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Direct screening of lyophilised biological fluids for bile acids using an evaporative light scattering detector

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Abstract

The usefulness of lyophilisation for the direct screening of biological fluids for bile acids was investigated. Human serum and urine were lyophilised without losses of the target compounds and further extracted with *n*-hexane in acidic medium under magnetic stirring. An integrated flow injection-liquid chromatographic system coupled to an evaporative light scattering detector (ELSD) was used for automated screening/confirmation. The continuous module allows sequential filtration of the organic phase, solvent changeover and solid-phase extraction for clean-up and preconcentration purposes. Retained bile acids were eluted with an acetonitrile–methanol (65:35, v/v) stream. For screening purposes, the effluent was directly introduced in the ELSD detector and the total bile acid content of the sample determined. For confirmatory analysis, another aliquot of the sample was processed in the screening module but the effluent was directed to the chromatographic columns, which provided the free bile acid profile of the sample. Fasting serum and urine samples obtained from healthy individuals were lyophilised and processed. Good agreement was obtained in the analysis of the two matrices assayed following the screening and confirmatory methods.
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1. Introduction

Lyophilisation can be defined as an operation by means of which water is separated by sublimation from a frozen system or phase [1]. Lyophilised samples are frequently employed in the preparation of control samples for instrument calibration/verification as well as reference materials to increase the stability of the analytes. An indirect objective of lyophilisation is analyte preconcentration in matrices with a high water content as a significant part of the matrix is discarded. Our research group has successively used this sample pretreatment in other application field such as the determination of residues of pesticides in horticultural samples [2–4], being possible the determination of these compounds at concentrations lower than their maximum residue limits without losses.

Biological fluids are among the most troublesome samples in terms of pretreatment because of the large amount of unknown compounds they may contain. Therefore, the analysis of such matrices poses many different analytical problems and frequently involves lengthy sample preparation procedures, which are usually the most complex and difficult to automate [5]. Preconcentration of the analytes is also important; undoubtedly, it helps to reduce limit of

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detection but time-consuming clean-up procedures have to be introduced to avoid contamination of the system and losing resolution due to the presence of matrix components. All these steps already increases the cost of the analysis [6].

The determination of primary (viz. cholic and chenodeoxycholic acids) and secondary (those obtained after enterohepatic circulation) bile acids in serum and other biological matrices is of increasing importance for the diagnosis of hepatobiliary diseases, and other gastro-intestinal problems [7–10]. Notwithstanding this, analytical methods for determination of bile acids in biological fluids are still laborious as the unfavourable bile acid to matrix ratio in the biological specimen requires an extensive pretreatment [11]. Determination of bile acids is usually carried out by using a chromatographic technique. Analytical methods based on either thin layer [12], liquid [13–16] or gas chromatography [17,18] have been reported. The applicability of capillary electrochromatography for the determination of bile acids in lyophilised animal bile has been recently reported [19].

In this work, the use of a simple, continuous method coupled on-line to an evaporative light scattering detector for the direct screening of lyophilised biological fluids for 10 bile acids is proposed for the first time. Biological fluids can be directly processed avoiding protein precipitation, centrifugation and liquid–liquid extraction of the analytes as it is required when working with fresh samples. After screening, the flow configuration is used as a continuous preconcentration/clean-up unit coupled on-line to a liquid chromatograph where only those samples providing an analytical signal close to or above the normal values established for these analytes in healthy individuals were processed. The configuration adopted is based on a manifold previously designed for the determination of six bile acids but requiring manual treatment of the biological fluids [20].

2. Experimental

2.1. Reagents and standard solutions

All reagents were of analytical grade or better. Bile acids (cholic, deoxycholic, glycocholic, lithocholic, chenodeoxycholic, taurochenodeoxycholic, taurodeoxycholic, glycodeoxycholic and glycochenodeoxycholic acids and sodium taurocholate) and Amberlite XAD-7 were purchased from Sigma–Aldrich (Madrid, Spain). HPLC-grade acetonitrile and methanol solvents (Scharlau, Barcelona, Spain) filtered through a Nylon 66 filter (0.45-μm pore size) were used to prepare both the eluent and mobile phase. All other reagents were obtained from Merck (Darmstadt, Germany).

Stock standard solutions of each bile acid at a concentration of 1 mg ml^{-1} were prepared in methanol and stored at 4 °C in the dark. Working standard solutions were prepared as needed by appropriate dilution of the stock in *n*-hexane or aqueous ammonia (pH 8).

2.2. Apparatus

The continuous flow system designed for screening lyophilised biological fluids for bile acids was constructed by using two low-pressure Gilson (Villiers-le-Bel, France) Minipuls-3 peristaltic pumps fitted with poly(vinyl chloride) and Solvaflex pumping tubes for aqueous and organic solutions, respectively, a Rheodyne (Cotati, CA, USA) 5041 injection valve and PTFE tubing (0.5-mm I.D.) for connectors and coils. A laboratory-made sorbent column was constructed by packing a commercial Omnifit glass column (2-cm×2.5-mm I.D.) with ca. 20 mg of Amberlite XAD-7 sorbent material; small cotton beads were used to prevent material losses. A glass column (3-cm×5-mm I.D.) packed with cotton wool was used for filtration purposes.

The chromatographic system consisted of an Hewlett-Packard 1050 high-pressure quaternary gradient pump for delivery of the mobile phase and a tandem-Nova-Pack® C_{18} of two LC cartridge columns (2×150×3.9 mm, 4 μm, Waters, Barcelona, Spain). Stainless steel tubing of 0.5 mm I.D. was used for all connections. An acetonitrile–methanol gradient from 65:35 to 70:30 (held for 5 min) (v/v) at a constant flow-rate of 0.35 ml min^{-1} allowed analyte separation in ca. 15 min at room temperature.

The FI and LC systems were interfaced by means of a six-port high pressure injection valve (Knauer 6332000) connected to a DDL 31 evaporative light scattering detector (Eurosep, Cergy-Portoise, France) through a 50×0.1-mm PEEK tube. The ELSD

detector used air as nebulising gas at a 2.0 bar; the photomultiplier gain was set at 600 V and the evaporation chamber was fixed at 55 °C; signals were registered on a Radiometer REC-80 Servograph recorder (Copenhagen, Denmark).

A Hetossic laboratory freeze-dryer, type CD-53-1 (Birkerod, Denmark) and a magnetic stirrer (Selecta, Barcelona, Spain) were also employed.

2.3. Biological fluids and sample preparation

Fasting urine and serum samples were obtained in the morning from healthy individuals with a negative serological test. Portions of 10 ml of urine or serum samples were placed on a laboratory freeze-dryer and lyophilised by freeze-drying at 6 Pa for 24 h, after which they were conserved in glass containers, at −20 °C in the dark, until analysis. Under these conditions, the concentration of the bile acids remained constant for at least 3 months. An accurately weighed amount of ca. 100 mg of the lyophilised biological fluid (serum or urine) was placed into a 10-ml glass tube and 2 ml of *n*-hexane plus 100 μl of HNO_3–H_2O (1:1, v/v) were added. After the tube was stoppered, the mixture was magnetically stirred for 10 min and allowed to settle. Then, 1 ml of the extract (*n*-hexane phase), containing the analytes, was continuously aspirated and filtered into the flow system depicted in Fig. 1.

2.4. Procedure

The integrated FI-ELSD arrangement designed for the direct screening of lyophilised biological fluids for bile acids and further confirmation of the positive results by FI-LC–ELSD is depicted in Fig. 1. In the screening step, a volume of 1 ml of *n*-hexane phase containing the lixiviated of bile acids was aspirated at a flow-rate of 1 ml min^{-1} (sampling time 1 min) and passed through a cotton filter to prevent suspended matter from reaching the continuous unit. The filtrate was continuously transferred to an evaporation–redissolution unit previously designed by our research group [21] were the *n*-hexane was evaporated under a nitrogen stream as it dropped into a 10-ml graduated glass tube containing 1 ml of weak aqueous ammonia solution (pH 8). Also, bubbling N_2 in the tube favoured homogenisation of the solution. Once evaporation–redissolution was completed, the second pump started the aspiration of the aqueous sample through the Amberlite XAD-7 column (located in the loop of the low pressure injection valve) at a flow-rate of 1 ml min^{-1}; bile acids were retained and the majority of the concomitants wasted. Then, the column was washed with 2 ml of methanol–distilled water mixture (2:8, v/v) to remove potentially adsorbed interferents. Bile acids were eluted by means of an acetonitrile–methanol (65:35, v/v) stream, pumped by the high-pressure pump at a flow-rate of 0.8 ml min^{-1}. In this step, the high-pressure line was directly connected to the ELSD detector by maintaining the HPIV in the load position. Peak height was used as analytical signal and a global response for the total bile acids (as free bile acids) content in the sample therefore obtained. In the confirmatory method, the FI system

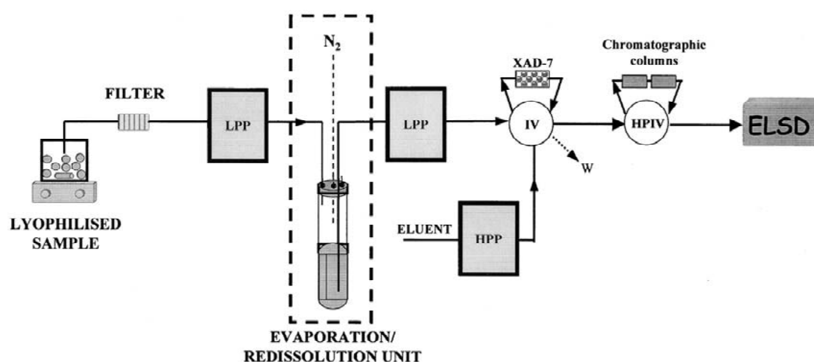

Fig. 1. Scheme of the integrated FI-LC–ELSD system designed for the continuous determination of bile acids in lyophilised biological fluids. LPP, low pressure pump; HPP, high pressure pump; IV, injection valve; HPIV, high pressure injection valve; ELSD, evaporative light scattering detector.

and the LC were connected by switching the HPIV to the injection position 1 min after elution from the Amberlite XAD-7 started; in this way, eluted free bile acids were loaded onto the LC cartridges. The mobile phase (acetonitrile–methanol, 65:35, v/v) gradient started at a flow-rate of 0.35 ml min^{-1} and separated analytes were individually detected and quantified in the ELSD detector.

3. Results and discussion

3.1. Instrumental parameters

This study was carried out by using a standard solution of the 10 bile acids (cholic, deoxycholic, glycocholic, lithocholic, taurocholic, taurochenodeoxycholic, taurodeoxycholic, chenodeoxycholic, glycodeoxycholic and glycochenodeoxycholic acids) in an equimolecular mixture at a global concentration of 50 μ*M* (ca. 25 μg ml^{-1}) in acetonitrile. A volume of 200 μl was injected into an acetonitrile (solvent providing the lowest baseline) stream and directly introduced in the ELSD detector at a flow-rate of 1 ml min^{-1} by means of the high-pressure pump. The three instrumental parameters affecting the sensitivity, namely: evaporation chamber temperature, nebulising gas pressure and photomultiplier gain, were optimised. The evaporation chamber temperature must be selected as a compromise between uniformity of particle size generated and complete solvent evaporation without analyte losses; the best results were obtained fixing this parameter at 55 °C. The nebulising gas pressure (air flow-rate) affects the uniformity and size of the droplets formed and was studied between 0.5 and 2.5 bar. Signal increased as the air pressure increased up to 1.8 bar, remaining then constant; therefore, a working pressure of 2.0 bar was selected as optimal. The photomultiplier gain was studied within the interval 400–700 V as no signal was obtained at lower values. A gain of 600 V was selected as it provides an average sensitivity with an adequate signal-to-noise ratio. The nebuliser was a cleaned monthly by passing an acetone stream at 2 ml min^{-1} through the detector for ca. 10 min, keeping an air pressure of 2.5 bar and an evaporation chamber temperature of 100 °C.

3.2. Sample lyophilisation

Pretreatment of biological fluids usually involves several extraction and clean-up steps to obtain the analytes under the optimum conditions (in terms of sensitivity and selectivity) for the analysis. One of the major limitation of such matrices is the presence of proteins which makes difficult the extraction procedure for two reasons, namely: protein-binding of the analytes of interest and formation of a broad interface that hinders quantitative extraction of the target compounds. When precipitation is used for proteins removal, further centrifugation, filtration of the supernatants and collection of the different extract are required. All these additional steps will potentially decrease the quality of the final results obtained, in terms of accuracy and precision. This negative effect can be dramatically reduced by including a sample lyophilisation step; in addition to the simplification of the extraction procedure, it facilitates both, the storage and conservation of samples. It is worth noting that lyophilised serum samples containing normal and abnormal levels of specific clinical parameters are usually employed to verify clinical analysers.

For this purpose, volumes of 5 ml of urine or serum samples were spiked with the 10 bile acids selected at a concentration of ca. 50 μ*M*; the resulting mixture was split into two fractions. One half (2.5 ml) was placed in a glass tube and mixed with 2.5 ml of methanol for protein precipitation; the mixture was vortex for 15 s, centrifuged (5 min), the supernatant collected, acidified (200 μl of 0.1 *M* HNO_3) and the bile acids extracted with 5 ml of *n*-hexane according to a previously proposed method [22]. The upper phase was transferred to a glass vial, evaporated under nitrogen and the residue redissolved in aqueous ammonia (pH 8) [20]. The aqueous extract was analysed by the proposed FI-LC–ELSD method. The other half was lyophilised, directly extracted with *n*-hexane–nitric acid mixture and the above-described method followed. Results of the experiments (repeated four times) provided the following conclusions: (i) studied bile acids were not evaporated during the lyophilisation process; (ii) although the results were similar for both subsamples, the precision was higher in lyophilised samples; and (iii) the direct extraction of the lyophilised

sample provided cleaner extracts than those of the fresh ones, then being directly processed into the proposed continuous flow system.

3.3. Solid-phase extraction unit

Despite the simplification of the analytical process via the lyophilisation of the biological fluids, a solid-phase extraction (SPE) is required before the analysis of bile acids to remove interfering endogenous compounds and preconcentrate the analytes. The SPE unit implemented in the proposed integrated FI-LC–ELSD configuration acts both as a preconcentration assembly for enrichment and clean-up of bile acids from biological fluids for screening purposes (FI-ELSD method global response) and as introduction system for FI-LC–ELSD where bile acids are separated and individually quantified. Taking into account the similarities with the device previously developed by our group [20] the optimal values for the chemical and flow variables were checked only for the new four bile acids included in this study (taurochenodeoxycholic, taurodeoxycholic, glycodeoxycholic and glycochenodeoxycholic acids).

Amberlite XAD-7 was selected as sorbent against RP-C_{18} as it offered higher selectivity for the bile acids in the biological matrices assayed (maximum retention of the analytes with minimum retention of interfering species). The optimum range of pH was 7–10, all the assayed being carried out at pH 8. The pH was adjusted with dilute ammonia for compatibility with the detector. The amount of sorbent did not affect bile acids retention between 10 and 70 mg, and a column packed with 20 mg of the solid was adopted. The sample flow-rate was fixed at 1.0 ml min^{-1}, while 0.8 ml min^{-1} resulted in the best performance for the eluent, acetonitrile.

Finally, a compromise has to be made between the optimum non-coincident values of the variables affecting both methods (screening and confirmatory) namely: composition and flow-rate of the eluent. Therefore, as a large proportion of acetonitrile (eluent in the screening method) hindered chromatographic separation of the bile acids assayed in the confirmatory method, retained analytes were eluted from the Amberlite XAD-7 column with acetonitile–methanol (65:35, v/v) for compatibility with the initial composition of the mobile phase. As no carry-over resulted from this modification, it was also used as eluent in the screening method. Concerning the change in the flow-rate, it was impossible to achieve a compromise between the two values required for elution and chromatographic separation; therefore, the pump was programmed to reduce the flow-rate from 0.8 to 0.35 ml min^{-1} exactly 1 min after elution of the analytes from the sorbent column started. Finally, the amount of bile acids introduced into the chromatographic column was reduced (to avoid saturation of the stationary phase) by switching the HPIV to the inject position 1 min after starting elution of the analytes from the Amberlite XAD-7 column, thus enabling the introduction of a sample plug of reduced volume (ca. 50%) into the chromatographic system. This operational procedure was followed to construct the individual calibration curves for bile acids using the FI-LC–ELSD method.

3.4. Sensitivity and precision

The manifold depicted in Fig. 1 was used to establish the analytical figures of merit of both, screening and confirmatory methods. The calibration graph for the global response of the 10 bile acids assayed was constructed by aspirating 1 ml of aqueous standard solutions, pH 8, containing different concentrations of the analytes between 2 and 200 μM (1–100 $\mu g\ ml^{-1}$), using three replicates for each standard and 10 standards for the calibration graph. The regression curve obtained was $\log Y = 1.04 \log X - 2.40$ ($r = 0.995$), where Y (V) is the analytical signal and X the total concentration of bile acids (μM). As no blank signal was obtained, the limit of detection was calculated as three times the standard deviation of the peak height for 10 determinations of same sample at the lowest concentration within the linear range up to 2 μM; it resulted to be 0.8 μM. The precision of the method as repeatability, expressed as relative standard deviation, was checked on 11 individual samples containing a total analyte concentration of 50 μM and was found to be ca. 1.0%.

Table 1 summarises the figures of merit for the confirmatory method, obtained under the optimum established conditions. The precision, expressed as relative standard deviation, was calculated for 11

Table 1
Analytical features of the integrated FI-LC–ELSD method used for confirmation of the results

Bile acid	Regression equation	Linear range (μ*M*)	Detection limit (μ*M*)	RSD (%)
T	log Y=1.35 log X−0.75	0.5–25	0.1	4.1
G	log Y=1.20 log X−0.90	0.5–50	0.2	4.5
C	log Y=0.98 log X−0.55	0.5–50	0.2	4.6
DC+CDC	log Y=1.60 log X−1.00	0.5–25	0.1	5.6
L	log Y=1.05 log X−0.85	0.5–50	0.2	3.9
TCDC	log Y=1.30 log X−0.80	0.5–50	0.1	5.1
TDC	log Y=1.38 log X−0.73	0.5–25	0.1	5.3
GCDC	log Y=1.02 log X−0.60	0.5–50	0.2	4.8
GDC	log Y=0.95 log X−0.48	0.5–50	0.2	5.2

T, taurocholic acid; G, glycocholic acid; C, cholic acid; DC, deoxycholic acid; CDC, chenodeoxycholic acid; L, lithocholic acid; TCDC, taurochenodeoxycholic acid; TDC, taurodeoxycholic acid; GCDC, glycochenodeoxycholic acid; GDC, glycodeoxycholic acid; *Y*, analytical signal (mV); *X*, concentration (μ*M*).

standards containing bile acids at individual concentration of 10 μ*M*, was acceptable in all instances.

3.5. Application to biological fluids

The application of the method to the determination of bile acids in lyophilised biological fluids requires the optimisation of the extraction procedure. The most important variables studied were the organic solvent used as extractant, the volume and the time required for quantitative extraction. All these variables were studied using lyophilised serum as model sample. In previous experiments [20] is was found that bile acids extraction from biological fluids using *n*-hexane is favoured in the presence of nitric acid and therefore, an *n*-hexane–nitric acid mixture was assayed for bile acid extraction from the lyophilised biological fluids. Good signals were obtained for a nitric acid concentration higher than 0.1 *M* and therefore, a volume of 100 μl of HNO_3 0.1 *M* was added. Under these conditions, only chromatographic signals for the free bile acids were obtained. Next, the volume of *n*-hexane–nitric acid mixture and the extraction time were optimised. For this purpose, several amounts of 100 mg of lyophilised serum samples were extracted with 2, 4, 6, and 10 ml of *n*-hexane–nitric acid and the mixture was magnetically stirred for different times (2–20 min). In each case, 1, 2, 3 and 5 ml of the extractant (to maintain constant the concentration of bile acids by aspirating one half of the total extract) were introduced in the continuous configuration. The influence of the volume of extractant was negligible, 2 ml thus being selected, and the optimum extraction time was 10 min. Finally, a recovery study of the 10 bile acids included in this study was carried out. They were spiked to lyophilised serum and urine samples at concentrations within the calibration interval. The global concentration provided by the screening and confirmatory methods were coincident. Notwithstanding this, the chromatographic results showed no signals for conjugated bile acids while those of the corresponding free bile acids increased proportionally. It can be ascribed to the hydrolysis of conjugated bile acids to the corresponding free forms under the acidic conditions employed for the extraction. In order to ensure quantitative hydrolysis of the compounds, the concentration of the nitric acid was increased, and thus 100 μl of HNO_3–H_2O (1:1, v/v) were added. The recovery study was then carried out for the free and conjugated bile acids separately. Thus, lyophilised serum and urine samples were analysed before and after spiking with variable amounts of conjugated bile acids and the previously described confirmatory procedure followed. No signal for the conjugated bile acids was obtained in the chromatograms for both unfortified and fortified samples, while the chromatographic peak for free bile acids proportionally increased in the spiked serum and urine samples. In a second step, lyophilised biological fluids were spiked with free bile acids and the samples were analysed following the proposed method. Quantitative results were obtained and the average values ranged from 90 to 95%. From

Table 2
Total concentration of bile acids found in lyophilised fasting serum and urine samples assayed

Sample	Screening method (μ*M*)	Confirmatory method (μ*M*)
Urine 1	2.8±0.1	2.5±0.1
Urine 2	2.1±0.1	1.9±0.1
Serum 1	4.5±0.2	4.6±0.3
Serum 2	7.3±0.4	7.2±0.5
Serum 3	6.4±0.3	6.7±0.3

the above, it can be concluded that the method allows the direct determination of the global bile acids content while in the confirmation of the results, the chromatogram showed that all of them were hydrolysed to their free form during sample treatment as consequence of the concentrated acid added for the quantitative extraction from the lyophilised biological sample.

Finally, fasting serum and urine samples were analysed by using the proposed integrated configuration. The average water content of the real biological fluids assayed was 96% for urine and 91% for serum; an amount of ca. 100 mg of lyophilised sample was taken in all analysis, which corresponded to 1 or 2.5 ml of the serum or urine sample, respectively. The results obtained are listed in Table 2. As can be seen, the values obtained for the screening method were in good agreement with the combined amounts of the individual free compounds found by the LC confirmatory method. In addition, the concentrations in the real biological fluids assayed are within the normal concentration ranges established for them. As an example, the individual concentrations of obtained in some of the samples assayed, for free cholic, deoxycholic+chenodeoxycholic and lithocholic acids after chromatographic separation were: 2.4, 0.3 and <0.2 μ*M* (urine 1); 2.7, 1.4 and 0.5 μ*M* (serum 1); and 2.7, 3.9 and 0.6 μ*M* (serum 2), respectively.

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