Fast LC–MS/MS analysis of free oxysterols derived from reactive oxygen species in human plasma and carotid plaque

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ABSTRACT

Background: A rapid liquid chromatography–tandem mass spectrometric (LC–MS/MS) method was developed and validated for the quantification of reactive oxygen species (ROS) derived free oxysterols and cholesterol in human plasma and atherosclerotic plaque.
Method: In vitro autoxidation of cholesterol during sample pretreatment was avoided by applying only one protein precipitation and re-concentration step using 80 µl plasma. For preparation of 10 mg atherosclerotic plaques an additional liquid–liquid extraction was included. Free 7-keto-, 7-α/ß-hydroxy-, 5,6-α-epoxy-, 5,6-β-epoxycholesterol, cholestane-3ß,5α,6ß-triol and cholesterol were separated within 7 min on a monolithic column. An API 4000 tandem mass spectrometer was applied in positive ionization mode using atmospheric pressure chemical ionization.
Results: The detection limit was 0.1 ng/ml and the linearity ranged from 0.5 to 0.75 to 2000 ng/ml for the oxysterols and from 50 to 1000 µg/ml for cholesterol. Recovery was between 80.9 and 107.9%. Between-run imprecision ranged from 7.9 to 11.7%. Analysis of plasma samples from additional 50 middle-aged volunteers revealed a large inter-individual variability (e.g. 7-ketocholesterol 2.63-30.47 ng/ml). Oxysterol concentrations normalized to cholesterol were about 43 times higher in carotid plaque compared to plasma (n = 5).
Conclusion: This rapid LC–MS/MS method enables reliable quantification focused on especially ROS-derived oxysterols in human plasma and atherosclerotic plaque samples under high-throughput conditions.

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1. Introduction

Analysis of oxysterols formed via reactive oxygen species (ROS) is of increasing interest for studying oxidative stress associated diseases such as atherosclerosis [1,2]. Notably, 7-ketocholesterol, 7-α- and 7-β-hydroxycholesterol, 5,6-α-epoxycholesterol, 5,6-β-epoxycholesterol, and cholestane-3ß,5α,6ß-triol have been shown to have cytotoxic properties and inflammatory effects in vitro [3–6]. Among these analytes 7-α-hydroxycholesterol is known to be formed both enzymatically and autoxidatively [7]. 7-ketocholesterol and 7-β-hydroxycholesterol were identified in atherosclerotic plaques that have been linked to the initiation and progression of atherosclerosis [1,2]. Oxysterols are transported as free and esterified form in lipoproteins. In particular the free oxysterols seem to get high importance as active metabolites regulating cholesterol biosynthesis, fatty acid synthesis, LDL receptor expression and cholesterol efflux [8–10]. The analysis of oxysterols is challenging due to their very low physiological concentrations, structural similarity, and the excess of cholesterol. Gas chromatography coupled to mass spectrometry (GC–MS) is widely used for oxysterol analysis in plasma and atherosclerotic plaques [1,2,11,12]. Sample pretreatment includes hydrolysis of esterified oxysterols, separation from cholesterol, derivatization and liquid–liquid extraction (LLE) [13–18]. In recent applications, LC–MS/MS was applied

after hydrolysis, derivatization, LLE or solid phase extraction (SPE) [19–23].

The susceptibility of cholesterol to in vitro autoxidation during sample pretreatment and storage plays an important role in oxysterol analysis [24]. It has been already discussed that some of the previously measured oxysterols like 7-β-hydroxycholesterol, 7-ketocholesterol, cholestane-3β,5α,6β-triol and 5,6-epoxycholesterols may be only the results of in vitro oxidation artifacts [25,26].

The aim of our study was the development of a single-step sample pretreatment protocol avoiding in vitro cholesterol autoxidation in combination with a rapid LC–MS/MS method for the simultaneous analysis of free oxysterols and cholesterol in human plasma and atherosclerotic plaque.

2. Materials and methods

2.1. Materials

Sterol standards were purchased from Steraloids (Newport, Rhode Island, USA), Euriso-Top, (Saarbrücken, Germany) and Avanti Polar Lipids (Alabama, USA) (see supplement Table 1). Methanol, acetonitrile and isopropanol (ULC-MS grade) were used from Biosolve (Valkenswaard, Netherlands). Water was purified using Nano Pure technology form Thermo Scientific (Waltham, USA). Butylated hydroxytoluene (BHT), sodium hydroxide (NaOH), hydrochloric acid (HCL) was purchased from Sigma-Aldrich (Munich, Germany). Ethanol, n-hexane (both LC grade) and sodium sulfate (Na_2SO_4) was obtained from Merck KGaA (Darmstadt, Germany). EDTA-K_3 containing tubes and polypropylene tubes were purchased from Sarstedt (Nümbrecht, Germany), glass vials from Wicom (Heppenheim, Germany) and 96-well plates from Nunc™, Thermo Fisher Scientific (Karlsruhe, Germany). Ultrasonic homogenizer UP 100H was purchased from Dr. Hielscher GmbH (Teltow, Germany). Mikro-Dismembrator S was from Sartorius AG (Göttingen, Germany).

2.2. Standards and solutions

Stock solution containing oxysterols (each 1 mg/ml in methanol) and cholesterol (10 mg/ml in isopropanol) were prepared. Calibration standards with oxysterol concentrations of 0.5, 0.75, 2, 5, 25, 100 and 500 ng/ml were prepared by diluting the stock solutions with methanol. For cholesterol, four calibrators (0.05, 0.1, 0.5, 1.0 mg/ml) were used. Isotope labeled standards for each oxysterol were reconstituted in methanol to a concentration of 1 mg/ml. All solutions included 0.05% (v/v) BHT to avoid autoxidation.

Pool plasma at five different concentration levels: native quality control (NQC), low concentrated quality control (LQC, spiked with 5 ng/ml oxysterol mix), medium concentrated quality control (MQC, spiked with 10 ng/ml oxysterol mix), high concentrated quality control (HQC, spiked with 20 ng/ml oxysterol mix), and pathological concentrated quality control (PQC, spiked with 75 ng/ml oxysterol mix) were prepared as quality control. Standards, calibrators and quality controls (QC) were aliquoted and stored at −80 °C until analysis.

2.3. Sample collection

EDTA-plasma from 50 (28 male/22 female) fasting healthy volunteers as well as EDTA-plasma and carotid plaques (n = 5) from patients undergoing carotid endarterectomy (CEA) were collected. Carotid plaques were separated from the surgical specimen under a stereomicroscope, split into tissue sample aliquots, and stored in liquid nitrogen until further preparation.

The study was approved by the ethic committee of the University of Leipzig 148-2005 and 082-10-190-42010. Each participant provided written informed consent. Plasma and plaque samples were immediately frozen after centrifugation at 3220 ×g for 10 min and stored in polypropylene cryotubes at −80 °C until analysis and thawed only once.

2.4. Sample preparation

80 μl of calibrator, QC, blank (methanol) or plasma sample were added to 1440 μl methanol/isopropanol 1:1 (v/v), containing the oxysterol internal standard mixture (each oxysterol c = 5 ng/ml) and CH-$^2H_7$ (c = 200 ng/ml). Samples were mixed thoroughly for 1 min and centrifuged for 10 min at 12500 ×g. Supernatants were transferred into glass vials and evaporated to dryness at room temperature under a stream of nitrogen. At −80 °C stored extracts were reconstituted with 80 μl methanol/water 3:1 (v/v), transferred into 96-well plates and centrifuged prior to analysis for 5 min at 3220 ×g.

The plaque extraction protocol was optimized using a single aliquoted plaque (10 mg aliquots). Plaque extraction was performed for 1 h at 4 °C employing a vortex mixer [27]. Efficiency of cholesterol extraction from plaque was compared for direct solvent extraction and solvent extraction after plaque homogenization for 0.5, 1, 2 h incubation time. Cholesterol autoxidation was investigated for a cholesterol solution (c = 500 μg/mL) following the same experimental conditions. The following solvent mixtures were compared: n-hexane/isopropanol (3:2, v/v) and acetonitrile/methanol (1:1, v/v), containing 0.01% (v/v) BHT, respectively [28,29]. Samples were homogenized for 1 min using an ultrasonic homogenizer or a mikro-dismembrator. After plaque extraction for 1 h with 1200 μl acetonitrile/methanol, the plaque extracts were directly evaporated after centrifugation for 10 min at 1000 ×g (4 °C). For plaque extraction with 1200 μl n-hexane/isopropanol, 50 μl water was added to each plaque sample. After extraction, 300 μl of 0.47 mol/l aqueous Na_2SO_4 solution was added, mixed thoroughly for 5 min, mixed for 20 min and centrifuged for 10 min at 1000 ×g (4 °C). The organic phase was evaporated. All dried plaque extracts were stored at −80 °C. Before LC–MS/MS analysis, the plaque extracts were reconstituted in 200 μl acetonitrile/methanol (1:1, v/v) and further processed according to the described plasma protocol. The plaque extracts were finally dissolved in methanol/water (3:1, v/v).

2.5. Chromatography and mass spectrometry

A PE binary micro pump system, series 200 autosampler, a column oven with a ten-port switching valve (Perkin-Elmer, Rotgau-Jügesheim, Germany) and an API 4000 triple quadrupole mass spectrometer with APCI (AB SCIEX, Toronto, Canada) was used. Oxysterols were analyzed in positive ion mode and multiple reaction monitoring (MRM) (mass transitions see supplement Table 2). APCI-source parameters were as follows: source temperature 500 °C, nebulizer current 3, curtain gas 25 psi, gas 1 25 psi and collision induced dissociation (CID) 5.

A Chromolith SpeedRod RP-18e monolithic column (50 × 4.6 mm) (Merck KGaA, Darmstadt, Germany) was applied. The flow rate was 1 ml/min with solvent A methanol/water (3:1, v/v) and solvent B 100% isopropanol. The gradient elution was as follows: 0–0.5 min 40% B, 0.5–3.5 min 40 to 70% B, 3.5-6.5 min 100% B, 6.5-7.0 min 40% B. During the first 0.5 min after sample injection, the eluent was switched by a valve to the waste to avoid source contamination from plasma matrix components.

2.6. Method evaluation

For investigation of in vitro oxidation processes plasma and methanol were spiked with isotope labeled cholesterol (c = 500 μg/ml) and processed according to the sample preparation protocols.

Matrix effects on oxysterol ionization were evaluated for each oxysterol (c = 1 μg/ml) by post-column infusion experiments by direct infusion with a flow rate of 0.01 mL/min to a HPLC flow rate of 1 mL/min and 10-fold injection of a plasma sample. Influence of excess cholesterol

was investigated by addition of cholesterol (100 to 1000 μg/ml) to an oxysterol standard mix (c = 20 ng/ml).

The limit of detection (LOD) was calculated from methanolic standard solutions after the described sample preparation using a signal-to-noise (s/n) ratio of 3. The lower and upper limits of quantification (LOQ) are stated as the concentration levels meeting the criteria of a coefficient of variation below 20%. Linearity of the oxysterols was tested up to a concentration of 2000 ng/ml, and for cholesterol in a range from 50 to 1000 μg/ml.

The accuracy of the method was assessed by recovery studies. Pool serum was spiked with standard solutions at appropriate concentration levels (range 5–75 ng/mL). The within-run and between-run imprecision was determined by preparing and analyzing each quality control 10 times per run and on 10 consecutive working days.

Stability of methanolic standard solutions (oxysterol standard mixture c = 10 ng/ml) and native and spiked plasma samples (c = 10 ng/ml) were investigated for three freeze–thaw cycles and for storage at −80 °C up to 12 weeks with and without addition of 50 μg BHT/ml sample, respectively.

3. Results

3.1. Sample pretreatment

Our established serum pretreatment protocol for plant sterol was modified for oxysterol analysis [29]. Protein precipitation was performed by diluting 80 μl of human plasma with 1440 μl methanol/isopropanol, (1:1, v/v) following evaporation. In vitro formation of oxysterols derived from isotopic labeled cholesterol was observed only in methanolic solutions, but not during plasma sample preparation (supplement Fig. 1.). In methanolic solutions oxysterol concentrations decreased during storage time and freeze–thaw cycles without BHT addition (data not shown). Thus, all methanolic oxysterol standard solutions were treated with 0.05% (v/v) BHT to prevent in vitro autoxidation. BHT addition to plasma did not enhance the stability of oxysterols in freeze–thaw cycles and storage at −80 °C (data not shown).

For plaque extraction a previous homogenization was omitted to minimize material loss and to prevent autoxidation of cholesterol. The best extraction yield was obtained by direct incubation and mixing of 10 mg plaque for 1 h at 4 °C in 1200 μl n-hexane/isopropanol (3:2, v/v) followed by LLE (supplement Fig 2. and 3.). In vitro formation of cholesterol oxidation products did not occur during plaque sample pretreatment by adding BHT.

3.2. Chromatography and mass spectrometry

The monolithic column was chosen due to the efficient chromatographic separation of the isomeric oxysterols 5,6-α-EC/5,6-β-EC and 7-OHC, Triol with MRM 385.3/367.3. The stereoisomers 7-α-OHC and 7-β-OHC could not be separated under the chosen chromatographic conditions and were indicated in the following as a sum parameter 7-α/β-OHC. An analytical run time of 3 min was needed for the separation of 5,6-β-EC, 5,6-α-EC, Triol, 7-α/β-OHC, 7-KC and cholesterol. Enzymatically formed oxysterols (20-α-OHC, 22-R-OHC, 22-S-OHC, 24-S-OHC, (25R)-27-OHC) were partially separated at a retention time between 1.2 and 1.4 min. In the end, a total run-time of 7.0 min was needed for the elution of the free oxysterols, cholesterol and the sterol ester fractions from plasma and plaque following column re-equilibration (Fig. 1). Mainly $[M + H-H_2O]^+$ ions (for 7-α/β-OHC, 5,6-β-EC, 5,6-α-EC), $[M + H-2H_2O]^+$ ions (for Triol), and $[M + H]^+$ ions (for 7-KC) were formed under the optimized conditions using APCI [30,31].

Influence of plasma matrix components was investigated by a post column infusion experiment. No signal suppression or signal enhancement was observed in the oxysterol retention time ranging between 1.5 to 3.0 min (supplement Fig. 4). In addition, peak area ratios of analyte to internal standard are comparable for spiked plasma and standard solutions for the sum parameter 7α/β-OHC. In the range from 100 to 1000 μg/ml, excess of cholesterol did not influence the quantification of 7-α/β-OHC, 7-KC, 5,6-β-EC and Triol, only for 5,6-α-EC a concentration increase up to 16% was observed at cholesterol concentrations at 1000 mg/ml.

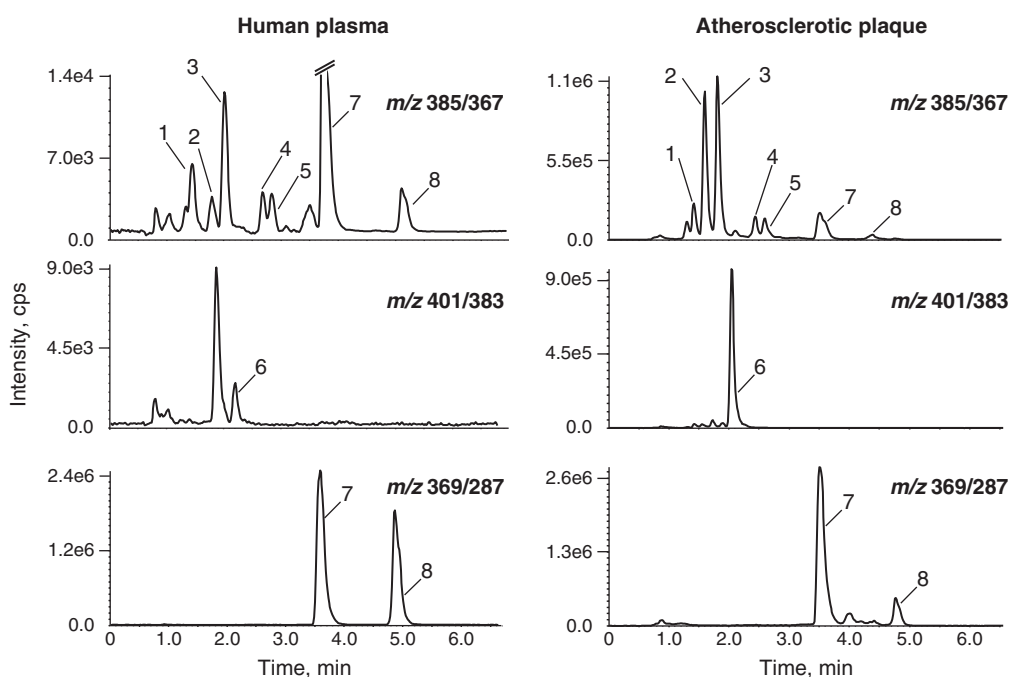**Fig. 1.** LC–MS/MS analysis of a human spiked plasma sample (c = 5 ng/ml) and of an extracted atherosclerotic plaque sample, mass transitions: *m/z* 385/367 (1) enzymatically formed oxysterols, (2) cholestane-3ß,5α,6ß-triol, (3) 7-α/ß-hydroxycholesterol, (4) 5,6-ß-epoxycholesterol, (5) 5,6-α-epoxycholesterol, *m/z* 401/383 (6) 7-ketocholesterol, and *m/z* 369/287 (7) cholesterol, (8) sterol ester fraction.

Table 1
Recovery for four spiked plasma levels with the corresponding coefficient of variation (CV) (n = 10).

Oxysterol (ng/ml)	Initial concentration a	Added concentration	Final concentration b	CV % n = 10	Difference b − a	Recovery rate %
7-ketocholesterol	2.7	5	7.4	6.3	4.7	103.9
	2.7	10	11.9	6.6	9.3	92.8
	2.6	20	22.8	2.2	20.2	101.0
	2.6	75	81.7	3.9	79.1	105.5
7-α/β-hydroxycholesterol	1.8	5	6.7	4.5	4.9	107.9
	1.8	10	11.6	2.4	9.7	97.4
	1.8	20	23.1	0.9	21.3	106.5
	1.8	75	82.5	3.3	80.7	107.6
5,6-α-epoxycholesterol	nd	5	4.3	9.3	4.3	94.2
	nd	10	8.5	4.3	8.5	85.0
	nd	20	17.1	0.4	17.1	85.5
	nd	75	70.4	2.3	70.4	93.9
5,6-β-epoxycholesterol	1.1	5	4.8	10.9	3.7	81.3
	1.1	10	9.2	5.1	8.1	80.9
	2.2	20	19.5	0.8	17.3	86.5
	2.2	75	74.7	2.9	72.5	96.7
Cholestane-3ß,5α,6ß-triol	nd	5	4.4	5.5	4.4	96.5
	nd	10	8.2	5.2	8.2	81.6
	nd	20	17.0	1.2	17.0	85.0
	nd	75	72.0	3.0	72.0	96.0

nd = not detectable.

3.3. Detection limit, linearity, accuracy and precision

The LOD was 0.1 ng/ml for all oxysterols and the LLOQ were 0.5 ng/ml for 7-α/β-OHC, 0.75 ng/ml for 7-KC, 5,6-α-EC, 5,6-β-EC, Triol as well as 50 μg/ml for cholesterol [19,23]. The standard curves were linear between 0.5 and 2000 ng/ml for oxysterols and 50–1000 μg/ml for cholesterol (see supplement Table 3). A chromatogram of the blank sample and chromatograms with analyte concentrations at the LLOQ are shown in supplement Fig. 5.

Accuracy, assessed by standard addition experiments, ranged between 80.9 and 107.9% (Table 1). The within-run imprecision ranged between 11.3 and 21.8% for native plasma specimens, and between 3.4 and 9.8% for the spiked quality control samples. The between-run imprecision was up to 26% in native plasma and between 7.9 and 11.7% for spiked plasma samples. Precision data are summarized in Table 2.

3.4. Oxysterols in human plasma and atherosclerotic plaque

The free oxysterol plasma concentrations of 50 middle-aged volunteers are summarized in Table 3. The plasma concentrations for free cholesterol were 471 ± 104 μg/ml (mean ± SD). 7-α/β-OHC was significantly correlated with cholesterol (r = 0.687, p = 0.009).

The oxysterol distribution in plasma was 7-KC (57.0%) > 7-α/β-OHC (20.7%) > 5,6-β-EC (11.7%) > Triol (10.5%) > 5,6-α-EC (<LLOQ) (Table 4). In contrast the oxysterol distribution in plaque differed: 7-KC (54.8%) > Triol (13.5%) > 7-α/β-OHC (11.6%) > 5,6-β-EC (10.1%) > 5,6-α-EC (10.0%). The concentrations of oxysterols normalized to free cholesterol, analyzed in the 5 human plaque samples are shown in Table 4. The oxysterol concentrations for plaque and the corresponding plasma samples normalized to total cholesterol are listed in supplement Table 4.

Table 2
Within- and between-day variability of native plasma and four spiked plasma levels (n = 10).

Analyte	Concentration (ng/ml)	Within-day CV (n = 10)		Between-day CV (n = 10)	
		Mean (ng/ml)	CV, %	Mean (ng/ml)	CV, %
7-ketocholesterol	Native	2.7	15.6	2.6	24.7
	5	7.4	4.2	7.3	10.6
	10	11.9	5.4	12.4	6.3
	20	22.8	9.5	24.6	7.4
	75	81.7	4.7	87.3	5.5
7-α/β-hydroxycholesterol	Native	1.9	11.3	1.8	9.4
	5	6.7	3.4	6.9	7.9
	10	11.6	2.1	12.4	8.8
	20	23.1	4.0	20.4	4.7
	75	82.5	4.0	75.7	4.7
5,6-α-epoxycholesterol	Native	<0.75		<0.75	
	5	4.3	9.8	4.6	9.7
	10	8.5	4.5	10.0	7.9
	20	17.1	2.5	19.0	6.7
	75	70.4	3.3	71.9	4.5
5,6-β-epoxycholesterol	Native	1.6	21.8	2.2	26.0
	5	4.8	8.9	6.2	11.3
	10	9.2	4.7	11.3	8.3
	20	19.5	4.0	19.6	7.7
	75	74.7	3.9	71.6	5.7
Cholestane-3ß,5α,6ß-triol	Native	<0.75		<0.75	
	5	4.4	5.8	4.3	11.7
	10	8.2	5.5	9.1	10.2
	20	17.0	6.8	15.5	7.2
	75	72.0	4.2	62.0	6.5
Cholesterol	Native	467 μg/ml	4.7	472.9 μg/ml	7.3

Table 3
Free oxysterol concentrations in EDTA-plasma samples from 50 volunteers (male = 28, female = 22).

Analyte	Human plasma, n = 50				
	Absolute concentration		Relative concentration		
	Median (ng/ml)	2.5–97.5 percentile	Median (ng/mg CH)	2.5–97.5 percentile	N[a]
7-ketocholesterol	6.19	2.63–30.47	13.08	7.26–51.62	50
7-α/β-hydroxycholesterol	3.50	1.17–20.64	8.18	2.81–45.69	50
5,6-α-epoxycholesterol	1.34	0.75–2.62	3.04	1.21–5.48	19
5,6-β-epoxycholesterol	6.02	2.68–13.03	12.89	6.40–28.31	50
Cholestane-3ß,5α,6ß-triol	1.42	0.76–8.05	3.07	1.79–11.67	38

[a] Number of plasma samples with quantifiable oxysterol concentrations.

Table 4
Free oxysterol concentrations adjusted to free cholesterol in atherosclerotic plaque and corresponding plasma samples (n = 5).

Analyte (ng/mg free cholesterol)	Atherosclerotic plaque		Human plasma	
	Median	Range	Median	Range
7-ketocholesterol/cholesterol	607.1	205.9–1407.6	14.6	5.9–29.7
7-α/β-hydroxycholesterol/cholesterol	128.0	75.6–216.8	5.3	3.1–13.7
5,6-α-epoxycholesterol/cholesterol	110.7	34.4–165.4	nd	
5,6-β-epoxycholesterol/cholesterol	112.2	95.5–334.2	3.0	1.6–7.6
Cholestane-3ß,5α,6ß-triol/cholesterol	149.4	57.7–340.8	2.7	1.8–5.2

nd = not detectable.

4. Discussion

The main challenge in oxysterol analysis is the excess of cholesterol and its potential to in vitro oxidation. Previous described analytical methods include LLE, SPE saponification and derivatization prior GC–MS or LC–MS/MS analysis. BHT is often added to avoid these effects but it has already been discussed that some of the previously measured oxysterols are only products of in vitro oxidation [25,26].

We developed a one step sample pretreatment protocol including protein precipitation for plasma and a simple extraction procedure for atherosclerotic plaques for oxysterol analysis. Plasma volume could be reduced to 80 µl and the required plaque amount was only 10 mg for oxysterol analysis [21,22]. Former described GC–MS application needed 70–240 mg plaque per analysis [1]. Applying this pretreatment protocol, in vitro formation of oxysterols derived from isotopic labeled cholesterol in plasma was not observed. Epoxycholesterol formation which was described during GC–MS sample pretreatment was not noticed [32]. The conversion of 5,6-EC to Triol by non-physiological temperatures and the use of acidic medium in the sample preparation could be avoided [33]. However, a three-fold increase of the $5,6-\alpha-EC-_2H^7$ was found in processing methanolic isotope labeled cholesterol solution without BHT addition probably due to a lack of antioxidants and stabilizers that are usually in physiological samples.

An additional important advantage of the developed LC–MS/MS method is the simultaneous analysis of free oxysterols and free cholesterol in a total run time of 7 min. This enables the normalization of plaque- and plasma oxysterol concentrations to cholesterol and therefore allows the comparison of oxysterol concentrations between blood and plaque as well as between morphologically heterogeneous plaque samples. However, 7-α- and 7-β-OHC can be analyzed only as sum parameter which is a limitation of the method. Because 7-α-OHC can also be derived enzymatically, the 7-hydroxycholesterol signal can be used only approximately for ROS activity.

The method development was focused on the free oxysterol fraction that represents the likely active form to investigate its effects on oxidative stress and atherogenic processes [7].

Due to the sensitivity of the developed LC–MS/MS method it can be used for the determination of free oxysterols in plasma and plaque samples. By using the sample preparation protocol autoxidation could be avoided so that the oxysterols present are in very low physiological concentrations. In this range the variability of free 7-ketocholesterol and free 5,6-β-epoxycholesterol concentrations were >20%.

The analyzed free oxysterol plasma concentrations of 50 middle-aged volunteers showed as previously described a high inter-individual variation [19,23,32]. However, 7-KC/CH and 7-α/β-OHC/CH concentrations in plaque samples were 31 and 45% lower compared to previously used methods applying more laborious sample pretreatment protocols [1]. Additionally free 5,6-α-EC and free 5,6-β-EC were quantified with 3–100-fold lower plasma concentrations as described by GC–MS methods. This may be the effect of the gentle sample pretreatment avoiding in vitro cholesterol autoxidation [13,14].

In carotid plaque the relative oxysterol concentrations were between 24- and 55-times higher compared to plasma. Interestingly, in carotid plaque Triol and 5,6-ECs seem to be more highly concentrated compared to other ROS-derived oxysterols. However, further investigations in a larger study cohort have to follow to clarify these findings.

5. Conclusion

We developed a fast LC–MS/MS method for the simultaneous quantification of five ROS-derived oxysterols: free 7-KC, 7-α/β-OHC, 5,6-α-EC, 5,6-β-EC, Triol, and free cholesterol in 80 µl human plasma or 10 mg atherosclerotic plaque. For the first time, complex sample pretreatment protocols of human plasma and extracted plaque samples could be reduced to a single protein precipitation and concentration step which avoids in vitro cholesterol autoxidation.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2013.06.022.

Conflict of interest

The authors declare that there are no conflict of interest.

Acknowledgment

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