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Case Report

Non-aqueous capillary electrophoretic separation of cholesterol and 25-hydroxycholesterol after derivatization with Girard P reagent

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ABSTRACT

Capillary electrophoresis (CE) can provide high separation efficiency with very simple instrumentation, but has yet to be explored regarding oxysterols/cholesterol. Cholesterol and 25-hydroxycholesterol (both are 4-ene-3-ketosteroids) were quantitatively transformed into hydrazones using Girard P reagent after enzymatic oxidation by cholesterol oxidase. Separation was achieved using non-aqueous capillary electrophoresis with UV detection at 280 nm; the "charge-tagging" Girard P reagent ensured both charge and chromophore (which are requirements for CE-UV). Excess reagent was also separated from the two analytes, eliminating the need for removal prior to the analysis. The compounds were separated in less than 5 min with excellent separation efficiency, using separation electrolytes fully compatible with mass spectrometry. The CE-UV method was used to optimize steps for charge-tagging, revealing that the procedure is affected by the analyte/reagent ratio and reaction time, but also the analyte structure.

1. Introduction

Cholesterol is one of the most important components of cells and especially cell membranes, involved in the synthesis of hormones such as vitamin D and bile acids (Myant, 1981). Elevated levels of cholesterol in the blood plasma can however lead to atherosclerotic plagues and blockage of the free flow of blood, resulting in strokes and heart attacks (Ross, 1993). Oxysterols are oxidized forms of cholesterol or its precursors having at least one oxygenated functional group in their chemical structure. They can be formed by enzymatic and/or radical processes and are involved in cholesterol homoeostasis and a plethora of other physiological processes (Griffiths et al., 2017; Mutemberezi et al., 2016).

25-hydroxycholesterol (25-OHC, systematic name cholest-5-en-3β,25-diol) is formed from cholesterol either by enzyme catalytic reactions (enzymes CYP27A1, CYP3A4, CH25H and/or CYP46A1) (Diczfalusy, 2013; Iuliano, 2011) or by a free radical oxidation mechanism (Brown and Jessup, 2009; Griffiths et al., 2016; Iuliano, 2011; Karu et al., 2011). Regarding chemical oxidation, a hydrogen atom is replaced at the C-7 position by reactive nitrogen species (RNS) or reactive oxygen species (ROS), $ONOO^-$ and $OH^\bullet$, respectively (Iuliano, 2011; Yin et al., 2011). 25-OHC is included in many biological functions, such as cellular cholesterol homeostasis (Brown and Jessup, 2009; Diczfalusy, 2013; Griffiths et al., 2006; Jakobsson et al., 2012; et al., 2010; Zhao and Dahlman-Wright, 2010) and atherosclerosis (Björkhem and Diczfalusy, 2002). Moreover, 25-OHC can stimulate growth effects in breast and ovarian cancer cells by activating ERα-mediated signalling and is able to up-regulate estrogen target genes (Lappano et al., 2011).

The measurement of oxysterols is a challenging task and high-performance separation techniques play a prominent role in this regard. An overview of state-of-the-art analytical methods for oxysterol analysis can be found in recent reviews by Griffiths et al. (Griffiths et al., 2017, 2016, 2013a). Besides the commonly used gas chromatography-mass spectrometry (GC–MS) methods (Dzeletovic et al., 1995; Matysik et al., 2012), liquid chromatography (LC) (Teng and Smith, 1995) and particularly LC–MS techniques with or without derivatization of oxysterols have been developed and applied (Ahonen et al., 2014; Griffiths et al., 2016, 2013b; Honda et al., 2009; Karu et al., 2011; Marcos and Pozo, 2015; McDonald et al., 2012; Pataj et al., 2016; Roberg-Larsen et al., 2014). Compared to the commonly used GC and LC techniques, capillary electrophoresis (CE) holds advantages regarding portability, simplicity, size, speed, and

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(in many cases) separation efficiency. In addition, separation capillaries are very inexpensive (e.g. empty fused silica capillaries are used as "columns"). Moreover, it requires very small sample amounts and separation electrolyte volumes, and is very suited for lab-on-chip systems/miniaturization (e.g. CE does not require a solvent pump). CE has however not been applied to oxysterol analysis. There are currently only two reports that discuss CE (related) separations of cholesterol related compounds: Thiam et al. (Thiam et al., 2000) were able to separate cholesterol and its 12 ester-derivatives using isocratic capillary electrochromatography and Xu et al. (Xu et al., 2002) used non-aqueous capillary electrophoresis (NACE) for quantification of cholesterol in foods. Because of the highly hydrophobic nature of most oxysterols, NACE seems to be a suitable candidate technique for oxysterols. In NACE, an aqueous separation electrolyte is replaced by an organic solvent, e.g. acetonitrile or methanol, in which the solubility of most oxysterols is much higher, the so-called 'levelling effect' (Hansen et al., 1996) of water is eliminated, and substances which are insoluble in water (for example lipids) can be separated (Geiser and Veuthey, 2009; Kenndler, 2014; Otieno and Mwongela, 2008).

We present a fast and simple NACE method for separation and detection of cholesterol and 25-OHC after their derivatization with Girard P (GP) reagent (reaction products are cholesterol-GP (CH-GP) and 25-OHC-GP (25-GP)). The developed method does not require removal of the excess of derivatization reagent, and the derivatized cholesterol and 25-OHC have sufficient charge to be efficiently separated within 5 min. We apply the method to critically evaluate the steps in the Girard P derivatization procedure.

2. Experimental

2.1. Chemicals and reagents

All chemicals were of reagent grade or better. Methanol was from Lach-Ner (Neratovice, Czech Republic) was used for stock solutions dilutions. Deionized (DI) water was from Purite (Neptune, Watrex, Prague, Czech Republic). Cholesterol (cholest-5-ene-3β-ol), 25-OHC, NaOH, Girard P, KH_2PO_4, K_2HPO_4, ammonium acetate, cholesterol oxidase from *Streptomyces* sp. were all purchased from Sigma-Aldrich (St. Louis, MA, USA). 2-propanol, glacial acetic acid, methanol and acetonitrile were from Lach-Ner (Neratovice, Czech Republic). 525.0 μM and 454.5 μM stock solutions of 25-OHC and cholesterol, respectively were prepared in 2-propanol (Lach-Ner, Neratovice, Czech) and stored at 4 °C.

2.2. Enzymatic oxidation

The stock solutions of 25-OHC and cholesterol (in separate vials) were first evaporated to dryness (Eppendorf Concentrator 5301, Eppendorf AG, Hamburg, Germany) and the residues were re-dissolved in 20 μL 2-propanol. Aliquots of 200 μl containing 100 μg/ml cholesterol oxidase dissolved in 50 mM phosphate buffer pH 7 were added to the standard solutions to convert the 3β-hydroxy-5-ene to a 3-oxo-4-ene. The oxidation was performed at 37 °C for a specific time interval using an Eppendorf Thermomixer (Eppendorf AG, Hamburg, Germany) set to 300 rpm. After the oxidation was finished the samples were derivatized with GP reagent.

2.3. Derivatization with Girard P solution

For derivatization, 220 μl of the solution from the first reaction step was mixed with 15 μl of glacial acetic acid and 500 μl GP reagent in methanol. The concentration of GP reagent in methanol was premixed so that 25-, 50-, 100- and 200-fold excess compared to the analytes was present in the mixture. Thus, in a final volume of 735 μl the concentration of cholesterol and 25-OHC was 100 μM. All derivatized samples were stored at 4 °C and analyzed within 1 week. Prior to the analysis, the resulting reaction product solution was diluted to required concentration by methanol.

2.4. Instrumentation

All capillary electrophoresis separations were performed with an Agilent 7100 CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with an on-column diode-array detector, a high voltage power supply able to deliver up to 30 kV and an autosampler. A CE Chemstation (Agilent Technologies) was used for CE control, data acquisition and data handling. All analyses were carried out in a polyimide coated fused-silica (FS) separation capillary (Polymicro Technologies, Phoenix, AZ, USA) with the following dimensions: L_{tot}, 50 cm and L_{eff}, 41.5 cm, inner diameter (ID) 50 μm and outer diameter (OD) 375 μm. Before the first measurement each day, the separation capillary was sequentially preconditioned at a pressure of 950 mbar for 5 min each with 1 M NaOH, DI water, methanol and background electrolyte (BGE) solution. Between two successive runs, the capillary was flushed at a pressure of 950 mbar for 1.5 min with BGE. BGE consisted of 20 mM ammonium acetate in methanol (if not stated otherwise). All measurements were performed at 25 °C and a potential of +30 kV was applied at the injection side of the separation capillary. When not in use, the capillary was washed with methanol and DI water and then stored in DI water.

Injections were performed hydrodynamically at 50 mbar for 15 s (if not stated otherwise). UV detection at 280 nm was used, which is the maximum absorption wavelength of the investigated analyte-GP derivate.

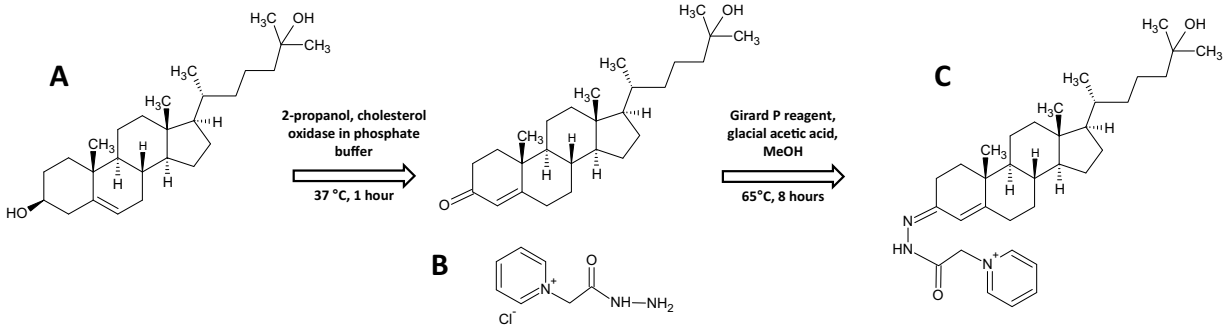

Fig. 1. Charge tagging reaction of 25-hydroxycholesterol. In the first step, the cholesterol oxidase enzyme converts the 3β-hydroxy-5-ene to a 3-oxo-4-ene. When this reaction is finished, in the second step of derivatization reaction with GP reagent occurs. 3-oxo group of 25-OHC reacts with terminal $-NH_2$ group of GP reagent in acidic conditions. The analogous reaction takes place with cholesterol and GP reagent. A: 25-OHC before reaction; B: GP reagent; C: final product, 25-GP.

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