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Preanalytical standardization for reactive oxygen species derived oxysterol analysis in human plasma by liquid chromatography–tandem mass spectrometry

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ABSTRACT

The analysis of the oxysterols 7-keto-, 7-α/β-hydroxy-, 5α,6α-epoxy-, 5β,6β-epoxycholesterol and cholestane-3β,5α,6β-triol derived from reactive oxygen species (ROS) is of interest as biomarkers in the field of atherosclerosis. Preanalytical validation is a crucial point to minimize the susceptibility of oxysterols to *in vitro* autoxidation. The aim of this study was to standardize a preanalytical protocol for ROS-derived oxysterol analysis by liquid chromatography–tandem mass spectrometry in human plasma.

Sample matrices were compared and stability of free oxysterols in whole blood and EDTA-plasma was investigated with regard to short-term storage until sample preparation, freeze–thaw cycles, addition of butylated hydroxytoluene and long-term storage up to 1 year at different temperatures (−20 °C, −80 °C and −130 °C) as well as different storage containers (safe-lock tubes, cryo tubes and straws). Sample preparation prior LC–MS/MS analysis was reduced to a simple concentration and protein precipitation step.

Storing EDTA-whole blood for 30 min at room temperature resulted in <25% concentration changes, within acceptable change limits (ACL). In freshly prepared plasma samples, free oxysterols were stable for 90 min stored at 4 °C with concentration changes <23.5% (within ACL). Up to nine freeze–thaw cycles did not affect analyte concentrations (concentration change −8.5% to +5.0%). 7-Ketocholesterol was stable for 2 years stored <−80 °C; concentration changes below 20.5% (within ACL). The remaining oxysterols were stored for a maximum of 2–4 weeks without exceeding ACL. The addition of BHT did not reveal improvement in analyte stability for storage at −80 or −130 °C.

We developed a standardized preanalytical protocol for oxysterol analysis based on LC–MS/MS, compared cryobanking conditions for each oxysterol and present data for long-term storage up to 2 years.

1. Introduction

Reactive oxygen species (ROS)-derived oxysterols are of increasing interest for studying oxidative stress associated diseases, including atherosclerosis [1,2]. However, the quantification of 7-ketocholesterol, (7-KC), 7-α/β-hydroxycholesterol (7-α/β-OHC), 5α,6α-epoxycholesterol (5,6-α-EC), 5β,6β-epoxycholesterol (5,6-β-EC) and cholestane-3β,5α,6β-triol (Triol) pose a challenge due to their sensitivity to *in vitro* autoxidation [3–5]. It is known that oxysterols analyzed in human plasma may be artificially formed by *in vitro* oxidation during sample storage and preparation [5–7]. Therefore, analysis of ROS-derived oxysterols in clinical studies requires a standardized preanalytical protocol for blood taking, storage and sample preparation prior to analysis.

To our knowledge, there is no published systematic investigation of preanalytical factors on ROS-derived oxysterol analysis. Sample preparation for gas chromatography mass spectrometric (GC–MS) analysis typically requires addition of the antioxidant butylated hydroxytoluene (BHT) to avoid autoxidation [1,5,8–11]. Due to simpler and gentler sample preparation with LC–MS methods, analogous addition of BHT may be avoided [12–15]. This distinction is significant because the use of BHT added prior to long-term sample storage is controversial [3,12,14,16].

The aim of our study was the establishment of a standardized preanalytical protocol for sample specimens, blood sample

Abbreviations: LC–MS/MS, liquid chromatography–tandem mass spectrometry; ROS, reactive oxygen species; 7-KC, 7-ketocholesterol; 7-α/β-OHC, 7-α/β-hydroxycholesterol; 5,6-α-EC, 5α,6α-epoxycholesterol; 5,6-β-EC, 5β,6β-epoxycholesterol; Triol, cholestane-3β,5α,6β-triol; BHT, butylated hydroxytoluene; GC-MS, gas chromatography mass spectrometry; RT, room temperature; NQC, native quality control; SQC, spiked quality control; ACL, acceptable change limit.

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collection, storage and processing procedures for the reproducible quantification of ROS-derived free oxysterols while minimization autoxidation. Long term storage with different cryobanking tubes and storage temperatures over a storage period of 2 years were investigated and reported.

2. Materials and methods

2.1. Chemicals and reagents

Oxysterol, cholesterol and isotope labelled internal standards were purchased from Steraloids (Newport, Rhode Island, USA), Euriso-Top (Saarbrücken, Germany) and Avanti Polar Lipids (Alabama, USA) (Supplement Table 1). BHT was used from Sigma–Aldrich (Munich, Germany). Multifly needle sets and polypropylene monovettes with and without anticoagulants (EDTA-K_3, citrate and lithium heparinate) and polypropylene safe-look tubes were purchased from Sarstedt (Nümbrecht, Germany), cryo tubes from Fluidx (Oakville, Ontario, Canada) and straws from Cryobiosystems (Paris, France).

2.2. Sample collection

Blood samples were collected freshly from six healthy normolipidemic volunteers (male $n = 3$, female $n = 3$) by venipuncture technique for each experimental question. The study of preanalytical standardization of oxysterol analysis was approved by the ethic committee of the University of Leipzig 082-10-190-42010. The collected blood samples were centrifuged at 2000*g* for 10 min. After centrifugation, plasma and serum were stored until analysis according to the research question. An overview of the preanalytical experiments is summarized in Fig. 1.

2.3. Sample pretreatment and quantification by LC–MS/MS

Sample preparation and LC–MS/MS analysis was based on our previously described procedures [13]. Briefly, 80 µl plasma was diluted with 1440 µl methanol/isopropanol 1:1 (v/v) including isotopically labeled standards for protein precipitation. Supernatants were evaporated to dryness at room temperature under a stream of nitrogen and dried extracts were stored at −80 °C until oxysterol analysis. An API 4000 triple quadrupole mass spectrometer equipped with APCI (AB SCIEX, Toronto, Canada) in positive ion mode was used. Chromatographic separation was performed on a Chromolith SpeedRod RP-18e monolithic column (50 × 4.6 mm) (Merck KGaA, Darmstadt, Germany).

In-house pooled plasma controls at two different concentration levels were used as quality controls: native quality control (NQC) and spiked quality control (SQC, spiked with 20 ng/ml oxysterol mix). Further, a freshly diluted methanolic standard mixture of 10 ng/ml of each oxysterol (stock solution $c = 10$ µg/ml) was used for quality checks.

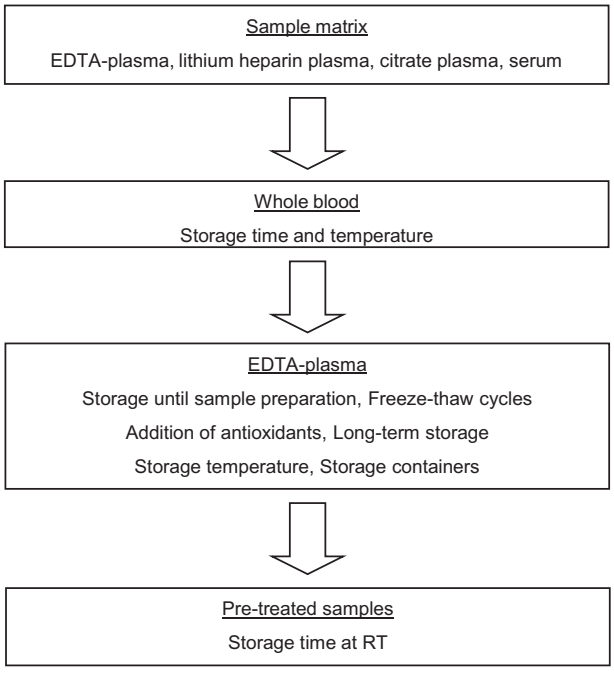

Fig. 1. Overview of the experimental set-up.

2.4. Comparison of plasma and serum

Human whole-blood samples from four healthy volunteers were collected in monovettes with no anticoagulants (serum) and with the anticoagulants EDTA-K_3, citrate and lithium heparin from Sarstedt (Nümbrecht, Germany) to determine the differences in oxysterol quantification.

2.5. Stability of oxysterols in EDTA whole blood until plasma separation

After collection of human whole-blood ($n = 4$) in EDTA-K_3 containing tubes, the samples were divided into a total of eight aliquots, for the storage at RT (4 aliquot) and at +4 °C (4 aliquot). Centrifugation of each individually stored aliquot took place after 30, 60, 90 and 120 min.

2.6. Stability of EDTA-plasma prior to long-term storage

Oxysterol stability was investigated in pooled EDTA-plasma specimen of 6 (3 male/ 3 female) healthy volunteers and a spiked plasma level ($c = 10$ ng/ml), each with and without BHT treatment (50 µg/ml plasma). Stability of oxysterol concentrations prior to sample processing was investigated immediately after 0.5, 1.0, 1.5, 2.0 and 6.0 h stored at +4 °C in triplicates.

2.7. Freeze–thaw cycles

Stability of oxysterol concentrations related to the number of freeze–thaw-cycles (at −80 °C and RT, respectively) was tested for up to 10 consecutive cycles in triplicates.

2.8. Storage of processed samples prior to LC–MS/MS analysis

Oxysterol concentration stability after sample pretreatment was tested through injection from the same vial 4-times within 9 h.

2.9. Cryobanking stability of oxysterols

Time-dependent influence of different air volumes (maximum-filled safe-look tube – 500 µl sample in 500 µl tube and low-filled safe-look tube with a 4-fold air volume – 100 µl sample in 500 µl tube) at −80 °C was compared for up to 1 year stored in triplicates. Long-term stability experiments for pooled EDTA-plasma and a spiked level ($c = 20$ ng/ml) were processed in triplicates. The analyte stability over a storage time of 12 months was tested at temperatures of −20 °C, −80 °C and −130 °C. Influence of different storage containers, safe-lock tubes, cryo tubes and straws were checked in triplicates. Treatment with and without BHT (50 µg/ml plasma) combined with overlay of nitrogen was investigated

for storing at −80 °C and −130 °C up to 12 months. Additionally, storing at −80 °C in safe lock tubes without addition of BHT was analyzed up to 2 years.

2.10. Statistics

Calculations were performed with IBM SPSS Statistics software (version 20). Results are expressed as median and 95% confidence interval. A mean percentage deviation greater than the Acceptable Change Limit (ACL = 2.77 CV) represents a presumed concentration change [17]. The ACL used were as follows: (i) for native plasma: 7-KC 68.4%, 7-a/b-OHC 26%, 5,6-b-EC 72.0% and cholesterol 20.4%, (ii) for spiked plasma (c = 10 ng/ml) 7-KC ± 17.5%; 7-α/β-OHC ± 24.4.0%; 5,6-α-EC ± 21.9%; 5,6-β-EC ± 23.0% and Triol ± 28.3%, and (iii) for spiked plasma (c = 20 ng/ml): 7-KC ± 20.5%; 7-α/β-OHC ± 13.0%; 5,6-α-EC ± 18.0%; 5,6-β-EC ± 21.3% and Triol ± 19.9%. Assessment of analyte concentration stability trends were performed by linear regression and one-way analysis of variance (ANOVA). Analytical concentration changes above the intra-assay CV in native plasma samples measured in one-batch were designated as substantial changes.

3. Results

3.1. Comparison of plasma and serum

Comparing citrate and lithium heparin stabilized plasma and serum with EDTA-plasma, serum showed higher 7-KC concentrations (mean +47%) after a clotting time of 30 min, whereas all the other analytes showed comparable concentrations. In citrate plasma lower concentrations of cholesterol (−20%) and oxysterols (−21% to −50%) were found. The oxysterol concentrations of lithium heparin plasma are similar to EDTA-plasma for all analytes except 7-KC, which shows a higher concentration (+20%) as shown in Fig. 2.

3.2. Stability of EDTA whole blood until plasma separation

Analyte stability in EDTA whole blood showed a high inter-individual variability. Therefore, EDTA whole blood has to be separated from cellular components within 30 min at RT after blood collection. Analytes were stable in whole blood specimens with storage at RT up to 120 min for cholesterol, up to 90 min for 7α/β-OHC and 7-KC, up to 30 min for Triol and 5,6-β-EC (Supplement Fig. 1). Storage at +4 °C did not increase stability of the analyte concentrations (data not shown).

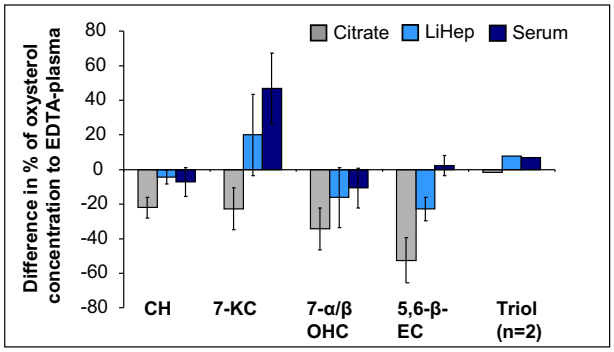

Fig. 2. Comparison of citrated plasma (citrate), lithium heparinated (LiHep) plasma and serum to EDTA-plasma for four probands (2 m/2 f), expressed as differences in % to EDTA-plasma (mean ± SD).

3.3. Stability of EDTA-plasma prior to long term storage

Oxysterol concentration in spiked plasma samples stored at +4 °C were stable up to 90 min for 5,6-β-EC (+20.1%) and up to 6 h for 7-KC (+15.7%), 7-α/β-OHC (+8.1%), 5,6-α-EC (+12.6%) and Triol (+5.5%). By addition of BHT, analyte stability of 5,6-β-EC increased up to 6 h (+13.3%, Supplement Fig. 2), too.

3.4. Freeze–thaw cycles

All investigated oxysterols were stable up to 9 freeze–thaw cycles (Supplement Table 2).

3.5. Storage of processed samples prior to LC–MS/MS analysis

Pretreated samples were stable over the period of one batch for 9 h at RT (Supplement Table 3).

3.6. Cryobanking stability of oxysterols

The air to sample volume ratio of a cryotube influences the stability of analyte concentration. In low-filled tubes with a 4-fold air volume analyte concentrations increased significantly and reached a maximum after 90 days: +39.3% for 7-KC; +34.2% for 7-α/β-OHC, +43.5% for 5,6-α-EC, 29.8% for 5,6-β-EC and no significant concentration change was observed for Triol (+14.1%). In comparison, analytes in maximum-filled tubes showed a tendency to become decomposed (Fig. 4).

Long-term storage of EDTA-plasma at −20 °C in safe lock tubes is not sufficient, due to oxysterol formation and significant concentration increase already after 30 days, of +47.4% for 7-KC, +27.5% for 7-α/β-OHC, +19.7% for 5,6-α-EC, +25.5% for 5,6-β-EC and +23.6% for Triol.

At −80 °C and −130 °C the analyte with the lowest stability was 5,6-α-EC for up to 2 weeks, followed by 5,6-β-EC, Triol and 7-α/β-OHC for up to 4 weeks. Cholesterol and 7-KC were stable up to 2 years in safe-lock tubes at −80 °C without BHT addition. Storage in cryotubes or straws showed similar results (data not shown). Generally, under experimental conditions, addition of BHT did not reveal an improvement in analyte stability for storage at −80 °C and −130 °C. Fig. 3 shows a representative change in analyte concentration over time for one long stable analyte (7-KC) and one short stable analyte (5,6-β-EC) at different temperatures.

4. Discussion

Defined preanalytical conditions are a prerequisite for oxysterol analysis in clinical studies. In particular, the quantitative analysis of ROS-derived oxysterols could be influenced by autoxidative formation of oxysterols as well as oxysterol degradation. Therefore a well-defined preanalytical protocol defining conditions for blood taking, storage and blood processing is required for oxysterol analysis.

Our results show that besides EDTA-plasma, lithium heparinated plasma is an alternative matrix for oxysterol analysis. However, levels of analytes differ from those in EDTA-plasma, why within one study, only one blood matrix should be compared. Citrated plasma is less suitable for oxysterol analysis because of decomposition of 7-α/β-EC and 5,6-β-EC. Serum showed a higher susceptibility to cholesterol autoxidation compared to EDTA-plasma, possibly due to the absence of a suited antioxidant [16,18]. Compared to plasma, serum has two additional limitations that have to be taken into account in oxysterol analysis: (i) blood samples have to coagulate at room temperature for at least 20 min and

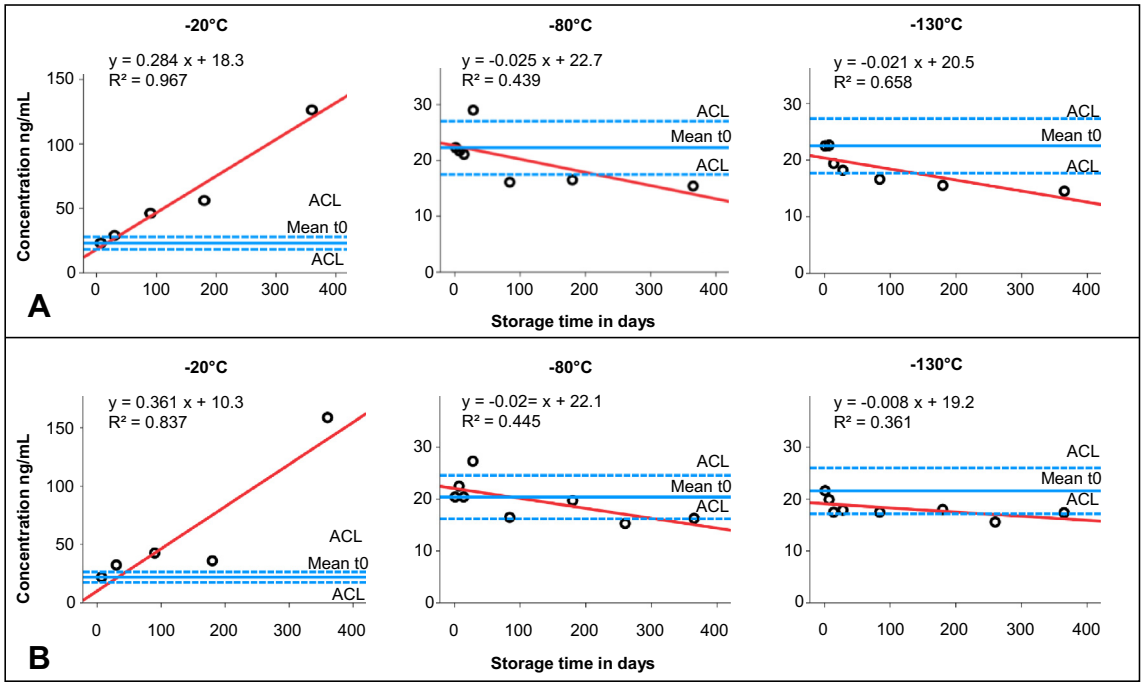

Fig. 3. Comparison of analyte concentration stability for storage at −20 °C, −80° and −130 °C in safe lock tubes in depending on storage time up to 1 year, analyzed in triplicates for (A) 5β,6β-epoxycholesterol and (B) 7-ketocholesterol. ACL – acceptable change limit, mean t1 – data are related to the mean value of a 1 day frozen sample under the mentioned condition.

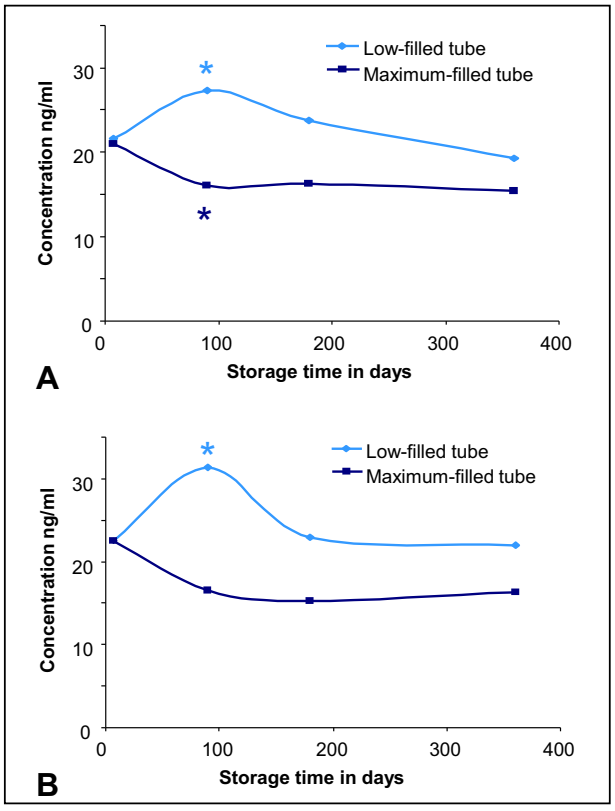

Fig. 4. Time-dependent influence of the air volume in the storage tube on oxysterol concentrations for (A) 5β,6β-epoxycholesterol and for (B) 7-ketocholesterol.

(ii) serum should be stored <−40 °C because it does not completely freeze at higher temperatures [18,19].

Since EDTA-plasma is most often used, the following standardization experiments were performed using EDTA-blood [16]. After blood taking, EDTA whole blood can be stored at room temperature for a maximum of 30 min, which is a crucial step in sample processing management. Inter-individually differences in storage stability at room temperature was observed which may reflect a different antioxidant buffer capacity of the blood [1].

In EDTA-plasma, oxysterols were stable for 90 min at 4 °C without BHT addition or up to 6 h at room temperature with addition of 50 μg BHT /ml plasma. If 5,6-β-EC is not being analyzed, the processing time window rise up to 6 h for EDTA-plasma stored at 4 °C. Our findings are in agreement with previous works where Triol and 7-KC were described as stable up to 15 h at RT [12].

We could show that oxysterol concentrations were stable for up to 9 freeze–thaw cycles, whereas previous works suggest only 3 [12].

After the protein precipitation step oxysterols were stable in methanolic solution for up to 9 h at room temperature, which was found for solvent extracts after liquid–liquid extraction and solid-phase extraction, too. This allows a batch wise sample pretreatment and analysis [12,14,20].

Long-term storage experiments are important for defining cryobanking conditions, in determining the validity of oxysterol analyses in retrospective studies and studies where different preanalytical settings have been used (e.g. meta-analyses). Long-term storage stability may be influenced by various factors, such as storage temperatures, the addition of BHT, the type of storage containers, and the filling level of the containers. With respect to the inter-assay CV of the analytical method ACL was calculated to identify significant concentration changes of oxysterols in spiked plasma [13,17]. A temperature of −20 °C is not appropriate for long-term sample storage and within 30 days the concentration of 7-KC increased twofold as a result of cholesterol autoxidation. Storage temperatures of −130 °C showed generally no advantage over −80 °C. At both temperatures, 5.6-α-EC was stable for only up to 2 weeks and 7-α/β-OHC, 5,6-β-EC and Trio for 4 weeks [5]. Only cholesterol and 7-KC were stable for up to 2 years at −80 °C in safe lock tubes, confirming previously reported results (223 days at −80 °C) [12].

Our findings support the former assumption that the measured concentrations after longer storage time did not reflect *in vivo* concentrations which has already been described for free cholesterol-5,6-epoxide [5]. However, it was not previously shown that this effect can be observed already within 2 weeks after blood sampling. We could not confirm the previously described stability of Triol (223 days at −80 °C) [12].

We speculate that shorter calculated stability of plasma samples stored at −80 °C with addition of BHT could be due to low amounts of methanol added to the sample. Methanol in turn could stabilize the active enzyme center and thus favor the enzymatic conversion of oxysterols [21]. However, this observation could not be seen with storage at −130 °C, indicating reduced enzyme activity at these temperatures. But under experimental conditions, addition of BHT did not reveal an improvement in analyte stability for storage at −80 and −130 °C, similar results were shown previously for 4-β-OHC [14].

Our data show no advantage of cryotubes and straws over safe-lock tubes for oxysterol analysis. The air volume in the storage tube has a strong impact on analyte concentrations. Therefore, storage tubes should be always fully filled according to manufacture information. Otherwise oxysterol concentrations increase (cholesterol autoxidation) and decrease (oxysterol decomposing) over time, thus showing a parabolic progression. However, after storage for 3 months, oxysterol concentrations reached a level that was stable up to the latest tested time point of 2 years.

To conserve *in vivo* oxysterol concentration in long-term stored samples, alternative storage strategies should be taken into account. Addition of high amounts of organic solvents may reduce enzymatic reactions and therefore reduce oxysterol concentration changes [22–25].

With respect to the performed stability experiments the following preanalytical protocol has to be considered regarding a reliable quantification of ROS-derived oxysterols in human plasma:

- Store EDTA whole blood at RT for a maximum of 30 min.
- Store EDTA-plasma at 4 °C or on ice for a maximum of 90 min.
- Freeze–thaw samples for a maximum of 9 times.
- Store pre-treated samples at RT for a maximum of 9 h.
- Fill tubes completely before freezing.
- Store EDTA-plasma for a maximum of 2 weeks <−80 °C; in prolonged storage, consider a consecutive concentration decrease.
- 7-KC at <−80 °C is stable for 2 years.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.087.

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