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Analysis of oxysterols and vitamin D metabolites in mouse brain and cell line samples by ultra-high-performance liquid chromatography-atmospheric pressure photoionization–mass spectrometry

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ABSTRACT

We have developed an ultra-high-performance liquid chromatography-atmospheric pressure photoionization-tandem mass spectrometric (UHPLC-APPI–MS/MS) method for the simultaneous quantitative analyses of several oxysterols and vitamin D metabolites in mouse brain and cell line samples. An UHPLC-APPI-high resolution mass spectrometric (UHPLC-APPI-HRMS) method that uses a quadrupole-time of flight mass spectrometer was also developed for confirmatory analysis and for the identification of non-targeted oxysterols. Both methods showed good quantitative performance. Furthermore, APPI provides high ionization efficiency for determining oxysterols and vitamin D related compounds without the time consuming derivatization step needed in the conventionally used electrospray ionization method to achieve acceptable sensitivity. Several oxysterols were quantified in mouse brain and cell line samples. Additionally, 25-hydroxyvitamin D_3 was detected in mouse brain samples for the first time.

1. Introduction

Vitamin D is well known for its importance in various functions in the human body and vitamin D deficiency or insufficiency has long been known to be a risk factor for bone metabolic diseases, such as rickets, osteomalacia, and osteoporosis [1,2]. More recently, it was shown, that vitamin D deficiency is also involved in other diseases such as certain cancers (for example leukemia), autoimmune diseases and neurodegenerative diseases [1,3,4]. Vitamin D exists naturally either as vitamin D_2 or as vitamin D_3. The main dietary sources of D_2 and D_3 are mushrooms and fish, respectively [1,5]. Vitamin D_3 is additionally biosynthesized in the skin from its precursor 7-dehydrocholesterol after exposure to ultraviolet light. Vitamin D_2, on the other hand, is supplied only via dietary sources or from supplements [6]. Vitamin D as such is biologically inactive but it is hydroxylated in the liver to 25-hydroxyvitamin D (25-OH-D), which is the major circulating form in the body [1,2,5]. 25-OH-D is then further metabolized in the kidney to the biologically active form, 1α,25-dihydroxyvitamin D (1α,25-OH-D). Additionally numerous other metabolites of vitamin D have been well known for decades and the metabolism route has previously been presented thoroughly [2,7].

Cholesterol is the precursor of hormonal steroids and bile acids, and it can be detected at high levels in the blood, brain and other steroidogenic tissues [8,9]. Cholesterol is also the starting material for the synthesis of oxysterols. The oxysterols can be formed enzymatically in phase I metabolism of cholesterol [8,10] and directly from cholesterol by reactive oxygen species [8,11]. Recently, oxysterols were shown to be biologically active molecules [8,12,13]. Structurally different oxysterols have specific characteristic biological activities whereas the same oxysterols might have different

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activities in different cells [8]. There is also evidence of changes in the quantities of oxysterols (higher or lower amounts depending on the compound) as a result of Alzheimer's disease compared to healthy subjects [14,15].

Numerous methods for analyzing vitamin D (and its metabolites) and oxysterols in biological samples have been described previously [16–19]. Many of the conventional methods are based on gas chromatography (GC) [8,20] or gas chromatography–mass spectrometry (GC–MS) [16,21], high performance liquid chromatography (HPLC) [16,19,21] or immunological methods [19,21,22]. The selectivity or quantitative performance of HPLC and immunological methods may, however, be limited in the analyses of complex biological samples [19,22,23]. GC–MS provides high selectivity and sensitivity, but requires time-consuming derivatization of the analytes [16,20,21]. LC–MS using electrospray ionization (ESI) [16,17] and atmospheric pressure chemical ionization (APCI) [16,17,24] are increasingly used in the analysis of vitamin D and oxysterols. ESI based methods also often require time-consuming derivatization procedures, because the ionization efficiency of the non-polar vitamin D and oxysterols as such is poor [16,17]. Derivatization is nowadays also often used in APCI based methods in order to enhance the sensitivity. Atmospheric pressure photoionization (APPI), on the other hand, provides high sensitivity for both classes of compounds without any additional derivatization steps [18,25,26]. This makes the LC-APPI–MS methods faster and easier to implement compared to ESI or APCI methods using derivatization for achieving the required sensitivity.

In this work we describe an ultra-high-performance liquid chromatography (UHPLC)-APPI–MS/MS and an UHPLC-APPI-HRMS method for the simultaneous analyses of several oxysterols and vitamin D derived compounds in biological samples. Both methods provide simple, fast, and sensitive analyses of these compounds without recourse to time-consuming derivatization procedures. As oxysterols and vitamin D may have a role in neurodegenerative diseases, the UHPLC-APPI–MS/MS method was developed for the analysis of these compounds in mouse brain samples. In order to study the role of oxidation of cholesterol in neurodegenerative diseases non-stressed cell line samples were compared to cell line samples that had been exposed to oxidative stress and thus were overexpressing alpha-synuclein (α-syn) aggregates (Parkinson's disease aggregates). This comparison is expected to provide information on the role of oxysterols in the formation of the aggregates. Furthermore, the performances of UHPLC-APPI–MS/MS and UHPLC-APPI-HRMS are compared in the analysis of cell line samples.

2. Materials and methods

2.1. Chemicals

The water was purified using a Milli-Q purification system (Millipore, Molsheim, France). LC–MS grade methanol (MeOH), isopropanol (IPA), and acetonitrile (ACN), and HPLC grade dichloromethane (DCM) and toluene were purchased from Sigma-Aldrich (Steinheim, Germany). The following compounds were also purchased from Sigma-Aldrich: progesterone (PROG, 4-pregnene-3,20-dione), deuterated vitamin D_3 (d_3-D_3, 6,19,19-deuterated cholecalciferol), 7-dehydrocholesterol (7-DHYD, 3β-hydroxy-5,7-cholestadiene), vitamin D_2 (D_2, ergocalciferol), 1α,25-dihydroxyvitamin D_3 (1α,25-OH-D_3, 1α,25-dihydroxycholecalciferol), and 25-hydroxyvitamin D_3 (25-OH-D_3, 25-hydroxycholecalciferol). Vitamin D_3 (D_3, cholecalciferol) was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA), 27-hydroxycholesterol (27-OH-Chl, cholest-5-ene-3β,26-diol) from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany), 24(S)-hydroxycholesterol (24-OH-Chl, cholest-5-ene-3β,24α-diol) from AH diagnostics Oy (Helsinki, Finland), desmosterol (DESMO, 3β-hydroxy-5,24-cholestadiene), 7-ketocholesterol (7-OXO, 3β-hydroxy-5-cholesten-7-one) and 27-hydroxycholesterol-d6 (d_6-27-OH-Chl, 25,26,26,26,27,27-hexadeuterocholest-5-ene-3β,27-diol) from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), and 7α-hydroxycholesterol (7α-OH-Chl, 5-cholesten-3β,7α-diol), 7β-hydroxycholesterol (7β-OH-Chl, 5-cholesten-3β,7β-diol) and 22(S)-hydroxycholesterol (22-OH-Chl, 5-cholesten-3β,22(S)-diol) from Fountain Limited (Naxxar, Malta). The structures of the compounds studied appear in Fig. 1.

2.2. Samples

2.2.1. Animals

Three months old male NMRI-mice were used in this study. The mice were kept in a 12/12 light/dark cycle at ambient temperature (20–22 °C), and between 2 and 5 animals per cage. All mice were given ad libitum access to standard mouse chow and to tap water. The protocol was accepted by the Animal Experiment Board of Finland and experiment was performed in accordance with Finnish legislation. The mice were anaesthetized using sodium pentobarbital (100 mg kg^{-1}, i.p.) and then transcardially perfused with phosphate buffered saline (PBS) solution for 4 min to purge all the blood out of the brain. Afterwards the brains were removed from the skull, frozen on dry ice and stored in −80 °C until assayed.

2.2.2. Cell line samples

Wild type (WT) SH-SY5Y human neuroblastoma cell line was purchased from ATCC (LGC Standards; Product # CRL-2266, Middlesex, UK) and cultured as described by Myöhänen et al. [27]. Stable cell lines expressing A30P and A53 T α-syn were generated using a lentiviral vector as described by Gerard et al. [28], and transfected cells were selected by their resistance to puromycin. The α-syn overexpressing cells were cultured as described by Myöhänen et al. [27]. Cell lines were used at passages 3–15 and grown at 37 °C and 5% CO_2 in a humidified atmosphere. In the oxidative stress treatment, 1×10^6 cells were seeded in T25-flasks and allowed to grow overnight. Thereafter, the aggregation process of α-syn was induced by adding 100 μM H_2O_2 and 10 mM $FeCl_2$ in the cell culturing medium for 3 days as previously described [27,28]. All cells (non-stressed and stressed) were homogenized as described by Myöhänen et al. using a 0.1 M Na–K-phosphate buffer at pH 7.0 [27]. The homogenates were centrifuged at 16,000 × *g*, at 4 °C, for 20 min, and thereafter the supernatant (soluble fraction) and pellet were separated and stored at −80 °C for further analysis.

2.3. Sample preparation

Stock solutions (1.0 mg mL^{-1}) of the analytes were prepared by dissolving the analytes in MeOH. The working standard solutions were prepared by diluting the stock solutions in MeOH to the appropriate concentrations. The standard solutions were used to prepare a calibration curve with the following concentration levels: 0.5, 1.0, 2.5, 5.0, 7.5, 10, 15, 25, 35, 50, 100, 250, 500 and 1000 ng mL^{-1}. 22 μL of a 1 μg mL^{-1} internal standard working solution containing d_3-D_3, and d_6-27-OH-Chl was added to 198 μL of each concentration level. Intra- and inter-day repeatabilities were measured using a freshly prepared working standard solution at a concentration level of 100 ng mL^{-1}.

2.3.1. Brain samples

The intact mouse brains were weighed and cut into four equal parts. A 2.5 μL volume of the 1 μg mL^{-1} internal standard working solution was added to each brain part. The samples