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Comprehensive quantitative analysis of fatty-acyl-Coenzyme A species in biological samples by ultra-high performance liquid chromatography–tandem mass spectrometry harmonizing hydrophilic interaction and reversed phase chromatography

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ABSTRACT

Fatty acyl-Coenzyme A species (acyl-CoAs) are key biomarkers in studies focusing on cellular energy metabolism. Existing analytical approaches are unable to simultaneously detect the full range of short-, medium-, and long-chain acyl-CoAs, while chromatographic limitations encountered in the analysis of limited amounts of biological samples are an often overlooked problem. We report the systematic development of a UHPLC–ESI-MS/MS method which incorporates reversed phase (RP) and hydrophilic interaction liquid chromatography (HILIC) separations in series, in an automated mode. The protocol outlined encompasses quantification of acyl-CoAs of varying hydrophobicity from C2 to C20 with recoveries in the range of 90–111 % and limit of detection (LOD) 1–5 fmol, which is substantially lower than previously published methods. We demonstrate that the poor chromatographic performance and signal losses in MS detection, typically observed for phosphorylated organic molecules, can be avoided by the incorporation of a 0.1% phosphoric acid wash step between injections. The methodological approach presented here permits a highly reliable, sensitive and precise analysis of small amounts of tissues and cell samples as demonstrated in mouse liver, human hepatic (HepG2) and skeletal muscle (LHCNM2) cells. The considerable improvements discussed pave the way for acyl-CoAs to be incorporated in routine targeted lipid biomarker profile studies.

1. Introduction

Fatty acids originating from the diet or *de novo* synthesized are intracellularly processed in their activated form following esterification with Coenzyme A. Fatty acyl-CoAs are key effectors at the crossroads of converging metabolic pathways [1] and thought to act as signaling intermediates in insulin resistance and related diseases [1,2]. Despite extensive research on the role of acyl-CoAs in mitochondrial dysfunction and the metabolic syndrome, details of direct mechanisms have not yet been identified, highlighting the need for more robust analytical methods to enable comprehensive profiling of acyl-CoAs in different matrices and models. While liquid chromatography followed by electrospray ionization tandem mass spectrometric detection (LC–ESI-MS/MS) has been routinely employed, most methods are capable of only detecting either short-, medium-, or long-chain acyl-CoAs [3–8]. Although their MS detection is well characterized, chromatography remains the most challenging part of the technique due to the structural complexity of the compounds. Even with advances in the latest generation of LC–MS systems and their superior analytical power *per se*, problems encountered with small amounts of biological samples requiring enhanced sensitivity have not been adequately addressed. The majority of published protocols rely on the well-established selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) scan mode and monitor the transition that yields the most abundant fragment of acyl-CoAs formed by the cleavage of the 3′-phosphate-adenosine-5′diphosphate subunit of the CoA part of the molecule (Fig. S1) [3,5,7,9–15]. Neutral loss [9,16,17] and precursor ion scanning [11,18] have also been utilized, enabling a non-targeted profiling approach. In comparison, reported chromatographic approaches vary considerably implying that an optimal method has not been established yet. To adapt for ESI-MS detection, phosphate buffer used in earlier protocols employing LC separation by UV at slightly

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acidic pH (4.5–5.5) is substituted with a volatile buffer(s) such as ammonium-formate [4,12,19] or highly alkaline mobile phase conditions (pH ~10–11) adjusted with NH_4OH solution [7,17]. Methods applying ion pairing chromatography using triethyl amine [10] or dimethylbutyl amine have also been reported [6].

Depending on chain length, acyl-CoAs vary greatly in polarity, and this has become the bottleneck in developing comprehensive methods [4,6,7,18,19]. In addition, severe peak tailing, signal deterioration and poor detection limits are often observed in LC–MS analysis of acyl-CoAs, especially for later eluting species [8,9,16,18–20]. In comparison, the potential of hydrophilic interaction liquid chromatography (HILIC) for the highly polar acyl-CoAs has not been fully explored [21].

We initially conducted experiments to better understand what causes the poor chromatographic behavior of acyl-CoAs. This approach facilitated the development of an improved automated UHPLC-ESI-MS/MS analytical protocol combining both reversed phase (RP) and HILIC separations in series, capable of resolving and quantifying acyl-CoAs in biological tissues ranging from short-chain (e.g. acetyl (C2)) to long-chain (e.g. arachidonoyl (C20)).

2. Experimental section

2.1. Chemicals

Crystalline malonyl-CoA (C3, referred to as malonyl), ($^{13}C_3$)malonyl-CoA, 3-hydroxybutyryl-CoA (3OH-C4), acetyl-CoA (C2), butyryl-CoA (C4), isovaleryl-CoA (*i*C5), *n*-heptanoyl-CoA (C7), *n*-ocanoyl-CoA (C8), lauroyl-CoA (C12), palmitoleoyl-CoA, *cis*-9 (C16:1), palmitoyl-CoA (C16), *n*-heptadecanoyl-CoA (C17), oleoyl-CoA, *cis*-9 (C18:1), stearoyl-CoA (C18), arachidonoyl-CoA (C20), high purity ammonia solution and glacial acetic acid (Trace SELECT), LC–MS grade ammonium bicarbonate, triethylammonium acetate (TEAA) and dexamethasone were purchased from Sigma-Aldrich (Dorset, UK). The *n*-hexanoyl-CoA (C6), *n*-decanoyl-CoA (C10), myristoyl-CoA (C14) and linoleoyl-CoA, *cis*-9, *cis*-12 (C18:2) were from Avanti Polar Lipids (Alabaster, AL, USA) and *n*-pentanoyl-CoA (C5) was from Advent Bio (Downers Grove, IL, USA). Newborn calf serum (Invitrogen 16010159), horse serum (Invitrogen 16050130) and basic fibroblast growth factor (bFGF, Gibco PHG6015) were from Thermo Fisher Scientific and epidermal growth factor (EGF, AF-100-15) was from PeproTech (London, UK). All solvents were of UHPLC–MS grade and water used was of high purity (18.2 MΩ cm^{-1}, Milli-Q, Merck Millipore).

2.2. Liquid chromatography

A Thermo Fisher Scientific Vanquish binary UHPLC (Thermo Scientific, Santa Clara, USA) system was used, with a Waters Acquity BEH C18 (2.1 × 50 mm, 1.7 μm) and a BEH HILIC (2.1 × 50 mm, 1.7 μm) column, in-line with guard columns (VanGuard, 2.1 × 5 mm) of matching stationary phases. During the initial stages of developing the chromatographic separations a UV-DAD detector was connected in line to monitor signals at 260 nm, the optimum wavelength for acyl-CoA species [22]. Columns were thermostatted at 30 °C, and a switching valve was used to divert mobile phase to one column at a time at a flow rate of 0.7 ml/min (Fig. 1). Mobile phase A1 consisted of ammonium bicarbonate (10 mM), adjusted to pH 8.5 with high purity ammonia solution, and acetonitrile (ACN) was used as mobile phase B1. Two solutions were introduced to clean the system between injections. Water/ACN/phosphoric acid 40/60/0.1 (v/v) (pH ≈ 2.5) was used as an 'acidic wash' (B2 channel), and 50/50 ACN/water (channel B3) as a 'neutral wash'. For RP separation, gradient elution started at 95% A1/5% B1 and after 0.2 min hold time, B1 was increased linearly to 45% at 1.0 min, 65% at 3.0 min and to 100% at 3.2 min and kept at 100% B1 until 5.0 min. At 5.01 min, 100% B1 was changed to 100% B2 to initiate the 'acidic wash' to pump B. At 5.1 min, eluent was diverted to waste for the cleaning protocol, bypassing the MS, by use of a diverter valve inserted in the flow path between the LC and the MS. After 3 min of acidic wash, at 8.0 min, the selection valve of pump B was switched to 100% B3 'neutral wash' to remove the acidic mobile phase from the system. Following rinsing for 3 min (11.01 min), 100% B1 was selected to exchange mobile phase to 'B1', and after 3 min initial mobile phase composition settings (95% A1/5% B1) were applied. The system was equilibrated for 3 min (until 17.0 min) before the next injection. Eluent flow was diverted back to the MS at 10.5 min.

The gradient program for the HILIC separation started at 5% A1/95% B1, and B1 was decreased linearly to 70% at 3.0 min, 50% at 3.2 min and kept at 50% until 5.0 min. Eluent was diverted to waste at 5.0 min and mobile phase composition was set to 100% B2 ('acidic wash'). After 3 min (8.0 min total), 100% B3 ('neutral wash') was introduced to remove the acidic solution from the system. Following 3 min rinsing, initial mobile phase composition settings (5% A1/95% B1) were applied and the system was equilibrated for 4 min (until 15.0 min) before the next injection and eluent flow was diverted back to the MS at 14.0 min. The total run time for both the analytical protocols in series and the wash step was 32 min.

2.3. Mass spectrometry

A TSQ Quantiva triple quadrupole MS/MS instrument (Thermo Scientific) equipped with a heated electrospray ion source (HESI) was used in positive ion mode with the following settings: spray voltage: 3500 V, sheath gas: 56 (Arb) ≈ 6.2 L/min, auxiliary gas 18 (Arb) ≈ 11.3 L/min, sweep gas: 2 (Arb) ≈ 2.7 L/min, ion transfer tube temperature: 368 °C, vaporizer temperature: 460 °C. When the eluent flow of the LC was not delivered to the MS, the spray voltage on the HESI needle was set to 0V. Mass spectrometer settings for each acyl-CoA were optimized by using commercially available standards (Table S1).

2.4. Sample preparation

Mouse liver tissue (C57BL/6) was purchased from Seralab (Sera Laboratories International Ltd, West Sussex, UK) and pulverized in a cryogenic tissue homogenizer (Cellcrusher Limited, Ireland). 10–30 mg of frozen powder was weighed in a polypropylene vial and an appropriate volume of ice cold extraction solvent mixture was added according to Minkler et al. [23]. Samples were spiked with 20 μL of 2.5 μM ISTD spiking mix (($^{13}C_3$)malonyl- and C17-CoA in MeOH containing 10 mM TEAA) and kept in a dry ice/ethanol cooling bath until further processing by homogenization (Omni TH, Camlab, UK) and solid phase extraction (SPE) as described below.

Human hepatocellular carcinoma cells, HepG2 (HB-8065, ATCC, LGC Promochem, UK), and LHCNM2 immortalized human skeletal muscle cells (a gift from Dr V. Mouly, UMR S 787, INSERM & UPMC, Institut de Myologie, Paris, France) were grown at 37 °C in humidified air with 5% CO_2 as per standard protocols. LHCNM2 cells were grown on 0.2% gelatin-coated tissue culture dishes and differentiation was induced at ~95% confluence by replacing growth medium (Dulbecco's minimum essential modified medium, DMEM, 10% heat-inactivated fetal calf serum, 10% newborn calf serum, EGF, bFGF, dexamethasone) with differentiation medium (DMEM, 2% horse serum) for a period of 5 days before cell harvesting. For analysis, cells were washed twice and collected in ~0.2 mL phosphate buffered saline (PBS) containing phosphatase and protease inhibitors (Roche Diagnostics, Sigma, UK). Cell suspensions were transferred to tubes containing 0.4 mL acetonitrile: isopropanol (3:1 v/v), and extracted for 30 s with a cell disruptor (Disruptor

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