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Determination of bile acids by hollow fibre liquid-phase microextraction coupled with gas chromatography

T. Ghaffarzadegan [a,*], M. Nyman [a], J.Å. Jönsson [b], M. Sandahl [b]

[a] Applied Nutrition and Food Chemistry, Department of Food Technology, Engineering and Nutrition, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden
[b] Centre for Analysis and Synthesis, Department of Chemistry, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden

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ABSTRACT

A method based on hollow-fibre liquid phase microextraction combined with gas chromatography was developed for determination of specific bile acids in caecal materials of rats. Nine unconjugated bile acids, including the primary bile acids (cholic acid, chenodeoxycholic acid and α-muricholic acid) and the secondary bile acids (lithocholic acid, deoxycholic acid, ursodeoxycholic acid, hyodeoxycholic acid, β-muricholic acid and ω-muricholic acid) were quantified. Extraction conditions were evaluated, including: sample pH, type of organic solvent and amount of caecal material to be extracted. To compensate for sample matrix effects during extraction the method of standard addition was applied. The satisfactory linearity ($r^2 > 0.9840$), high recovery (84.2–108.7%) and good intra-assay (6.3–10.6%) and inter-assay (6.9–11.1%) precision illustrated the good performance of the present method. The method is rapid, simple and capable of detecting and determining bile acids with limit of detection (LOD) ranged from 0.002 to 0.067 μg/mL and limits of quantification (LOQ) varied from 0.006 to 0.224 μg/mL. The results indicated that the concentration of some secondary bile acids, which usually are associated with health problems, were lower in rats fed with fermentable dietary fibre compared with a fibre free control diet, while the concentration of primary bile acids, usually connected with positive health effects, were higher in rats fed with diets containing dietary fibre. Of the dietary fibres, guar gum and to some extent the mixture of pectin + guar gum had the most positive effects. Thus, it was concluded that the composition of bile acids can be affected by the type of diet.

1. Introduction

Bile acids (BAs), mainly cholic acid and chenodeoxycholic acid, are formed in the liver from cholesterol. These are referred to as primary BAs and they are conjugated with the amino acids glycine and taurine to form water-soluble molecules, before they are secreted into the small intestine via the bile. In the large intestine the microbiota remove glycine and taurine forming the secondary BAs deoxycholic and lithocholic acid [1]. In rodents, alternative reactions give rise to differently structured BAs, particularly α, ß and ω-muricholic acids. The liver produces bile for its digestive function and the BAs are necessary for solubilization and degradation of dietary fat. BAs are the most important degradation products formed from cholesterol and therefore of highest significance for regulation of the amount of cholesterol in man.

In recent years, there has been a great nutritional interest in BAs, and there are suggestions that they are involved both in the development of colon cancer and of metabolic diseases. Thus, a low faecal concentration of secondary BAs has been associated with low incidence of colon cancer in humans [2]. In this respect, it should be mentioned that the colonic formation of secondary BAs from primary BAs is lower at a low pH, caused by a high intake of fermentable dietary fibre [3]. Some secondary BAs have also been associated with positive health effects, which complicate things further [4]. Most studies, however, mainly focus on the effect of dietary fibre on total faecal BAs excreted and not on specific BAs [5,6]. Epidemiological data have also shown an association between a high dietary fibre intake and reduced risk for coronary heart diseases. Different mechanisms are suggested, but one could be an increased faecal loss of BAs due to binding of BAs by the dietary fibre, and as a consequence less amounts of BAs can be reabsorbed in the enterohepatic circulation. Since the enterohepatic pool of BAs has to be constant there must be a renewed synthesis of BAs from cholesterol; thus, reducing body cholesterol and blood cholesterol values. Whether the composition of BAs is of importance in this case, we know less about. However, numbers of interesting physiological effects have been associated with BAs. Some, for example, have been shown to inhibit diet-induced obesity and prevent the development of insulin resistance [7]. With this background, it is

* Corresponding author. Tel.: +46 46 222 8321; fax: +46 46 222 45 32.
E-mail address: Tannaz.Ghaffarzadegan@appliednutrition.lth.se (T. Ghaffarzadegan).

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important to identify suitable methodologies for extraction and determination of the total formation and pattern of individual BAs.

BAs can be extracted by several extraction techniques; including C_{18} solid phase extraction cartridge, ethanol extraction, enzymatic extraction and Soxhlet extraction [8–15]. These methods are, however, all relatively time-consuming. Further, in most above-mentioned methods large amounts of solvents are used, which are more hazardous to health and to the environment and indirectly also expensive [8,9]. So, there is a need for another method. An alternative technique for this purpose is the hollow fibre liquid-phase microextraction technique (HF-LPME) [16] or solvent bar microextraction (SBME) [17]. The advantages of such a technique are; high selectivity, low consumption of organic solvents, good clean-up efficiency, low costs and high enrichment factors [18].

In this work we present for the first time a two-phase hollow fibre liquid-phase microextraction (HF-LPME) method [19,20] for quantitative analysis of BAs, in which the methodology developed is applied on caecal materials from rats. A fibre-free control diet as well as three diets including substrates known to become highly fermented in the large intestine giving different short-chain fatty acid (SCFA) patterns have been fed to rats. Pectin (mainly giving acetic acid), guar gum (propionic acid) and a mixture of pectin + guar gum (butyric acid) were selected for the study [21]. The different SCFA patterns also reflect that different types of microbiota are involved in the fermentation process. The method for BA analysis is based on one-step HF-LPME followed by gas chromatography (GC) for final quantification after derivatization with a mixture of N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), ammonium iodide (NH_4I) and dithioerythritol (DTE) in the ratio (MSTFA:NH_4I:DTE (500:4:2, v/w/w)) [22,23]. The concentration of each BAs in caecum samples was determined by using the method of standard addition. The BAs were extracted from non-spiked and spiked samples with different amounts of caecum material in a similar way as was earlier applied to sewage sludge samples [23]. This analytical approach permits repeated analysis of the limited sample amount that can be obtained from the caecum of a single rat (about 730 mg of dry weight) for quantifying all the relevant BAs in one step.

2. Experimental

2.1. Chemicals and reagents

The following BAs: cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA), hyodeoxycholic acid (HDCA) and 5β-cholanic acid (internal standard, IS) were purchased from Sigma-Aldrich Chemicals Co (Steinheim, Germany), while α-muricholic acid (α-MCA), β-muricholic acid (β-MCA) and ω-muricholic acid (ω-MCA) were obtained from Steraloids, Inc. (Newport, RI, USA).

Methanol HPLC grade, sodium hydroxide and hydrochloric acid were from Merck (Darmstadt, Germany). Di-n-hexylether (DHE), N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA, derivatization grade), tri-n-octylphosphine oxide (TOPO), ammonium iodide (NH_4I), and dithioerythritol (DTE) were supplied by Sigma. Ultra-pure reagent water purified by a Milli-Q gradient system (Millipore, Bedford, MA, USA) was used. The Q3/2 Accurel PP Q3/2 polypropylene hollow-fibre membranes (HF) (200 μm wall-thickness, 600 μm inner diameter, 0.2 μm pore size) obtained from Membrana (Wuppertal, Germany) were used to extract BAs from standard solutions and caecal samples. Microsyringes (with 25 μL volume) from Hamilton (Bonaduz, Switzerland) were applied to collect the solvent in the fibre. The sample pH was measured with a MeterLab mod 210 microprocessor pH-metre (Lund University, Sweden).

2.2. Preparation of standard stock solutions

Stock solutions of each BA were prepared in methanol at a concentration of 1 mg/mL. From the stocks a standard mixture of all BAs was prepared in water at a concentration of 100 μg/mL. The mixture was used for method validation and standard addition in the extraction process. Internal standard (5β-cholanic acid) was prepared in methanol at a concentration of 100 μg/mL. Then 250 μL of this solution was evaporated to dryness with a Mivac concentrator (Lund University, Sweden) and the remaining part was dissolved in 100 μL of the derivatization mixture. All solutions were stored refrigerated at −20 °C until used.

2.3. Preparation of biological samples

Freeze-dried caecal contents from rats were used to validate the present method. The rats had been fed different diets during 28 days to give rise to different patterns of SCFAs and also to different compositions of the microbiota [24]. The selected dietary fibres for the experiment were pectin, guar gum, and a mixture of the two (8 g/100 g, dry weight basis (dwb)), which were included in high fat diets (30 g/100 g (dwb)). A dietary fibre-free control was included. The animals had been fasting for 3–6 h before being killed. Seven animals per diet were analyzed for BAs. Samples of the caecum contents were freeze-dried and stored at room temperature before analysis. The Ethic Committee for Animal Studies at Lund University approved the experiment (application number: M 30–09).

Five caecal samples from each rat were prepared for the standard addition experiment. Sodium hydroxide (0.01 M–5 mL) was added to each of five samples of 20 mg of freeze-dried caecum and incubated for 1 h at 80 °C in order to hydrolyse the sample. Four samples were then spiked with a mixture of BAs to a final concentration of 0.5, 1, 1.5 and 2 μg/mL, respectively. One sample was analyzed without spiking. In the next step, hydrochloric acid (HCl, 0.01 M) was added to the solutions and reagent water to reach the sample volume of 20 mL. The pH of these samples was 6. For extraction, one HF was placed in each sample.

2.4. Hollow-fibre liquid-phase microextraction

The HFs were cut in ca. 8.5 cm pieces and each piece was heat-sealed at the two ends using a hot surface (soldering iron). Then, they were cleaned with acetone and dried overnight. Prior to extraction, the fibres were filled by sonication for 1 h by soaking in the organic solvent (DHE containing 10% (w/v) TOPO). The filled HFs were taken from the organic solvent and shaken in reagent water to wash away any excess of organic solvent from the surface. Each HF was placed in an aqueous caecum sample suspension (20 mL) for extraction. During the extraction, the solution was stirred at 600 rpm on a magnetic stirrer (IKA-WERKE, Staufen, Germany), permitting up to 10 samples to be simultaneously extracted. After 2 h extraction, the HF was taken out, the ends were cut and the solvent in the lumen was collected by pushing through one end of the fibre with a micro syringe. Normally, 18 μL of acceptor solvent was obtained from each fibre. Into 10 μL of this solvent, 8 μL of derivatization mixture (MSTFA:NH4I:DTE (500:4:2, v/w/w)) and 2 μL of internal standard (dissolved in the derivatization mixture) were added for derivatization at 60 °C in a water bath for 30 min. Afterwards, 1 μL of the derivatized extract was injected into the GC for final analysis.

2.5. Apparatus and instrumental conditions

All analyses were performed using an Agilent 6890 N series gas chromatograph equipped with a split/splitless injector and an Agilent 7683 B series auto-sampler (Agilent Technologies Inc., Santa