Biochemical and Biophysical Research Communications 446 (2014) 756–761

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Evaluation of novel derivatisation reagents for the analysis of oxysterols

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ARTICLE INFO

Article history:
Available online 10 February 2014

Keywords:
Girard P reagent
Derivatisation
Liquid chromatography
Mass spectrometry
Sterols

ABSTRACT

Oxysterols are oxidised forms of cholesterol that are intermediates in the synthesis of bile acids and steroid hormones. They are also ligands to nuclear and G protein-coupled receptors. Analysis of oxysterols in biological systems is challenging due to their low abundance coupled with their lack of a strong chromophore and poor ionisation characteristics in mass spectrometry (MS). We have previously used enzyme-assisted derivatisation for sterol analysis (EADSA) to identify and quantitate oxysterols in biological samples. This technique relies on tagging sterols with the Girard P reagent to introduce a charged quaternary ammonium group. Here, we have compared several modified Girard-like reagents and show that the permanent charge is vital for efficient MS^n fragmentation. However, we find that the reagent can be extended to include sites for potential stable isotope labels without a loss of performance.

1. Introduction

Oxysterols are neutral lipids formed by oxidation of cholesterol either by enzymatic oxidation (especially by enzymes of the cytochrome P450 family) or by autoxidation. As well as acting as key components of the bile acid biosynthesis pathways [1], oxysterols have been shown to bind to liver X receptors (LXRs) [2,3], retinoic acid related orphan receptors (RORs) [4] and the G protein-coupled receptor Epstein–Barr virus induced gene 2 (EBI2) [5,6]. Very recently (25R)26-hydroxycholesterol has been shown to promote proliferation of breast cancer cell lines by binding to the estrogen receptor (ER) α [7,8].

Sterols are typically under-represented in global lipidomics studies which are dominated by charged species (e.g. phospholipids) or those readily ionised due to the presence of a functional group such as an amine (e.g. sphingosine, sphingomyelin). The sterolome itself is dominated by cholesterol which is present at a level three orders of magnitude higher than the most abundant oxysterols. In addition, the lack of a strong chromophore and poor ionisation characteristics in mass spectrometry (MS) make the analysis and accurate quantitation of oxysterols challenging. Gas chromatography (GC)–MS with prior derivatisation to trimethylsilyl ethers is considered the 'gold standard' for oxysterol analysis but a number of liquid chromatography (LC)–MS approaches have also been reported (for a recent review see Ref. [9]).

Chemical derivatisation is commonly used in LC–MS methods to improve the ionisation characteristics of the biological molecules of interest [10]. An effective derivatisation reagent should: (1) react in near-quantitative yield with the analyte of interest (e.g. by 'click' chemistry [11]); (2) incorporate a permanently charged or readily ionised functional group (e.g. an amine); and preferably (3) be amenable to stable-isotope labelling for use in relative and absolute quantitation experiments.

We have developed an LC–electrospray ionisation (ESI)–MS method including enzyme-assisted derivatisation for sterol (or steroid) analysis (EADSA) [12]. Sterols are enzymatically oxidised using bacterial cholesterol oxidase before 'charge-tagging' with the Girard P (GP) reagent (Fig. 1A). This incorporates a permanently charged quaternary ammonium group which increases solubility in reversed-phase solvents, improves ionisation by several orders of magnitude, and directs fragmentation of the sterol backbone to provide structurally informative MS^n (MS with multistage fragmentation) spectra.

Abbreviations: 3β-HCA, 3β-hydroxycholest-5-en-(25R)-26-oic acid; 4-DMAP, 4-dimethylaminopyridine; API, atmospheric pressure ionisation; EADSA, enzyme-assisted derivatisation for sterol (or steroid) analysis; EBI2, Epstein–Barr virus induced gene 2; ER, estrogen receptor; ESI, electrospray ionisation; GC, gas chromatography; GP, Girard P; LC, liquid chromatography; LIT, linear ion trap; LXR, liver X receptor; MRM, multiple reaction monitoring; MS, mass spectrometry or mass spectrum; MS^n, MS with multistage fragmentation; RIC, reconstructed ion chromatogram; ROR, retinoic acid related orphan receptor.

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http://dx.doi.org/10.1016/j.bbrc.2014.01.173
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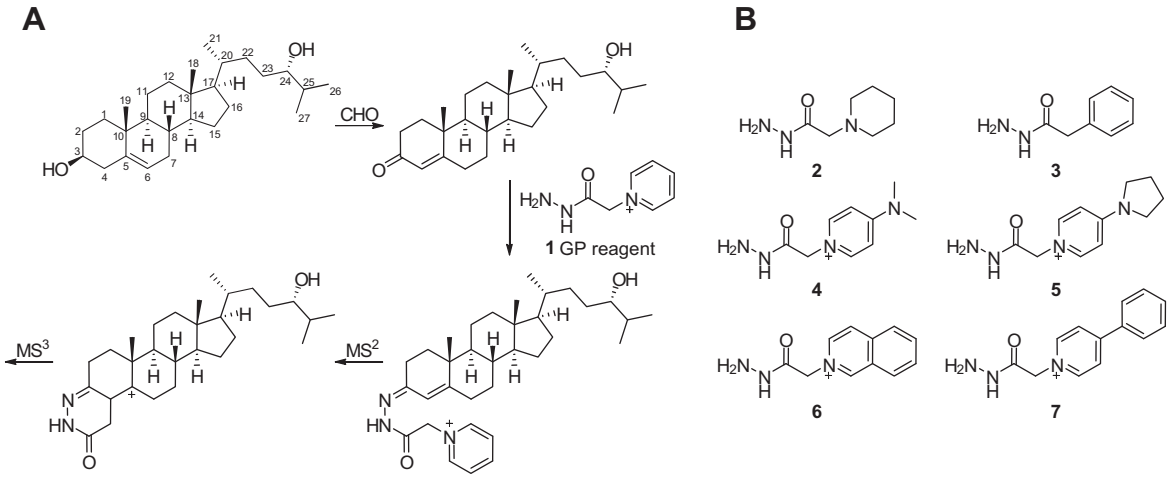

Fig. 1. (A) Numbering of the cholesterol backbone and outline of EADSA exemplified with 24S-hydroxycholesterol. (B) Novel derivatisation reagents **2**–**7** used in this study. CHO: cholesterol oxidase; GP: Girard P.

As part of a project to incorporate isotope labels into the GP reagent (Crick et al., manuscript in preparation [13,14]) we have synthesised and tested a range of charge tags incorporating a variety of functional groups. Here we evaluate the performance of these reagents and discuss their merits and limitations.

2. Materials and methods

A list of the sources of reagents and equipment along with a detailed protocol for EADSA is given in Ref. [12].

2.1. Solvents and reagents

HPLC grade solvents were from Fisher Scientific (Loughborough, UK). GP reagent (1-(carboxymethyl)pyridinium chloride hydrazide) was from TCI Europe (Oxford, UK) and 2-(1-piperidinyl)acetohydrazide was from Fluorochem (Hadfield, UK). Phenylacetic hydrazide and cholesterol oxidase from *Streptomyces* sp. were from Sigma–Aldrich (Dorset, UK). Certified Sep-Pak tC18 200 mg and Oasis HLB 60 mg cartridges were from Waters (Elstree, UK). Pooled serum from male AB plasma was from Sigma–Aldrich. Chemicals used for the synthesis of derivatisation reagents **4**–**7** were from Sigma–Aldrich.

2.2. Synthesis of derivatisation reagents

Charge tags were synthesised using a modification of the procedure reported by Girard and Sandulesco [15]. A solution of heterocycle (4-dimethylaminopyridine, 4-pyrollopyridine, isoquinoline or 4-phenylpyridine, 12.6 mmol) and ethyl bromoacetate (1.4 mL, 12.6 mmol) in absolute ethanol (10 mL) was heated at reflux for 4 h. The mixture was slowly cooled to 0 °C and hydrazine hydrate (788 μL of 78–80% solution in H_2O, ~12.6 mmol) was added dropwise. The resulting white precipitate was recovered by filtration and dried under reduced pressure to afford the desired derivatisation reagent (**4**, **5**, **6** or **7**) in 80–95% yield.

2.3. Extraction of oxysterols from serum

Serum (100 μL) was added dropwise to a solution of 24(R/S)-[25,26,26,26,27,27,27-$^2H_7$] hydroxycholesterol (20 ng) in absolute ethanol (1.05 mL total volume) in an ultrasound bath. After 5 min, water (350 μL) was added, ultrasonicated for a further 5 min then centrifuged at 14,000*g* for 30 min at 4 °C. A Waters Sep-Pak tC18 cartridge was rinsed with 4 mL of absolute ethanol followed by 6 mL of 70% ethanol and the serum in 70% ethanol (1.5 mL) was applied to the column. The flow-through was collected and combined with a wash of 5.5 mL of 70% ethanol to give SPE-1-Fr-1 containing oxysterols. The cartridge was washed with a further 4 mL of 70% ethanol to give SPE-1-Fr-2, before cholesterol was eluted with 2 mL of absolute ethanol (SPE-1-Fr-3). Finally, the column was washed with 2 mL of absolute ethanol to give SPE-1-Fr-4. The fraction containing oxysterols (SPE-1-Fr-1) was dried under reduced pressure using a vacuum concentrator.

2.4. Enzyme-assisted derivatisation

The dried fractions were reconstituted in 100 μL propan-2-ol and 1 mL potassium phosphate buffer (50 mM, pH 7) containing 3 μL of cholesterol oxidase (2 mg/mL H_2O, 44 U/mg protein) was added. The mixture was incubated at 37 °C for 1 h when the reaction was stopped by addition of methanol (2 mL). Acetic acid (150 μL) was added followed by 0.8 mmol of the derivatisation reagent to be tested. The mixture was vortexed until all of the solid was dissolved then incubated at room temperature overnight in the dark.

2.5. Removal of excess reagent

To remove excess reagent while retaining all of the compounds of interest, a recycling procedure was used. The procedure is similar to that previously reported [12] but a Waters Oasis HLB 60 mg cartridge was used in place of the Sep-Pak tC18.

An Oasis HLB cartridge was pre-conditioned with methanol (6 mL), 10% methanol (6 mL) and finally 70% methanol (4 mL). The reaction mixture (in ~3 mL 70% methanol) was loaded onto the cartridge and the flow-through collected. The column was washed with 70% methanol (1 mL) followed by 35% methanol (1 mL). Water (4 mL) was added to the combined eluent to give ~9 mL 35% methanol. This solution was applied to the same cartridge and the flow-through collected, along with a wash of 17% methanol (1 mL). Water (9 mL) was added to the eluent to give ~19 mL 17% methanol. This solution was applied to the column followed by a wash of 10% methanol (6 mL). Finally, the oxysterols were eluted from the cartridge using methanol (3 × 1 mL) then ethanol (1 mL) to give SPE-2-Fr-1, Fr-2, Fr-3 and Fr-4. Aliquots of SPE-2-Fr-1 and Fr-2 were combined and diluted to 60% methanol for analysis by LC–MS.