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Underivatized oxysterols and nanoLC–ESI-MS: A mismatch

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ABSTRACT

Due to their non-charged character, liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS) measurements of oxysterols are often performed after derivatization with e.g. charged Girard reagents. However, derivatization reactions are time-consuming and may require numerous steps to remove excess reagent. In addition, extensive sample handling can be associated with cholesterol autoxidation, resulting in analyte artifacts and hence false positives.

Nano scale liquid chromatography in combination with electrospray-mass spectrometry (nanoLC–ESI-MS) is a powerful tool for analyzing limited samples, due to substantially increased sensitivity compared to conventional LC–ESI-MS. The signal enhancement may compensate for the poor ionization of the oxysterols; hence we have explored the possibility to quantify oxysterols without derivatization using nanoLC–ESI-MS.

Non-derivatized oxysterols and nanoLC were however not compatible, due to persistent and large carry-over. This was attributed to the extended contribution of surface to volume ratio in such miniaturized systems and interactions with the materials of the nanoLC instrumentation (e.g. adsorption to the fused silica tubing).

Two contemporary MS instruments (Q-Exactive™ hybrid quadrupole-Orbitrap and TSQ Quantiva™ triple quadrupole) were used. However, both the MS and MS/MS spectra of non-derivatized oxysterols were ambiguous and/or unrepeatable for both of the instruments employed.

Derivatizing oxysterols is more cumbersome, but provides more selective and reliable results, and Girard derivatization + nanoLC–ESI-MS continues to be our recommended choice for measuring oxysterols in very limited samples.

These investigations also indicate that extra care should be taken to remove lipids prior to nanoLC of other analytes, as adsorbed oxysterols, etc. can compromise analysis.

1. Introduction

Oxysterols are hydroxylated cholesterols, formed either enzymatically or by autoxidation. Oxysterols have a great number of biological roles, dealing with e.g. regulation of cholesterol homeostasis [1], acting as selective estrogen receptor modulators [2], activating liver X receptors [3] and possibly the hedgehog signaling pathway [4], in addition to being a biomarker for Niemann–Pick type C1[5]. The neutral nature of oxysterols such as 24S-, 25- and 27-hydroxycholesterol (24S-OHC, 25-OHC and 27-OHC) makes their measurements challenging with ESI-MS, the standard instrumentation for identifying and measuring compounds separated by LC. Consequentially, several strategies for derivatizing oxysterols into chargeable species have been developed, e.g. derivatization with Girard reagents [6]. Such approaches can enable mass detection limits in pg–fg levels [6–10].

NanoLC (LC columns with inner diameters (ID) less than 0.1 mm) is a tool for increasing sensitivity in combination with ESI-MS (or other concentration-sensitive detectors), as compounds undergo significantly less radial dilution during the separation process compared to conventional LC; for instance a 0.1 mm ID column should give a theoretical 400-fold signal increase compared to a 2.1 ID mm column. Additional sensitivity can be achieved by large volume injection using an online solid phase extraction

Abbreviations: LC, liquid chromatography; ID, column inner diameter; nanoLC, nano scale liquid chromatography; microLC, micro scale liquid chromatography; MS, mass spectrometry; ESI, electrospray ionization; nanoESI, nano electrospray ionization; 25-GT, girard T derivate of 25-hydroxycholesterol; 25-OHC, 25-hydroxycholesterol; 24S-OHC, 24S-hydroxycholesterol; 27-OHC, 27-hydroxycholesterol; 22S-OHC, 22S-hydroxycholesterol; MeOH, methanol; FA, formic acid; IPA, 2-propanol; AF, ammonium formate; AF/FA, 2.5 mM ammonium formate and 0.25% formic acid; SPE, solid phase extraction.

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(SPE) column [11]. Nanospray ESI (nanoESI) allows for a larger percentage of the analytes to enter the MS, enhancing sensitivity [12,13], in addition to produces smaller charged droplets, which gives less ion suppression [14]. Although nanoLC–ESI-MS is more technically demanding to operate it is increasingly employed, particularly in proteomics, where it has become a standard approach. In metabolomics, the use of nanoLC is still very limited. However, nanoLC–ESI-MS can allow analysis of very small samples, and attomolar concentrations of 24S-OHC, 25-OHC and 27-OHC (derivatized with Girard T reagent) could be measured in just 10,000 pancreatic cancer cells, with excellent repeatability [8].

A disadvantage of charge-tagging oxysterols is that it can be time consuming (reactions can require elevated temperatures and overnight treatment), and may require considerable manual efforts, although several steps e.g. SPE clean-up (for removal of excess derivatization reagent) can be automated [8,15]. We therefore wanted to investigate whether the enhanced sensitivity of nanoLC–ESI-MS could compensate for the poor ionization of underivatized oxysterols, to allow sufficient detection of oxysterols in limited samples (e.g. million-scale cell numbers) in a shorter time than our present method [8]. This was partly inspired by the study of McDonald et al. [10] who could monitor oxysterols-salt complexes (along with a number of other lipids) in biological samples using ESI and an AB Sciex API 5000 triple quadrupole mass spectrometer without derivatization. Other ionization sources such as APPI [16] and APCI [17,18] were not considered as they are not compatible with the low flow used in nanoLC.

In the present study, the traits of nanoLC–ESI-MS for detecting Girard "charge-tagged" oxysterols and underivatized oxysterols have been compared, using two contemporary mass spectrometers (Q-Exactive™ hybrid quadrupole-Orbitrap and TSQ Quantiva™ triple quadrupole). MicroLC–ESI-MS methods for native and derivatized oxysterols using a column with similar reversed phase material were included for comparison.

2. Experimental

2.1. Chemicals and reagents

For microLC and nanoLC of derivatized oxysterols mobile phase A consisted of 0.1% Formic acid (FA) in type 1 H_2O (Millipore, Billerica, MA, USA) and mobile phase B consisted of 0.1% FA in MeOH (Hipersolv grade, VWR, Radnor, PA, USA). For determination of native oxysterols, mobile phase A consisted of 2.5 mM ammonium formate (LC–MS quality, Fluka, Sigma Aldrich, St. Louis, MO, USA) and 0.25% FA (AF/FA) in type 1 H_2O and mobile phase B consisted of AF/FA in MeOH, ethanol (EtOH, absolute, AnalaR Normapure, VWR) or 2-propanol (IPA, p.a, Sigma Aldrich). Ammonium acetate and acetic acid (both of LC–MS quality, Fluka, Sigma Aldrich) were also used as mobile phase additives.

A stock solution of 188 μg/mL cholest-5-ene-3β, 25-diol (25-OHC, Sigma Aldrich) was prepared by dissolving 25-OHC in 2-propanol (Rathburn chemicals Ltd., Walkerburn, Scotland, UK). This stock solution was diluted with mobile phase (50% B) to suitable concentrations before injections.

Other standard solutions for Girard T derivates were prepared as previously described in [8].

2.2. Direct infusion

A solution of 1 μg/mL 25-OHC in IPA was derivatized to 25-GT with Girard T reagent as described elsewhere [8]. To remove excess Girard T reagent, a 100 mg C_{18} Isolute (Biotage, Uppsala, Sweden) SPE column was used. The SPE column was conditioned with 1 mL MeOH followed by 1 mL type 1 H_2O and 2 mL $MeOH^+$ type 1 H_2O (1 + 1). The derivatized standard solution (700 μL in MeOH) was applied and the flow-through collected. The flow-through was diluted to approximately 30% MeOH and reapplied on the SPE column followed by a wash with 2 mL type 1 H_2O. The 25-GT was eluted using 1 mL MeOH and 1 mL chloroform (AnalaR, VWR), evaporated into dryness and re-dissolved in 700 μL 0.1% FA in MeOH.

Solution for direct infusion of the native oxysterol 25-OHC was diluted from IPA stock solution with AF/FA in MeOH.

The solutions were infused with a flow rate of 1 μL/min using a direct infusion pump, 30 μm ID silanized fused silica tubing (see below), 30 μm ID stainless steel emitters and a nanospray flex ion source on both Q-Exactive™ hybrid quadrupole-Orbitrap and TSQ Quantiva™ triple quadrupole mass spectrometers (Both from Thermo Scientific, Waltham, MS, USA).

2.3. LC–MS

MicroLC of Girard T derivates was performed with the same chromatographic condition as described in [15], but detection was performed with the Q-Exactive™ hybrid quadrupole-Orbitrap. The injected solution for microLC contained 14 nM 24S-GT, 25-GT, 27-GT and 22S-GT. For nanoLC the injected solutions contained 30 pM 24S-GT, 25-GT, 27-GT and 22S-GT and nanoLC of Girard T derivates of oxysterols were performed with the same equipment and same chromatographic conditions as previously described [8].

For native oxysterols a 1 mm ID × 100 mm ACE 3 C_{18}-PFP column was used in microLC, while a 0.1 mm ID × 100 mm ACE 3 C_{18} column was used in nanoLC, as C_{18}-PFP was not available in nano-dimensions at the time of study. The microLC flow rate was 40 μL/min and 1 μL of 800 ng/mL 25-OHC was injected. For nanoLC of native oxysterols, a column switching system as described in [8] was used. Loading mobile phase was mobile phase A (5 μL/min) and all tubing were silanized by flushing them with 5% chlorotrimethylsilane (Sigma Aldrich) in n-heptane (p.a, Merck, VWR) as described in [19]. The nanoLC flow rate was 500 nL/min and 2 μL of 15 ng/mL 25-OHC was injected. In micro LC the mobile phase composition went from 70% to 100% B in 15 min and was held at 100% B for 10 min, while in nanoLC the mobile phase composition went from 70% to 100% B in 20 min and was held at 100% B for 20 min.

2.4. Carry-over

To localization carry-over effects, a variety of solutions, columns and equipment were used. For details see Supplementary Table S1.

3. Results

The goal of our study was to develop a fast and easy nanoLC–MS method for determination of oxysterols in small cell samples. Preliminary experiments were conducted with different side-chain hydroxylated oxysterols (e.g. 24S-OHC, 27-OHC) and deuterated internal standard. However, as all these compounds behaved similar in the nanoLC–MS system, only results for 25-OHC is shown in the following section.

3.1. Adduct formation and fragmentation of native and derivatized oxysterols

Direct infusion of 25-GT in 0.1% FA in MeOH using a Q-Exactive™ hybrid quadrupole-Orbitrap with full MS mode (Fig. 1A) showed ample signal of the desired molecular ion m/z 514.436 $[25\text{-GT}]^+$. MS/MS fragmentation with the HCD collision