter paper (90 mm diameter, circles, ash less) (Maidstone, UK)
All chemicals and materials were purchased from Fisher Scientific (Hampton, USA).

Table 1
HPLC operation conditions used in the HPLC methods.

	Mobile phases*	Column temperature	Flow rate	Running time
Method A	100mL ultra-pure water 700mL methanol 200mL phosphate buffer (0.05M, pH6.5) (70:20:10, v/v/v)	Isothermal 25 °C	1 mL/min	25min
Method B	1L mobile phase A added with 1mL formic acid (0.1% formic acid in the final solution)	Isothermal 25 °C	1 mL/min	25min
Method C	450mL ultra-pure water 500mL acetonitrile 1mL 0.5M tetrabutylammonium phosphate (45:50:1, v/v/v)	Isothermal 40 °C	1 mL/min	10min

*Mobile phase solutions were vacuum-filtered with Whatman ®filter paper and degassed using an ultrasonic cleaner (F320, Fisher Scientific, VA, USA).

Bile salts including sodium taurocholate (NaTC, T4009), sodium glycocholate (NaGC, G7132), sodium taurochenodeoxycholate (NaTCDC, T6260), and sodium glycochenodeoxycholate (NaGCDC, G0759) were purchased from Sigma-Aldrich (St Louis, USA).

Bile salt standard solutions

Bile salt standard solutions (NaTC, NaGC, NaTCDC and NaGCDC) were first prepared with ultra-pure water at the molar concentration of 20mM. Individual solutions were then mixed together at the ratio of 1:1:1:1 (v/v/v/v) and the BS mixture (containing 5 mM of each BS) was analyzed by three HPLC methods (Table 1) to compare the separation efficiency of each one. Each individual bile salt solution was analyzed to confirm the corresponding retention times.

HPLC system and operation conditions

The HPLC apparatus used was an Agilent 1100 high performance liquid chromatograph (Agilent Technology, Santa Clara, CA, USA) equipped with a G1311A Quaternary Pump, G1313A Autosampler, G1316A Column Thermostat and G1315B Diode Array Detector). Bile salt analysis and separation was carried out using a C18 Gravity Nucleodur reversed-phase column (250×4.6mm, i.d., 5 µm) (Macherey-Nagel GmbH & Co. KG, DÜren, Germany) and a UV detector set at a wavelength of 200nm. The mobile phases were eluted in isocratic mode at the flow rate of 1 mL/min with the column operating at 25 °C or 40 °C depending on the method. The HPLC operation conditions (mobile phases, column temperature, flow rate and running time) used in each method are detailed in Table 1.

HPLC analysis and method comparison

A 5uL sample was injected for analysis and the Agilent Chem-Station LC&LC/MS Systems Software package (Agilent Technologies, Santa Clara, CA, USA) was used to monitor HPLC conditions and collect the data. The chromatographic results obtained by means of the three HPLC methods are shown in Fig. 1. Bile salts have weak chromophores with absorption around 200nm, which was the UV detector wavelength used [5]. The three mobile phases tested shared some similar BS separation trends. The order of elution was NaGC, NaTC, NaGCDC and NaTCDC using either mobile phase . Conjugates of cholic acid (i.e. NaGC and NaTC) showed faster elution and greater detection response than the more hydrophobic dihydroxyl chenodeoxycholic conjugates (i.e. NaGCDC and NaTCDC). Glycine-conjugated BS eluted faster than taurine-conjugated BS.

The three methods were further compared and validated regarding their retention factor (K), separation resolution (Rs), peak symmetry (As) (Table 2), which were calculated according to the

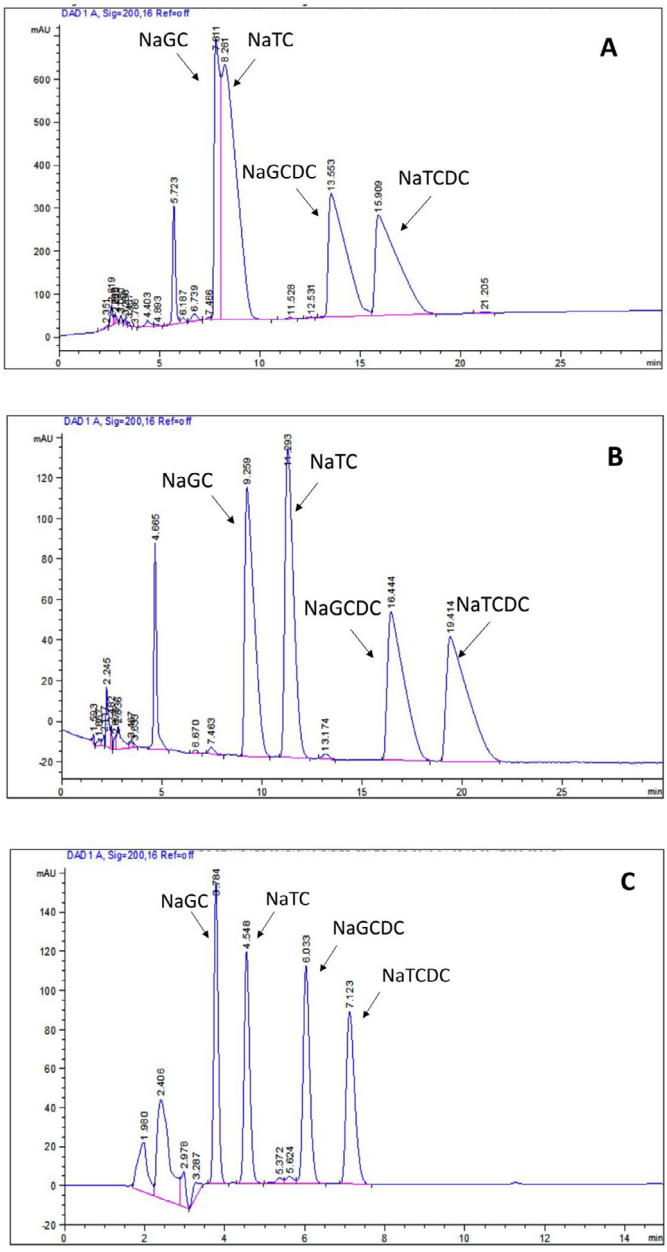

Fig. 1. Representative HPLC chromatograms showing the separation of primary bile salts (NaGC, NaTC, NaGCDC and NaTCDC) by using method (A) mobile phase: methanol, phosphate buffer (0.05M, pH6.5), and water (70:20:10, v/v/v) ; (B) mobile phase: methanol, phosphate buffer (0.05M, pH 6.5), and water (70:20:10, v/v/v) with 1% formic acid; (C) mobile phase: water, acetonitrile and 0.5M tetrabutylammonium phosphate (45:50:1, v/v/v).

Table 2
Chromatographic parameters (retention factor, t efficiency -number of theorical plates-, resolution and asymmetry factor) corresponding to the HPLC methods A, B and C.

Treatment	Method A				Method B				Method C			
	K	N	Rs	As	K	N	Rs	As	K	N	Rs	As
NaTC	1.98	568	-	3.375	4.66	3757	-	2.59	0.92	994	-	1.29
NaGC	2.15	636	0.32	3.375	7.36	9001	7.66	1.78	1.30	1094	1.42	1.35
NaTCDC	4.18	3710	5.95	4.64	9.87	5079	4.10	2.85	2.05	1378	2.28	1.21
NaGCDC	8.01	1209	1.29	9.62	11.97	4615	2.72	4.64	2.60	1565	1.51	1.30

K: Retention factor; 1-10 indicates good separation.
N: Number of theorical column plates; higher N values indicate more efficient columns which result in a narrower peak at a given T_R.
Rs: Resolution; >1.5 indicates good separation between two adjacent peaks.
As: Asymmetry; <1.5 indicates good peak symmetry.

following equations [6]:

$$Retention\ factor\ (K) = \frac{T_R - T_0}{T_0} \tag{1}$$

$$Selectivity\ (\alpha) = \frac{K_{peak2}}{K_{peak1}} \tag{2}$$

$$Plate\ number\ (N) = 16\left(\frac{T_R}{W_b}\right)^2 \tag{3}$$

$$Resolution\ (Rs) = \frac{1}{4}\sqrt{N} \times \frac{\alpha - 1}{\alpha} \times \frac{K}{1+K} \tag{4}$$

$$Asymmetry\ (As) = \frac{B}{A} \tag{5}$$

Where T_R is the retention time, T_0 is the void time, W_b is the width of the peak, N is theoretical plate number which determine the column efficiency, B is the tail portion (right half) of the peak width at 10% peak height, A is the front portion (left half) of the peak width at 10% peak height.

Method A, with mobile phase of methanol, phosphate buffer (0.05M, pH6.5) and water (70:20:10, v/v/v), did not completely separate NaGC and NaTC, showing considerable low resolution (Rs 0.32 $\ll$1.5) and peak symmetry (As $\gg$1.5) (Fig. 1A, and Table 2). The method B, which was developed from method A, improved the peak separation and selectivity (Rs >1.5), particularly well for cholate BS (NaGC and NaTC); nevertheless, the peak symmetry of peaks corresponding to chenodeoxycholate BS (NaGCDC and NaTCDC) was still poor (As $\gg$1.5). Method C which increased the column temperature to 40°C, led to the best peak separation (Rs $\geq$1.5) and peak symmetry (As <1.5) among the three methods. In this method, the addition of an ion-pairing reagent (i.e. tetrabutylammonium phosphate), facilitated separation due to its charge, which is opposite to the BS charge, and its long hydrophobic chain, that allows interacting with the stationary phase, plus associated counter-ions. Method C not only led to the best separation efficiency and peak shape, but also shortened the run time to hardly 10 minutes, decreasing by more than half the run times required by methods A and B (25 min) (Fig. 1). Therefore, because of these improvements and advantages, the method C was chosen to develop the standard curves and to perform the separation and quantification of permeated free BS (NaTC, NaGC, NaGCDC and NaTCDC) into the dialysate [3].

Development of standard curves

Bile Salt standard curves using method C were drawn for each BS (NaGC, NaTC, NaGCDC and NaGCDC) and the BS mixture at a range of concentrations from 0.03125 to 10 mM BS (Fig. 2). Since the initial BS mixture used to perform the *in vitro* digestion experiments consisted of a solution of

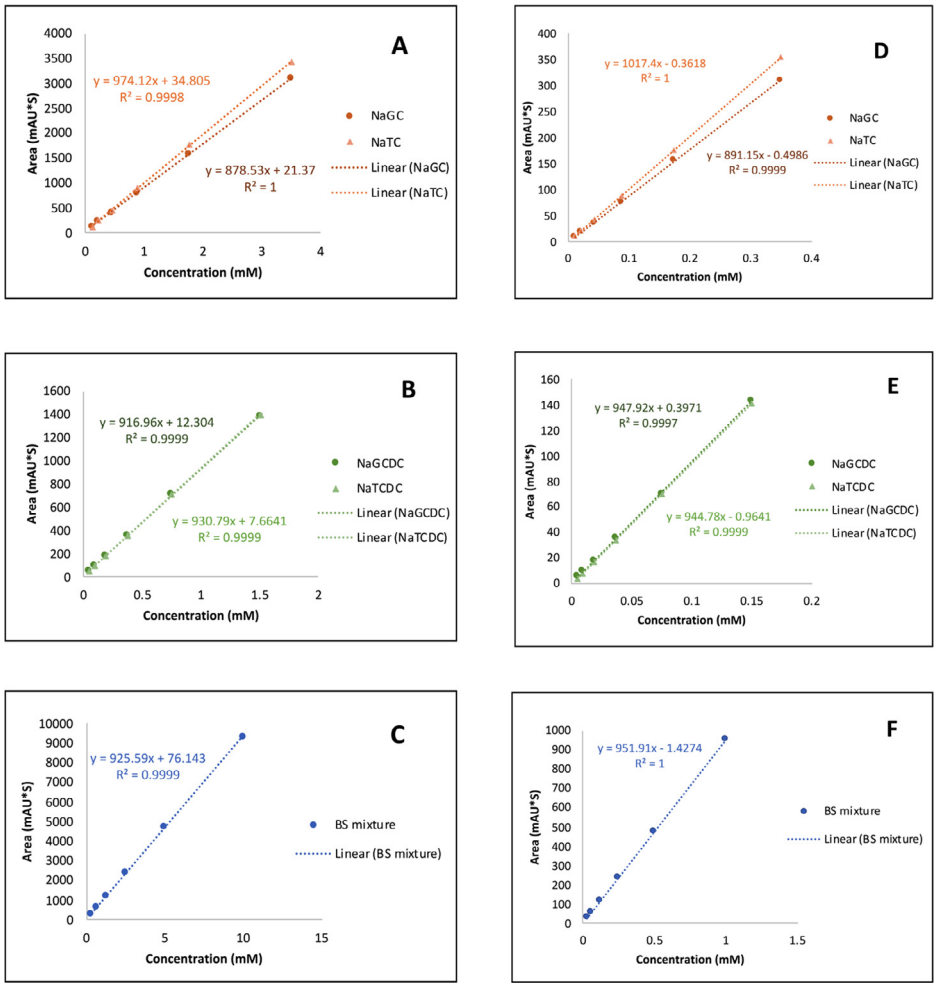

Fig. 2. Standard calibration curves (n= 3) for quantification of (tauro- and glyco-) cholate BS (A), (tauro- and glyco-) chenodeoxycholate BS (B) and a mixture of cholate and chenodeoxycholate BS (C) in the initial samples (0.3125 –10 mM) and for quantification of (tauro- and glyco-) cholate BS (D), chenodeoxycholate BS (E) and a mixture of cholate and chenodeoxycholate BS (F) in the dialysates (0.03125 –1 mM). Each point represents bile salt detection at 200 nm using method C (ultra-pure water, acetonitrile and 0.5M tetrabutylammonium phosphate (45:50:1, v/v/v)). The slope, y-intercept values and regression coefficients used for the determination of bile salt concentrations in dialysate samples are shown in each graph.

35% NaGC, 35% NaTC, 15% NaGCDC and 15% NaGCDC (molar ratio) in phosphate buffer [7], a stock solution of the BS mixture (10mM) with the same molar ratio was prepared. BS 10 mM was the upper concentration used as it is the representative concentration found in the adult intestine under fed state conditions. Using phosphate buffer, the BS stock solutions were serial diluted to 10, 5, 2.5, 1.25, 0.625, 0.3125mM (Fig. 2A, B, C). Standard curves ranging BS concentrations (1mM - 0.03125mM) ten times lower than the initial dilutions were also drawn (Fig. 2D, E, F) to account for the dilution occurred to the digested samples during the dialysis step. The samples were analyzed by injecting 5uL and the corresponding peak area values were recorded. The calibration curves were drawn by representing the values of peak area obtained vs concentration of the sample for each BS individually and for the BS mixtures. The linear regression of the curves showed good quantification results ($R \approx 1$),

which indicated that the minimum concentration of BS measured by Method C could be as low as 0.03125mM.

Conclusions

Three HPLC methods were compared to show the process of development of the most suitable method for our study. We aimed to underline differences among the methods and cases involving BS analysis, where the application of each method would be acceptable and satisfactory. According to our results, method A would be appropriate for BS analysis when just a single type of BS is used in the study. Regarding developed method B, it might be applied in studies that use only a mixture of cholate BS. Finally, the adapted method C is shown to be the optimal method to accurately achieve the best peak symmetry and a successful separation of a mixture of cholate and chenodeoxycholate BS.

Additional information

In the current method, extraction of bile salts was not performed as the (free) BS non sequestered or bound by bean materials permeated from the simulated chyme through a dialysis membrane into a buffer which could be directly analyzed by HPLC. Alternatively, a centrifugation method can also be used to evaluate the retention of bile salts by fiber-rich foods. Unbound BS in the supernatant can be identified and quantified by the HPLC method described here after filtration of the supernatant through a 45 µm syringe filter. However, if a dialysis or centrifugation method is not used and the unbound bile salts are mixed with chyme, other digestive fluids or blood, a procedure to extract BS is needed before HPLC analysis. In those cases solid-phase extraction is commonly used and can be referred to other studies [1,8]. After extraction of BS, the HPLC analysis of BS can be performed following the procedure (method C) detailed in the current article.

Background information

Bile salts are amphiphilic molecules derived from cholesterol in the liver. They not only regulate cholesterol metabolism and function as biological detergents in lipid digestion, but also behave as modulators in many key metabolic responses. Bile salts are conjugates of bile acids with the amino acids glycine and taurine. There are four types of primary BS in humans, including taurocholate, glycocholate, taurochenodeoxycholate, and glycochenodeoxycholate [9]. After each meal, primary BS are secreted by the liver via the gall bladder and then flow into the small intestinal where they participated in lipid digestion and transportation. Almost 95% of BS are reabsorbed in the ileum (the last section of the small intestine) and returned to the liver in a process called enterohepatic circulation; the remaining 5% will continue to the colon, where they are transformed into secondary BS by gut bacteria [10]. The bile salts pool and profile is partially determined by the pattern of food intake. Retention of primary BS by dietary fibers and fiber-rich foods in the small intestine has been linked to several health benefits such as lowering blood cholesterol and lipid levels and thus to a decreased incidence of cardiovascular disease [11]. Therefore, there is an increasing interest to study the capacity of isolated dietary fibers and fiber-rich foods to sequester or retain BS. The BS-binding or BS-retaining ability of foods or food components can be evaluated by quantifying the free BS non-retained or bound by the food. For BS detection, we can differentiate between *in vivo* studies utilizing human or animal plasma samples and *in vitro* studies using chyme samples, with HPLC analysis as the routine tool for the identification and quantification of these analytes. Despite mobile phases consisting of methanol and phosphate buffer [1,12] (e.g. method A) were commonly used in analyzing bile salts in *in vitro* digesta samples, they have showed poor separation of a mixture of bile salts. Recently, we reported on the ability of dry beans and bean main components to sequester or bind four primary BS under conditions simulating the upper gastro-intestinal tract. Our study utilized a previous HPLC method used originally in human plasma samples (method C) to determine concentrations of free or unbound BS in *in vitro* digested samples. We were able to successfully adapt this method

previously developed for serum examination of BS into a simplified and faster protocol for the analysis of chyme concentrations of primary bile salts.

Declaration of Competing Interests

The authors confirm that there are no conflicts of interest.

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