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Determination of bile acids in human serum by on-line restricted access material–ultra high-performance liquid chromatography–mass spectrometry

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ABSTRACT

This paper describes a new, fully automated on-line method combining restricted access material (RAM) extraction and ultra high-performance liquid chromatography (UHPLC) with mass spectrometric (MS) detection for determining congeners of bile acids (BAs) in human serum. In this method, low-pressure RAM and high-pressure UHPLC–MS are hyphenated by using a 2.5-mL loop-type interface. The compatibility problem between the large volume (1.2 mL) of strong solvent (methanol) used for RAM elution and the need for a weak solvent in UHPLC injection has been addressed by using an auxiliary pre-column cross-flow of 0.1% aqueous formic acid. In this way, the complete 2.5 mL loop volume can be injected into the UHPLC system, thereby maximizing sensitivity while maintaining good chromatographic performance. The optimised method allows the simultaneous analysis of 13 bile acids in a single run, including glycine- and taurine-conjugated bile acids, cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), and litocholic acid. The complete analysis of a 100-μL single serum sample is performed in 30 min, providing detection limits in the pg range (corresponding with clinically relevant concentration levels) for all of the analytes except lithocholic acid, intra-day precision values (%R.S.D.) below 4% (except ursodeoxycholic acid) and inter-day precision lower than 15% (except ursodeoxycholic, glycoursodeoxycholic acid (GUDCA) and lithocholic acid).

1. Introduction

Bile acids (BAs) are produced in the liver and, as the main components of bile, they are involved in several processes, including cholesterol homeostasis, absorption of dietary lipids and fat-soluble vitamins by formation of micelles, and both the excretion and recirculation of drugs and toxins. In total, about 20–30 g of bile acids are excreted daily into the small intestine via the bile duct and 95% are reabsorbed and recycled, so concentrations in the ng/g range are found in peripheral blood. During this recirculation, called enterohepatic circulation, BAs undergo multiple metabolic reactions. BAs produced by direct biosynthesis in the liver, called primary bile acids, are cholic acid (CA) and chenodeoxycholic acid (CDCA), whereas those produced by microbial flora are called secondary bile acids. These latter are less soluble in water and, therefore, toxic to cells. BAs are synthesized from cholesterol, and their major structural components are a steroid nucleus and a sidechain. In hepatocytes, BAs undergo amino acid conjugation at the sidechain with glycine, taurine or (less frequently), sulphate or glucuronide, which increases their water solubility.

The composition of bile and the concentrations of BAs in biological fluids, such as serum or urine, can provide indications regarding an individual's metabolism, and potentially useful information about hepatobiliary and intestinal disease states. In addition, since BAs play an important role in the elimination of cholesterol from the body, unusually low or high concentrations could be directly related to various hyper- or hypo-cholesterolemia diseases. Furthermore, since BAs have therapeutic applications, e.g. CDCA and ursodeoxycholic acid (UDCA) are used to dissolve cholesterol gallstones, their determination in serum provides a useful means for monitoring therapy progress.

Several analytical methods have been reported for the determination of BAs in serum, based on liquid chromatography (LC) with UV detection [1–3] or LC coupled to evaporative light scattering detection (ELSD) [4,5] but they have limited sensitivity and are prone to interference by various components of biological interferences. Moreover, since BAs do not absorb UV radiation significantly, pre-column labelling is required to allow their detection by UV and fluorescence detection systems [6]. These problems can be overcome by using mass spectrometric (MS) detection. However, GC–MS analysis requires several steps – including preliminary

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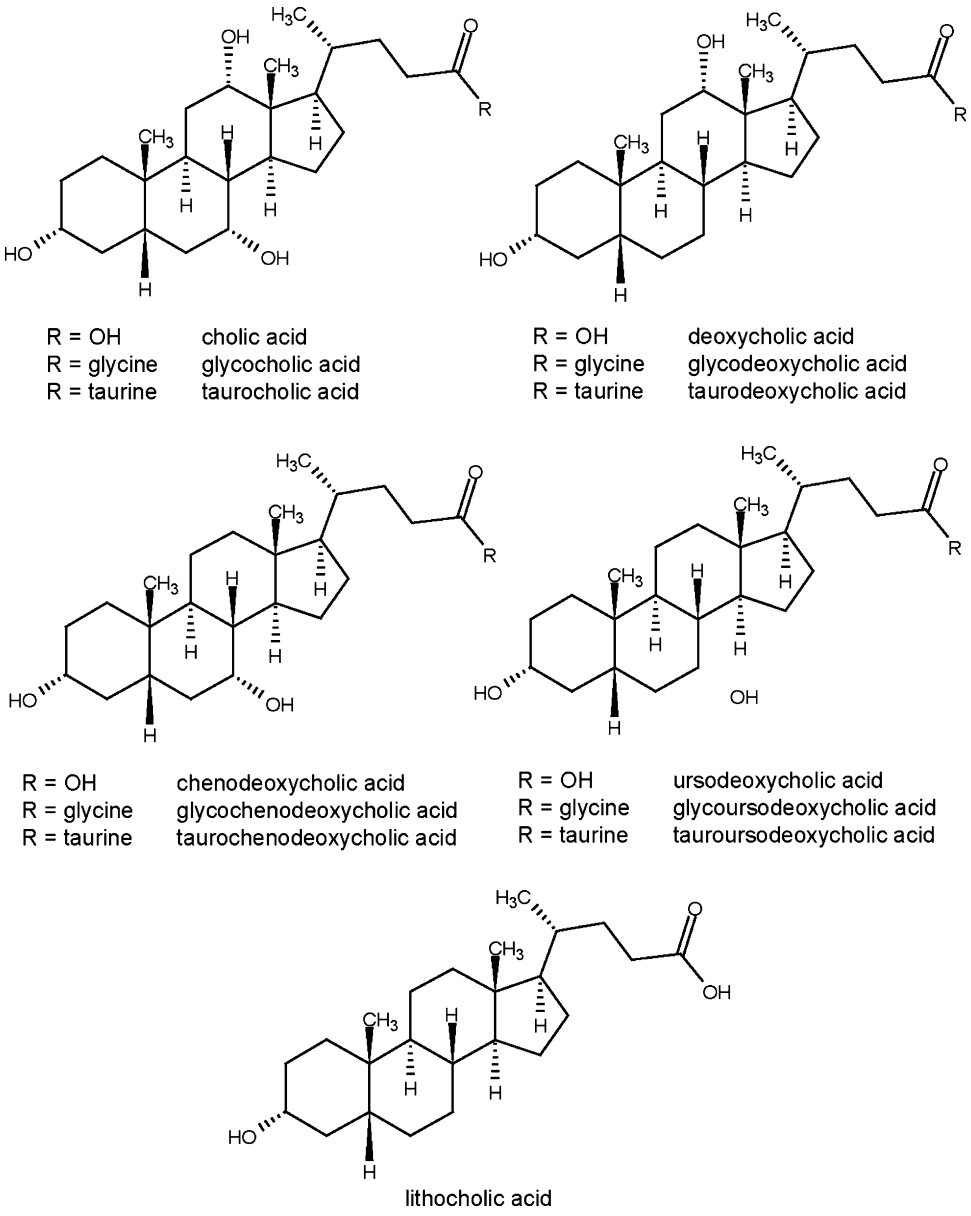

Fig. 1. Structures of the bile acids included in the study.

group fractionation, hydrolysis of conjugates and preparation of volatile derivatives [7–10] – which are labour intensive and time consuming. Therefore, LC–MS is the most suitable technique for determining BAs in serum because no derivatization steps are needed and it provides appropriate sensitivity [2,9,11–17] Prior to LC–MS, samples must be pre-treated, in order (*inter alia*) to remove proteins from them to avoid the system clogging and/or interfering substances affecting the detection. Serum samples are often cleaned by solid phase extraction (SPE), usually using C18 or anion-exchange cartridges [1–4,9,11–13,17]. The determination of BAs in serum, in clinical applications, requires analytical methods that are capable of handling very low volume samples while providing large sample throughputs. These requirements can be addressed using on-line methods but, to the best of our knowledge, no such method including LC–MS determination has previously been reported in the scientific literature for analysing BAs in serum.

Restricted access material (RAM) are being increasingly often used in biological applications since they have useful combinations of size exclusion and in-pore reversed stationary phase properties and, furthermore, they can be conveniently packed in cartridges and used in on-line systems [18–20]. There are clear attractions for clinical applications in coupling such cartridges to ultra high-performance liquid chromatography (UHPLC) systems, in which stationary phases with smaller particles are used than those in "traditional" high-performance LC, providing faster run times and, hence, higher sample throughputs. However, direct hyphenation of UHPLC with any sample pre-treatment procedure is highly challenging, and no such coupled systems appear to have been previously described in the scientific literature. There are two main reasons for this. First, UHPLC has extreme pressure (up to 1000 bars) and low flow (usually less of 0.6 mL/min) requirements, which are not directly compatible with typical on-line extraction methods and, secondly, the large amounts (several mL) of strong solvent (typically methanol) needed for SPE or RAM elution cannot be directly introduced into UHPLC systems while maintaining retention and peak shape.

Therefore, this contribution has several aims. First, to design and test an automated on-line setup that can be used to hyphen-

ate extraction (RAM, low-pressure) and separation-detection (UHPLC–MS, high-pressure) steps and to make compatible the respective flow requirements in terms of total volume, flow and solvent identity. Then, to use the developed setup to analyse bile acids in human serum in order to introduce an on-line method able to fulfil the analytical requirements (mainly sensitivity and sample throughput) needed for clinical analysis.

2. Experimental

2.1. Chemicals and reagents

The following bile acids were included in the study: glycodeoxycholic acid (GDCA, purity 97%, Chemical Abstract Service identification number: 360-65-6), taurocholic acid (TCA, 97%, 345909-26-4), cholic acid (CA, 98%, 81-25-4), chenodeoxycholic acid (CDCA, 98%, 474-25-9), deoxycholic acid (DCA, 99%, 83-44-3), lithocholic acid (LCA, 99%, 434-13-9), glycocholic acid (GCA, 98% dried, 475-31-0), glycochenodeoxycholic acid (GCDA, 97%, 16564-43-5), taurochenodeoxycholic acid (TCDA, 95%, 6009-98-9), taurodeoxycholic acid (TDCA, 95%, 207737-97-1), and ursodeoxycholic acid (UDCA, 99%, 128-13-2), all purchased from Sigma–Aldrich (Madrid, Spain); and tauroursodeoxycholic acid (TUDCA, 99%, 14605-22-2) and glycoursodeoxycholic acid (GUDCA, 96%, 66480-66-6), purchased from Calbiochem (San Diego, CA, US). Stock solutions of ~1,000 μg/g were prepared for each analyte in methanol, and a working solution of the bile acids, each at a concentration of approx. 10 μg/g in Milli-Q water, was prepared weekly and stored at −20 °C in darkness. Fig. 1 displays the structures of the selected analytes.

The solvents used as mobile phases were methanol (gradient grade), isopropanol (LC–MS grade) and formic acid (reagent grade) from Scharlau (Barcelona, Spain). Milli-Q water was obtained from a Milli-Q Plus 186 device from Millipore (Billerica, MA, USA). Trichloroacetic acid was purchased from Riedel-de-Haën Fine Chemicals (Seelze, Germany).

2.2. Sample preparation

Serum samples were obtained from informed healthy volunteers and stored at −20 °C in the dark. Shortly before analysis, the samples were brought to room temperature and 10% (w/w) of a 10% (v/v) aqueous trichloroacetic acid solution was added to the raw serum. The resulting mixture was centrifuged at 3400 × *g* for 30 min, the supernatant was filtered through a 0.22-μm syringe Nylon filter, and a 100 μL portion was directly injected into the on-line system described below.

2.3. On-line setup

The on-line setup, illustrated in Fig. 2, included an ACQUITY™ UPLC (UHPLC PUMP in the figure, Waters, Milford, MA), an HP 5010 Series pump equipped with an HP 1050 Series injector (AUX 1, Hewlett-Packard, Palo Alto, CA), and a Kontron 322 System auxiliary binary pump (AUX 2, Kontron Instruments, Neufahrn, Germany). Analytes were detected using a Quattro micro™ API Mass Spectrometer (ESI MS, Waters) and an ACQUITY™ UPLC Tunable UV (TUV) Detector (UV, Waters). The coupling between the "low-pressure loading-extraction" path (max. 50 bar) and the UHPLC "high-pressure LC" path (approx. 500 bar) was achieved via a VICI EHMA six-port valve controlled by a microelectric actuator ("primary valve" hereafter, VICI® Valco Instruments, Houston, Texas); a 2.5-mL loop; and a six-port valve integrated in the MS spectrometer ("secondary valve"). The complete clean-up, separation and detection process (total run time from sample to sample is 31 min, as illustrated in Fig. 2) can be divided into three steps.

2.3.1. Loading

The 100-μL-treated serum samples were injected using AUX 1 device and introduced into a 20 mm × 4 mm RAM cartridge (Biotrap 500MS, ChromTech Ltd., Cheshire, UK) using a 1.0-mL/min flow of Milli-Q water:isopropanol:formic acid (96:4:0.45%, w/w). Elution of the large, non-retained molecules was monitored by recording UV absorption at 254 nm. Their elimination was considered to be complete when the UV signals corresponding to proteins and other macromolecules had fallen to less than 0.05 UA (after 4.0 min). The valve was then automatically switched to the elution position.

2.3.2. Elution

Retained analytes were removed from the RAM in backflush mode into the 2.5-mL loop by a 1.0-mL flow of methanol. Before entering the loop, an additional acidified cross-flow of 0.1% (w/w) aqueous formic acid was added. Thus, the composition of the solvent in the loop matched that of the initial UHPLC mobile phase, so its introduction did not provoke any baseline disturbance or weaken the analyte retention, thereby improving the peak shape and pre-concentration of the analytes. After 6.5 min, the position of the secondary valve was changed and the analytes were conducted from the loop to the column in backflush mode for 8.9 min. At the same time, the RAM cartridge was cleaned with methanol and subsequently equilibrated with the loading flow for a total time of 9.5 min. The primary valve is then switched back to the loading position; mobile through the cartridge is adjusted to the loading composition and flow; and injection of the following sample takes place in 31 min.

2.3.3. Detection

Analytes were separated using a BEH ACQUITY™ 100 mm × 2.1 mm × 1.7 μm C18 column (Waters) and a mobile phase consisting of 0.1% (w/w) aqueous formic acid (A) and 0.1% (w/w) formic acid in methanol (B). Column temperature and flow rate were set at 50 °C and 0.3 mL/min, respectively. The gradient stated with a 50:50 A:B for 20 min while the sample was being loaded and the macromolecules were eliminated, followed by a linear gradient to 60% B over 1 min, then to 75% B over 1.5 min, which was held for 1.5 min, followed by a further linear rise to 83% over 0.8 min, held for 1 min, and finally a linear rise to 100% B over 8.3 min, held to 34.5 min, then back to the initial composition over 0.5 min to the initial composition. The elution span from the first to the last peak was 4.8 min, as illustrated in Fig. 3. An ESI probe was used as the ionization source in the MS system and MASSLYNX 4.0 software (Waters) was used to acquire and process the data generated. The detection parameters were: negative ionization mode, capillary voltage 3.00 kV, source temperature 125 °C, desolvation temperature 300 °C, desolvation nitrogen flow 400 L/h and cone nitrogen flow 40 L/h. The deprotonated form of the molecules $[MW-H]^-$ has been used for quantifying all the bile acids. Hence, the ions determined have been 498.6 (TUDCA, TCDA, TDCA), 448.6 (GUDCA, GCDCA, GDCA), 514.6 (TCA), 464.6 (GCA), 391.6 (UDCA, CDCA, DCA), 407.6 (CA) and 375.6 (LCA).

3. Results and discussion

3.1. On-line setup

As explained in the Introduction, one of the main aims of the work presented here was to develop an on-line analytical method to increase sample throughput. To achieve this goal, the sample

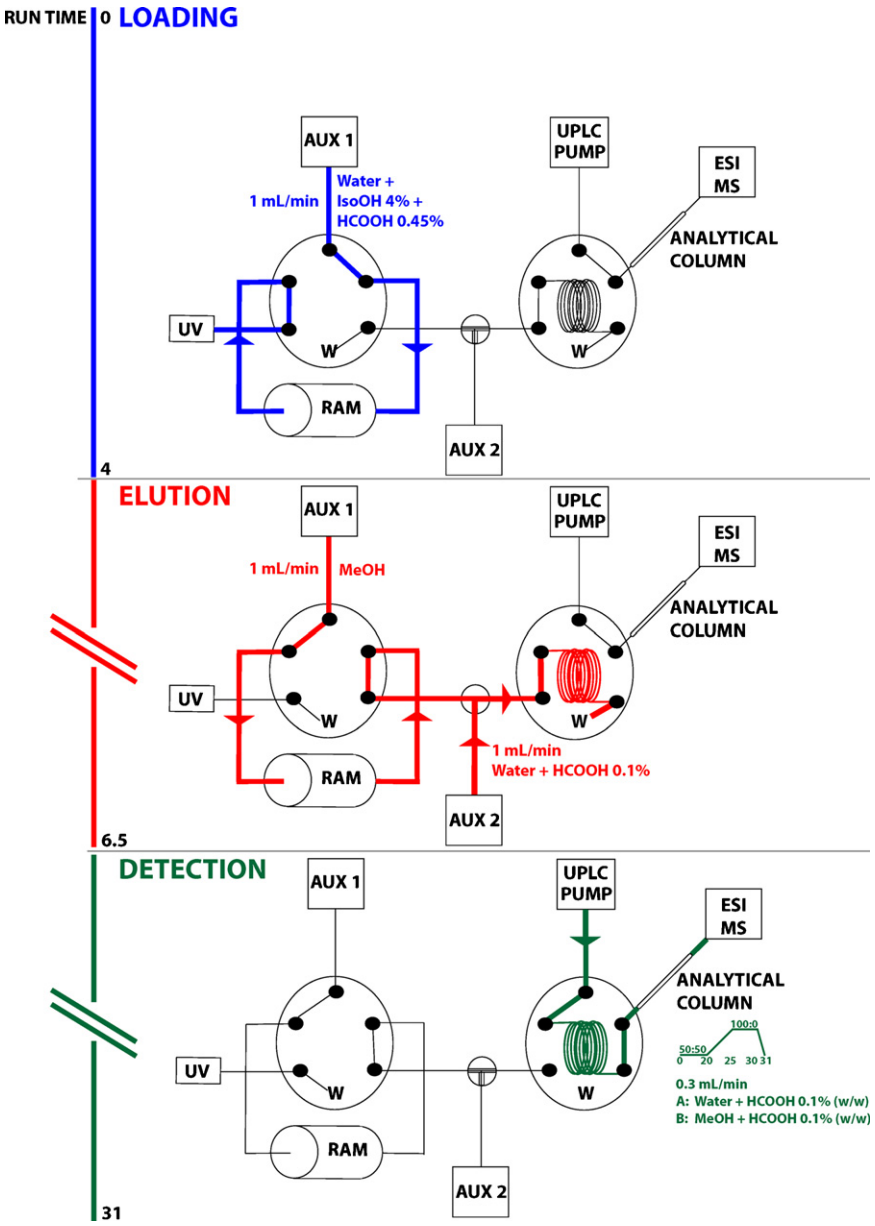

Fig. 2. Schematic diagram of the on-line RAM–UHPLC–MS setup. For a more detailed explanation of the acronyms used, see Section 2.3.

pre-treatment steps had to be reduced. The use of a RAM cartridge allows the injection of serum samples into the system with very little pre-treatment, but it is highly recommended to remove cryoproteins and particulate impurities in order to prevent damage to the cartridge and clogging. Removal is performed by acidifying serum with aqueous trichloroacetic acid, followed by centrifugation and filtration of the supernatant; low pH in the supernatant also helps extraction in the RAM cartridge, since it keeps acids in their protonated forms, which is a must for retention.

The extraction step has been designed using methanol as eluting solvent due to its compatibility with the following chromatographic separation. It has been determined (data not shown) that 1.2 mL of methanol is needed for quantitative recovery of the analytes. Unfortunately, since the stationary phase in the analytical column is similar to that of the RAM cartridge (reversed phase), such a large volume of strong solvent (equivalent to approx. 5 times the inner volume of the UHPLC column) containing the extracted analytes cannot be directly introduced into the column because it would adversely affect analyte retention in the column, leading to peak broadening, fronting and unsatisfactory analytical results. To overcome this problem, an auxiliary cross-flow of 1.0 mL/min of acidified water (0.1% formic acid) is coupled to the eluting flow (see Fig. 2) so the final in-loop solvent composition is 50% methanol and 50% 0.1 formic acid, which has proven to be sufficiently weak to prevent elution of the least strongly retained analyte, TUDCA. When the sample band of 2.5 mL has filled the loop, the secondary valve switches position, and the analytes are transferred to the analytical column at a flow rate of 0.3 mL/min (compatible with the optimum mobile phase composition), lasting approx. 9 min. It should be mentioned that this step is the bottleneck of the proposed procedure, but it could be significantly reduced by extracting samples in a parallel fashion, i.e. injecting another serum sample into the RAM cartridge when the preceding sample is being transferred.

Separation has been achieved by a conventional gradient of water and methanol, both acidified with formic acid. It is worth

Table 1
Detection features of bile acids

Analyte	Acronym	MW (Da)	SIR ion [MW−H]⁻	Retention time (min)[a]
Tauroursodeoxycholic acid	TUDCA	499.6	498.6	22.4
Glycoursodeoxycholic acid	GUDCA	449.6	448.6	23.2
Taurocholic acid	TCA	515.6	514.6	23.3
Glycocholic acid	GCA	465.6	464.6	23.9
Taurochenodeoxycholic acid	TCDA	499.6	498.6	24.1
Ursodeoxycholic acid	UDCA	392.6	391.6	24.3
Taurodeoxycholic acid	TDCA	499.6	498.6	24.4
Glycochenodeoxycholic acid	GCDCA	449.6	448.6	24.7
Cholic acid	CA	408.6	407.6	24.8
Glycodeoxycholic acid	GDCA	449.6	448.6	25.1
Chenodeoxycholic acid	CDCA	392.6	391.6	26.0
Deoxycholic acid	DCA	392.6	391.6	26.2
Lithocholic acid	LCA	376.6	375.6	27.2

[a] Retention times refer to the times from the start of the UHPLC gradient.

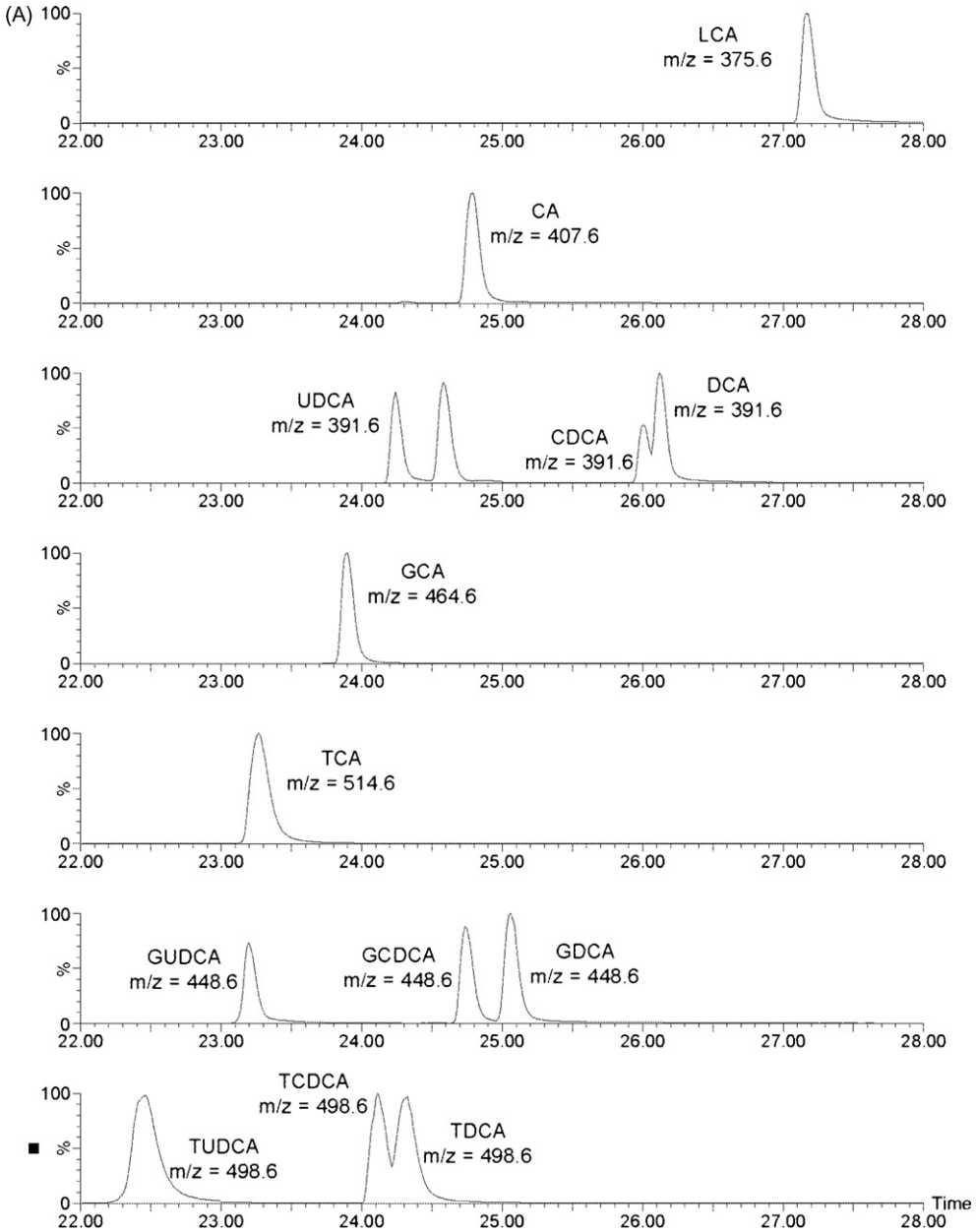

Fig. 3. Typical chromatograms obtained using the on-line method. (A) Standard solution in water (50 ng/g) and (B) pool of serum samples (individual concentrations from 1.9 to 355 μg/g). Peaks are identified as in Table 1.

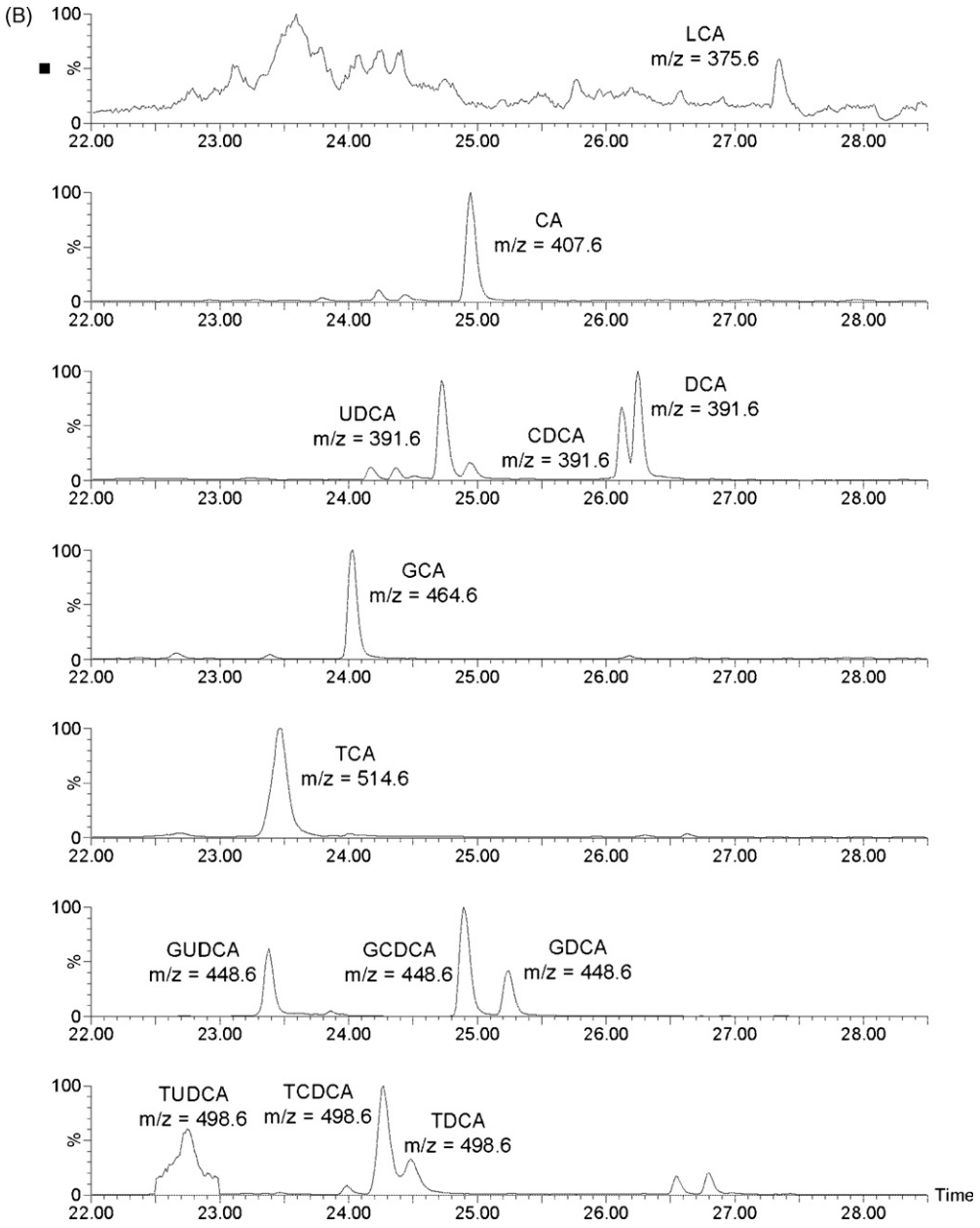

Fig. 3. (*Continued*).

mentioning that the use of a 1.7-μm instead of a 3-μm particle UHPLC column is a must to achieve sufficient resolution to separate "cheno" from "non-cheno" isomers (especially CDCA and DCA as well as TCDCA and TCA; see Fig. 1 for structural information) in a short time span without the use of a complicated mobile, as demonstrated in the chromatograms depicted in Fig. 3 and data provided in Table 1. As described in Section 2, analytes have been detected using MS detection with electrospray ionization in negative mode. Not all the compounds could be fragmented in the second quadrupole to obtain parent–daughter transitions (which increases selectivity) so major ions detected in the first quadrupole were used to detect all of them. As usual when detecting acids, the deprotonated molecule $[MW-H]^-$ were found to be the more abundant ions and were used for detection, providing additional qualitative identification when analysing diagnostic serum samples. The detection features are summarised in Table 1.

3.2. Analytical performance

Table 2 summarises the results obtained. The linear detection range of the BAs was determined by injecting standard solutions with concentrations of the acids ranging from 1 to 1000 ng/g. Since the injected volumes represent serum volumes of up to 100 μL, the most dilute concentration represents an absolute injected amount of ca. 100 pg per acid, which is clinically relevant, and generally sufficient for successful mass spectrometry.

It could be argued that external calibration is not appropriate for samples with matrices as complex as serum. This is true, and its shortcomings could be overcome by performing standard addition analysis, but we discarded this strategy for three reasons. Firstly, as highlighted several times in this communication, sample throughput is of paramount relevance and is not compatible with the standard addition protocol, which necessitates several analysis

Table 2
Analytical parameters of bile acids detection

Analyte	LOD (pg)	LOQ (pg)	Dynamic range (ng/g)	Calibration curve (R^2)	Intra-day precision (% R.S.D., $n = 5$)	Inter-day precision (% R.S.D., $n = 3$)	Average serum concentration (ng/g)	Usual concentration intervals (ng/g)
Tauroursodeoxycholic acid	23	76	1.9–838	$y = 1806x + 6824$ ($R^2 = 0.997$)	9.9	9.5	1.9	–
Glycoursodeoxycholic acid	15	51	10–314	$y = 956.7x + 11139$ ($R^2 = 0.996$)	1.9	11	41.9	–
Taurocholic acid	31	104	2.3–1020	$y = 1943x + 7378$ ($R^2 = 0.998$)	2.0	9.0	54	10.8–223
Glycocholic acid	14	47	10–311	$y = 870.6x + 91.90$ ($R^2 = 0.995$)	1.8	3.7	135	27–452
Taurochenodeoxycholic acid	427	1.4[a]	2.8–653	$y = 1437x + 1535$ ($R^2 = 0.999$)	3.7	9.2	116	11.0–310
Ursodeoxycholic acid	55	182	1.8–127	$y = 793.1x + 1298$ ($R^2 = 0.997$)	7.9	19	21.3	–
Taurodeoxycholic acid	406	1.4[a]	59–1180	$y = 2094x - 29805$ ($R^2 = 0.999$)	3.3	7.6	15	4.0–88.5
Glycochenodeoxycholic acid	53	176	2.3–317	$y = 1130x + 3672$ ($R^2 = 0.999$)	0.9	2.6	355	63.9–1538
Cholic acid	11	38	2.3–156	$y = 1250x + 3490$ ($R^2 = 0.996$)	2.2	8.0	126	3.7–203.1
Glycodeoxycholic acid	63	210	2.5–349	$y = 1245x + 4598$ ($R^2 = 0.999$)	1.4	2.5	130	5.9–409
Chenodeoxycholic acid	98	327	2.5–347	$y = 626.2x + 2157$ ($R^2 = 0.998$)	2.3	16	197	11.0–494
Deoxycholic acid	142	473	2.9–415	$y = 1170x + 5837$ ($R^2 = 0.998$)	1.9	6.8	163	5.1–627
Lithocholic acid	4.4[a]	14.7[a]	14–200	$y = 193.7x + 1579$ ($R^2 = 0.995$)	2.4	23	21.3	2.7–12.8

[a] Data expressed as ng.

(in addition to the analytical replicates) for a single determination. Secondly, the amount of samples needed for a complete standard addition protocol is not compatible with the intrinsically limited nature of serum samples. Thirdly, the rationale for developing the method was to acquire a technique for detecting anomalous levels of BAs (including presence/absence anomalies) in serum samples compared to healthy patients, therefore measurements will always be compared to "reference" standards and, consequently, matrix effects will be compensated.

Nevertheless, the selectivity/specificity of the proposed method was assessed by means of the standard addition method to compare the slopes of the standard addition and the aqueous calibration line. If there are not major matrix interferences, both lines will have the same (or similar) slope. Both calibrations were built with standards in concentrations within the linear range reported previously. The parameters of both regressions were determined following the method described in the next paragraph and the sensitivities were compared using the *t*-test as described by García et al. [21]. The statistics calculated were smaller than the tabulated ones for the significance level set to 0.05 but bigger than the ones tabulated for a significance of 0.1. It can be therefore concluded that no major mistakes are committed when the calibration is carried out in aqueous media and the method is appropriate taking into account the discussion provided in the previous paragraph.

It is well known that, when considering dynamic ranges as wide as that described in the previous paragraph, common least squares regression is not effective for determining linearity. Therefore, our linearity criterion was based on the residuals of the calibration curve: residuals exceeding 5% of the predicted value were discarded. The calibration curves for the bile acids determined were linear in two–three orders of magnitude with coefficients of correlation higher than 0.995 in all cases.

Limits of detection and quantification were determined by means of the injections of Milli-Q water just after the 1-μg/g standard solution. They were calculated by multiplying the standard deviation of the area of the peaks observed, by 3 and 10, respectively. The values obtained vary from 11 to 427 pg (LOD) and from 38 to 473 pg (LOQ), which are in accordance with the expected ones in mass spectrometry. It has been observed some carry-over for taurochenodeoxycholic acid, taurodeoxycholic acid and lithocholic acid, and therefore, the limits of detection and quantification calculated for this compounds result higher than those of the others bile acids. In any case, areas observed for these compounds in blanks have been always smaller than those corresponding to the 1 ng/g standard solution, so that, it can be assure that carry-over is less than 0.1% of the amount injected.

Intra-day precision values (also known as repeatability, precision where independent test results were used with the same method, equipment, operator, on identical test items and within short intervals of time). The results calculated range from 1.0 to 7.6%. Inter-day precision (also known as intermediate precision since only a single factor (change of day) has been investigated) were determined from replicated analysis of serum samples. The values obtained vary from 2.5 to 9% for most of the bile acids, being higher for CDCA (16%), UDCA (19%) and LCA (23%).

The parameters obtained were in the same range as those reported by authors who likewise used HPLC–MS/MS for bile acid quantification in serum [13,17].

The method here developed has been used to determine the concentration of the target analytes in a pool of serum samples obtained from informed healthy volunteers. As the concentrations resulting do not correspond to any of the volunteers, but represent the average values of all of them, it has been included in Table 2

as the "*average serum concentration*" of the analytes determined. A representative chromatogram is depicted in Fig. 3. As demonstrated by the directly comparable standard chromatogram also shown, the peak shape and resolution are acceptable, and there is no apparent interference due to matrix components. For comparison, the usual ranges found in the bibliography for these chemicals are also presented in the table. As can be seen, determined concentrations of the BAs were at levels commonly described in the scientific literature.

4. Conclusions

An on-line RAM–UHPLC–MS method has been developed for determining BAs in serum with reduced sample pre-treatment steps, allowing the complete processing of a single sample in 30 min, which could be further shortened by using a simultaneous sample treatment strategy.

The proposed method allows a low-pressure RAM extraction step to be coupled to an UHPLC–MS separation and detection step, by using a loop-type interface to collect the eluting flow and add a cross-flow to reduce the elution strength of the solvent to be transferred to the head of the chromatographic column. This configuration, which has been developed for BAs, can be adapted to different analytes in serum, such as drugs and small biomolecules, with little setting up time. It could also be used without any major modification for hyphenating UHPLC with other methods commonly used in on-line method applications, such as solid phase extraction.

Further work will be focused on reducing the transfer time of the loop volume into the analytical column. Two different strategies are currently being evaluated. The first exploits the low limits of detection obtained, to split the flow from the loop interface before it enters the column, allowing the use of displacing flows higher than 0.3 mL/min and reducing the time required accordingly. The second is to replace the loop interface by a solid chromatographic trap (i.e. an UHPLC precolumn) capable of withstanding UHPLC pressures and thus to be directly included in the chromatographic path without any detrimental effect on peak shape and resolution.

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