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Short communication

Identification of conjugated linoleic acid elongation and β-oxidation products by coupled silver-ion HPLC APPI-MS

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Abstract

Atmospheric pressure photoionisation (APPI) was used in combination with silver-ion (Ag^+)-HPLC for detection of (conjugated) fatty acid methyl esters (FAME) by tandem-mass spectrometry. APPI-MS of methyl esters of conjugated linoleic acid showed an increase in signal-to-noise ratio by a factor of 40 compared to atmospheric pressure chemical ionization in the positive mode. It was possible to identify double bond position, configuration and chain length of FAME based on chromatographic separation and mass detection. The developed LC–MS method is useful for the analysis of CLA elongation and β-oxidation products, especially with *trans*,*trans*-configuration, which are difficult to analyze by conventional GC–MS techniques.
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1. Introduction

Conjugated linoleic acids (CLA) are a naturally occurring minor group of positional and geometric isomers of linoleic acid (c9c12-C18:2), which have been shown to have potentially positive nutritional effects such as fat mass reduction in humans [1], anti-atherosclerotic effects in mice [2] and anti-cancerogenic effects in mice [3]. These conjugated fatty acids are formed during the biohydrogenation of linoleic and linolenic acid by microorganism *Butyrivibrio fibrisolvens* in ruminants [4]. Main CLA isomer is the c9t11-C18:2 (rumenic acid) [5] which amounts for nearly 90% of total CLA content in beef and milk and some 19 CLA isomers have been identified in milk fat with double bond position between Δ12Δ14 and Δ7Δ9 in *cis*- and *trans*-configuration [6,7]. The biological mechanisms of action are still subject to intensive research and there is evidence for significant differences in biologic activity of single isomers [8]. Highly sensitive methods for the analysis of fatty acids and their metabolites (such as prostaglandins and isoprostanes) from food and biological matrices have been established in the past such as negative chemical ionization (NCI) GC–MS of their pentafluorobenzyl (PFB) derivatives [9] or isobutane positive chemical ionization (PCI) GC–MS/MS of fatty acid methyl esters (FAME) [10]. Characterization of double bond position and configuration of FAME was established by acetonitrile PCI GC–MS/MS [11]. However, CLA isomers cannot be completely resolved by GC [12] and mass spectral data for structural determination of the fatty acids are often compromised due to co-eluting compounds. Alternatively, silver ion HPLC (Ag^+-HPLC) has become the most favourable method for CLA analysis as it is capable of resolving most isomers [13,14]. Advantage of Ag^+-HPLC is that compounds are separated into a *trans*,*trans*-, a *cis*,*trans*/*trans*,*cis*- and a *cis*,*cis*-group depending on the configuration of double bonds and on the double bond position within each group. The drawback, the carbon chain length has no influence on the separation within such a

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group. This results in co-elution of elongation (C20:2) and β-oxidation (C16:2) metabolites of CLA with other conjugated fatty acids (e.g. c11t13-C18:2 co-elutes with c11t13-C20:2) as shown recently in human coronary artery smooth muscle cells (HCASMC) after incubating with different CLA isomers [15]. Differences in metabolisation of *trans* fatty acids depending on the configuration and position of double bond may explain positive or negative nutritional effects [15–17]. Therefore, Ag^+-HPLC is a very powerful technique to study the fatty acid elongation and desaturation metabolism of conjugated fatty acids but so far additional GC–MS measurements for confirmation of the absence of co-eluting compounds were necessary. Aim of this study was to hyphenate Ag^+-HPLC for separation of CLA and their elongation and β-oxidation metabolites with selective mass-spectrometric detection using lipids from HCASMC as model system. Atmospheric pressure photoionization (APPI) was tested for efficient ionisation of the non-polar methyl ester derivatives and compared with atmospheric pressure chemical ionization (APCI).

2. Experimental

2.1. Samples

c7t9-C16:2 and c11t13-C20:2 β-oxidation and elongation products of CLA have been identified in a previous study by Ag^+-HPLC-DAD, GC–FID and GC–MS as main metabolites in human coronary artery smooth muscle cells (HCASMC) incubated with 50 μM c9t11-CLA [15]. Unfortunately, these metabolites are not commercially available as pure standard compounds at the moment. Therefore, lipid extracts from HCASMC were used as model lipids for HPLC-MS method development. The metabolites were absent in control cells. Lipids were extracted from the cells three times with chloroform/methanol (2/1, v/v) after addition of 100 μL internal standard solution in *n*-hexane (100 μg heptadecenoic acid methyl ester, c10-C17:1). Lipids were *trans*-methylated with 5% potassium methylate solution in methanol (30 min at 60 °C) and subsequent acidic esterification of free fatty acids by 0.5 M sulphuric acid in methanol (15 min at 60 °C) [18]. After addition of saturated sodium chloride solution FAMEs were extracted from the aqueous phase with hexane. Internal standard c10-C17:1 and CLA methyl ester mix (98%) were purchased from Sigma, Seelze, Germany. All solvents, sulphuric acid (96%) and sodium chloride were of analytical grade and purchased from Merck, Darmstadt, Germany. Potassium methylate was obtained as 30–35% solution in methanol from the same supplier.

2.2. Liquid chromatography

HPLC system consisted of an Agilent 1100 LC binary pump, an Agilent 1100 column oven (20 °C), a CTC PAL autosampler and an Agilent 1100 diode-array detector (234 nm). Separation was performed using three Chromspher 5 Lipids columns in series (250 mm × 4.6 mm, 5 μm) with a pre-column (50 mm × 4.6 mm, 5 μm) of the same column material (Varian, Darmstadt, Germany). 0.2% propionitrile in *n*-hexane (both Merck, Darmstadt, Germany) was used as eluent [19] at a flow rate of 0.6 mL min^{-1}. Analysis was performed after cleaning the columns with 8% propionitrile in *n*-hexane for 1 h and equilibrating the columns with the eluent for 1 h.

2.3. Mass-spectrometry

The LC-system was equipped with an API 4000 QTrap mass spectrometer detector (Applied Biosystems, Darmstadt, Germany/MDX Sciex, Toronto, Canada). APPI and APCI interfaces were tested. Experiments were performed either by direct flow injection or by Ag^+-chromatography with an injection volume of 10 μL. For APPI experiments toluene was used as dopant for the ionization. The toluene was co-injected into the APPI ion source via the auxiliary gas (gas2) at a flow rate of 30 μL min^{-1}. The source temperature was 300 °C for APPI and APCI experiments. Needle current for APCI was set to 3 μA. Detector/interface-parameters: full scan parameters: enhanced MS (EMS, Linear Ion Trap (LIT) capability of the tandem MS was used), polarity: positive, scan rate: 4000 amu s^{-1}, LIT fill time: dynamic, scan range: 200–400 amu, curtain gas 10 (arbitrary units), ion transfer voltage IS: 730 V, nebulizer gas (gas 1): 45 psi, auxiliary gas (gas 2): 30 psi, interface heater: on, system pressure 4.8 10e−5 Torr, declustering potential: 50 V. Multiple reaction monitoring (MRM) parameters: polarity: positive, curtain gas: 10 (arbitrary units), ion transfer voltage IS: 730 V, nebulizer gas (gas 1): 45 psi, auxiliary gas (gas 2): 30 psi, interface heaeter: on, collision gas (nitrogen): medium (system pressure 3.8 10e−5 Torr). Precursor/product ions and MRM parameters, which were optimized automatically by injection of CLA methyl ester standard mix using Analyst 1.4.1 software, are listed in Table 1.

Table 1
Multiple reaction monitoring parameters for different FAME

Fatty acid	Precursor ion $[M]^+$ (amu)	Product ion $[M - CH_3OH]^+$ (amu)	Dwell time (ms)	DP	CE	CXP
C18:2	294.26	262.1	70	51	13	16
C16:2	266.30	234.3	70	50	15	13
C18:3	292.30	260.3	70	50	15	13
C20:2	322.33	290.2	70	46	13	16
C20:3	320.30	288.3	70	50	15	13
C20:4	318.30	286.3	70	50	15	13

DP, declustering potential; CE, collision energy; CXP, collision cell exit potential.

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