Pre-analytical stability of novel cerebrospinal fluid biomarkers

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Abstract

Stability of the cerebrospinal fluid (CSF) composition under different pre-analytical conditions is relevant for the diagnostic potential of biomarkers. Our aim was to examine the pre-analytical stability of promising CSF biomarkers that are currently evaluated for their discriminative use in various neurological diseases.

Pooled CSF was aliquoted and experimentally exposed to delayed storage: 0, 1, 2, 4, 24, 72, or 168 hours at 4°C or room temperature (RT), or 1-4 months at –20°C; or up to 7 freeze/thaw (f/t) cycles, before final storage at –80°C. Eleven CSF biomarkers were screened using enzyme-linked immunoassays, (ultra-)high-performance liquid chromatography, LC-MS/MS, or enzymatic methods. Data were normalised to the concentration at time zero per marker. Mean relative concentration and 95% confidence interval per data point were presented.

Levels of neurogranin (truncP75), chitinase-3-like protein (YKL-40), beta-site amyloid precursor protein cleaving enzyme 1 (BACE1), acetylcholinesterase (AChE) enzymatic activity, theobromine, secreted protein acidic and rich in cysteine-like 1 (SPARCL-1) and homovanillic acid (HVA) levels were not affected by the applied storage conditions. 3-Methoxy-4-hydroxyphenylglycol (MHPG) levels linearly and strongly decreased after 4 hours at RT (–10%) or 24 hours at 4°C (–27%), and with 6% after every f/t cycle. 5-Methyltetrahydrofolate (5-MTHF) (–29% after 1 week at RT) and 5-hydroxyindoleacetic acid levels (5-HIAA) (–16% after 1 week at RT) were reduced and 3,4-dihydroxyphenylacetic acid (DOPAC) levels (+22% after 1 week at RT) increased, but only after >24 hours at RT.

Ten out of eleven potential CSF novel biomarkers showed very limited change under common storage and f/t conditions, suggesting that these CSF biomarkers can be trustfully tested under the pre-analytical conditions present across different cohorts.
Introduction

Biomarkers in cerebrospinal fluid (CSF) have a high potential to support the diagnosis, monitoring and prognosis of neurological diseases to eventually improve patient care. Studies aimed at the identification and clinical validation of novel biomarkers often make use of samples originating from historical cohorts, collected before the establishment of clear biobanking protocols, or from multiple centres, between which the variation in pre-analytical conditions can be substantial. Pre-analytical variation can occur during collection, processing, and storage of the CSF and could result in non-biological variation of biomarker levels. Excluding pre-analytical conditions from significantly influencing the biomarker levels is of great importance, as biomarkers are used as clinical tools influencing medical decisions and patient care. As such, knowledge on the pre-analytical stability of potential CSF biomarkers is an essential prerequisite before proceeding to clinical validation of the biomarker assay. For the classical CSF biomarkers amyloid-β and tau isoforms, used for diagnosis of Alzheimer’s disease, these stability tests have been performed and have constituted the CSF collection and processing consensus protocol.

To facilitate a universal method for immunoassay validation, a standard operating procedure (SOP) was recently developed, which includes a section on storage stability to ensure that relevant pre-analytical conditions for CSF are addressed during the technical validation of a novel biomarker. Upon application of this SOP, stability studies will be better aligned, resulting in better interpretable outcomes and more certainty regarding analyte stability. The SOP was a result of the BIOMARKAPD project, a part of the EU Joint Programming - Neurodegenerative Disease Research (JPND) focussing on standardisation of biomarker assays for dementia (www.jpnd.eu), and was applied in this study.

The following CSF proteins and molecules were selected for stability testing, based on their potential as biomarker candidates for neurological diseases. Neuroragin and beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) are both synaptic proteins, and neurogranin CSF levels are currently being explored in a number of clinical trials, e.g. in the gantenerumab trial. BACE1 protein levels have been explored in elderly healthy participants after chronic treatment with a BACE inhibitor. CSF Acetylcholine esterase (AChE) has been proposed as a potential biochemical marker for cholinergic function in Alzheimer’s disease (AD), decreasing modestly as dementia progresses, but also current AD therapy is mostly based on inhibitors of AChE which only have modest and transient therapeutic effects. Chitinase-3-like protein (YKL-40) in CSF increases upon neuroinflammation in several neurodegenerative diseases, and YKL-40 levels were found to be decreased upon immunosuppressive treatment in multiple sclerosis (MS). Theobromine, a metabolite of caffeine, has been explored in CSF after traumatic brain injury for its potential neuroprotective effects, and might have a neuroprotective effect in AD.
as well. Secreted Protein Acidic and Rich in Cysteine-like 1 (SPARCL-1) is a secreted protein involved in extracellular matrix organisation and has been studied in CSF as diagnostic biomarker for subtypes of MS and to discriminate AD patients from controls. The monoamines, 3-methoxy-4-hydroxyphenylglycol (MHPG), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA), are the principal metabolites of neurotransmitters and have been studied in CSF as biomarkers for AD or dementia with Lewy bodies. 5-Methyltetrahydrofolate (5-MTHF) in CSF reflects the methionine–homocysteine transmethylation cycle in the brain and has been studied as a diagnostic marker for various CNS diseases, including AD.

Our aim was to assess the storage and freeze/thaw stability of eleven CSF proteins or molecules that are currently evaluated in our centre and in collaborative projects for their potential as novel biomarkers for neurological diseases.

Methods

Samples

CSF samples surplus (e.g. samples having inadequate clinical information for use in clinical validation) from the Alzheimer Center Biobank at the VU University Medical Center (VUmc, Amsterdam, the Netherlands), stored at -80°C, were used to prepare pools for storage and freeze/thaw stability testing. Aliquots of pooled CSF were typically 500 µl and were stored in 1.5 ml polypropylene tubes with screw caps (Sarstedt, Nümbrecht, Germany). The aliquoted CSF pools were exposed to storage conditions as described in the SOP for sample stability, i.e. storage up to 1 week at either 4°C or room temperature (RT); storage at -20°C up to 1 or 4 months, before final storage at -80°C; or exposure up to 7 freeze/thaw cycles. A minimum of three different CSF pools were measured per marker, only for SPARCL1 the sample size was extended to 5 in view of the variation of the assay. For delayed storage of 5-MTHF, fresh CSF was centrifuged at 2000 g for 10 minutes and then stored for 30 minutes, 1 hour, 1 day, or 1 week at 4°C or RT; other aliquots underwent 1 or 4 f/t cycles before storage at -80°C. Reference samples for all markers were stored at -80°C directly at time point zero. All samples were blinded and donors gave informed consent. This study was in line with the institutional research code and the biobank was approved by the local ethical committee. Samples were stored at -80°C for maximum one year before measurement of the biomarker of interest.

Assays

Neurogranin

Neurogranin levels were measured using an enzyme-linked immunosorbent assay (ELISA) that specifically detects the neurogranin epitope that is C-terminally truncated at P75 (ADx).
Neurosciences, Ghent, Belgium) according to the manufacturer’s instructions. Intra- and inter-assay variabilities reported by the manufacturer were <5% and <7%, respectively.

**YKL-40**

YKL-40 levels were measured at the University of Gothenburg using ELISA according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA). Intra- and inter-assay variabilities were established as <10% and 6%, respectively.

**Theobromine**

Theobromine levels were quantified using liquid chromatography coupled to tandem mass spectrometry and compared with an internal standard at the Center for Neurosciences and Cell Biology, University of Coimbra (modified from as described in the supplementary material). The coefficient of variation reported for this assay was 6% and 17% for intra and inter-assay, respectively.

**SPARCL-1**

SPARCL-1 levels were measured at VUmc Amsterdam using a DuoSet ELISA Development kit according to the manufacturer’s instructions (R&D systems, Abingdon, United Kingdom). Intra- and inter-assay coefficients of variation reported in that study were 6% and 12% respectively.

**BACE1**

BACE1 levels were measured using ELISA according to the manufacturer’s instructions (Adx Neurosciences, Ghent, Belgium). Intra- and inter-assay variabilities reported by the manufacturer were 3.8% and 8.5%, respectively.

**AChE enzymatic activity**

AChE activity was determined by a modified microassay version of the colorimetric Ellman’s method at the University Miguel Hernández. AChE was assayed with 1 mM acetylthiocholine and 50 µM tetraisopropyl pyrophosphoramide (Iso OMPA), a specific inhibitor of butyrylcholinesterase, a second cholinesterase that co-exists with AChE in brain and CSF. The coefficient of variation reported for this assay was 3%.

**Monoamine metabolites**

The principal metabolites of noradrenaline, dopamine and serotonin, i.e. 3-methoxy-4-hydroxyphenylglycol (MHPG), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and, 5-hydroxyindoleacetic acid (5-HIAA), respectively, were determined using a reversed
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Phase ultra-high pressure liquid chromatography system coupled with electrochemical detection (RP-UHPLC-ECD) (Alexys® Neurotransmitter Analyzer, Antec Leyden, Zoeterwoude, Netherlands). The previously validated and optimized method was applied with modifications regarding the installed column (BEH C18 Waters column, 150 mm x 1 mm, 1.7µm particle size), pump preference (LC110S pump, 487 bar; isocratic flow rate of 75µL/min) and mobile phase composition (11% MeOH, 100mM citric and phosphoric acid, 2.8 mM octane-1-sulfonic acid sodium salt; pH 3.0). To remove excess proteins, pre-column purification of CSF samples was conducted using Amicon® Ultra 0.5 Centrifugal Filters (cut-off 3,000 Da, Millipore, Ireland; 14,000 x g, 40 min, 4°C), after which samples were injected. Intra- and inter-assay precisions were reported <3%.

5-MTHF

5-MTHF, a metabolite of folic acid, was quantified through a compound-specific redox reaction after separation of chemical compounds in CSF using RP-HPLC-ECD. This method was established with an intra-assay variation of 4.2% and an inter-assay variation of 5.7% (Metabolic Laboratory, Dept. of Clinical Chemistry, VUmc).

Validation of the stability SOP

The laboratories at the University of Antwerp and at the University Miguel Hernández prepared and tested their own CSF stability sets next to the VUmc-prepared samples according to the standardised guidelines and stability SOP. This was to validate the sample preparation, exposures, and part of the findings.

Data analyses

Biomarker concentrations were normalised to the value of the reference sample (t=0), and 95% confidence intervals (CIs) of the mean per time point, per temperature were reported. Markers for which the CI includes 100% were considered stable for that time point; markers for which the CI did not include 100% were considered unstable.

Results

Storage stability of potential biomarkers

When we analysed storage stability over 1 week storage at 4°C or RT, or 1 month at -20°C compared to the reference sample stored at –80°C, levels of neurogranin, YKL-40, theobromine, SPARCL-1, BACE1, AChE and HVA did not change after (figure 1A, C, E, G, I, K, M). Similarly, levels of these seven biomarkers were not changed after up to 7 freeze/thaw cycles (figure 1B, D, F, H, J, L, N).

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5-HIAA levels did not change after 1 week at 4°C, 3 days at RT, or 2 months at −20°C, but started decreasing after longer periods at RT or −20°C storage (figure 1O). After 1 week storage at RT levels decreased with −16%, after 4 months storage at −20°C levels decreased with −18%. 5-HIAA levels did not change after 7 freeze/thaw cycles (figure 1P).

In contrast, MHPG levels steadily decreased with −10% after 4 hours at 4°C, and −27% after 4 hours at RT, whereas at −20°C storage for 1 month, a larger reduction of −58% was observed (figure 1Q). Similarly, after 7 freeze/thaw cycles, MHPG steadily decreased with an average of −6.4% per freeze/thaw cycle (figure 1R).

In addition, DOPAC levels slightly increased with +8% after 24 hours storage at 4°C, and with +13% after 24 hours at RT, accumulating to +14% after 1 week storage at 4°C, and +22% after one week storage at RT (figure 1S). At −20°C storage, DOPAC levels similarly increased after one week, starting from +8% up to +30% after 2 months, where DOPAC levels reached a plateau (+31% after 3 and 4 months storage at −20°C). No change in DOPAC levels after 7 freeze/thaw cycles was observed (figure 1T).

5-MTHF acid levels remained unaltered after 1 week of storage at 4°C, but showed a steep decline so that, after 1 week at RT, only 70% of the initial 5-MTHF concentration was detected (figure 1U). 5-MTHF levels were not changed after 4 freeze/thaw cycles (figure 1V).

Validation of the stability SOP
To validate the sample preparation and exposures according to the SOP at VUmc and to validate part of the findings, the laboratories at the University of Antwerp and the University Miguel Hernández prepared and tested their own CSF stability sets next to the VUmc-prepared samples. In these sets, again, no changes were observed in neurogranin, BACE1, or AChE activity levels when CSF was exposed to up to 1 week of storage at 4°C or RT, or, 1 month at −20°C (Supplementary material 1).
Figure 1. Storage (left) and freeze/thaw stability (right) of potential novel biomarkers measured in individual
assays. Circles and solid lines show RT results, squares and broken lines show 4°C results, and triangles show
theobromin results. Dotted lines represent the reference line at 100%. Symbols represent the mean of 3-5 measurements
normalised to the reference value. Error bars show the 95% confidence interval. RT = room temperature.

Storage stability of SPARCL-1 over time (hours)

Storage stability of Neurogranin over time (hours)

Stability of YKL-40 over freeze/thaw cycles (n)

Stability of theobromine over freeze/thaw cycles (n)
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Error bars show the 95% confidence interval. RT = room temperature.

Figure 1 (continued).

Circles and solid lines show RT results, squares and broken lines show 4°C results, and triangles show -20°C results. Dotted lines represent the reference line at 100%. Symbols represent the mean of measurements normalised to the reference value. Error bars show the 95% confidence interval.
Figure 1 (continued). Storage (left) and freeze/thaw stability (right) of potential novel biomarkers measured in individual assays. Circles and solid lines show RT results, squares and broken lines show 4°C results, and triangles show -20°C results. Dotted lines represent the reference line at 100%. Symbols represent the mean of 3-5 measurements normalised to the reference value. Error bars show the 95% confidence interval. RT = room temperature.

Discussion

We here gained insight in the storage stability of several CSF proteins and metabolites that are currently relevant and evaluated as potential novel biomarkers for various neurological diseases. Under the pre-analytical conditions tested in the present study, which are extreme compared to common biomarker research practice, the CSF proteins and compounds neurogranin, YKL-40, BACE1, AChE activity, theobromine, SPARCL-1 and HVA were stable to all subjected storage and freeze/thaw conditions. However, a few compounds did not show complete stability: MHPG levels showed a moderate decrease at 4°C, RT and -20°C, as well as upon multiple freeze/thaw cycles. As for 5-MTHF and 5-HIAA, we found decreased, and for DOPAC increased, CSF

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levels after 1 week of delayed storage time at RT. The standardised duration of laboratory
handlings with CSF is recommended to be maximum 2 hours at 4°C between collection and
centrifugation, maximum 2 hours between centrifugation and freezer storage at −80°C, and
maximum 2 freeze/thaw cycles. Apart from MHPG, none of the markers tested showed any
alterations under these recommended pre-analytical conditions.

Some biomarkers that we analysed here have been previously evaluated for their stability. A
previous study showed that full length CSF neurogranin levels were stable at 4°C and −20°C
for one week, while after 2 days of storage at RT a decrease of 20% was observed, which
contrasts with our results. Our finding, i.e. no decline in truncated neurogranin levels at any
temperature, could be explained by the use of a different assay that targets the P75 truncation
of neurogranin specifically. Surprisingly, others reported slightly reduced levels of truncated
P75 neurogranin (on average ~3.2%) already after one freeze/thaw cycle, although this small
change remains within the intra-assay variation. YKL-40 levels were previously reported to be
stable in CSF after short-term (< 2 hours) storage at RT, which is in agreement with the stable
YLK-40 levels we observed. ACHE activity levels were previously found to be unchanged during
repeated freeze/thaw cycles, similarly to results from the current study. In addition, 5-HIAA
and HVA levels were previously published to be stable under delayed storage conditions up to 3
days. Again, this is in line with our results, which extended the findings for delayed storage
from 3 days to one week at RT, thereby showing stable levels of HVA, but a 16% decrease
in 5-HIAA levels. Overall, this study expands the current knowledge on CSF stability, as we
examined many storage time intervals at regular intervals, and a high number of freeze/thaw
cycles. Moreover, these conditions were measured within the same CSF pools, thus examining
all conditions in parallel.

Remarkably, MHPG levels have previously been found to be stable for up to 72 hours at RT
using HPLC-ECD, while we detected a substantial decrease of MHPG levels after 4 hours at
RT already. No other studies so far investigated the stability of MHPG in CSF; however, its overall
low (patho)physiological concentration in CSF and very early elution of the column (ca. 2 mins;
polar compound) make MHPG a rather difficult metabolite to detect (own observations (YV)).
On the contrary, the chromatographic method used in the current study has been thoroughly
optimised for specific MHPG detection, amongst others by sample pre-purification to remove
the MHPG bound to the sulphate conjugate, in combination with optimal column length,
mobile phase and detector settings (E<sub>190</sub> detection potential of 670 mV). These methodological
differences could underlie the conflicting results. Also, the study of Langlais et al. analysed a
mere 6 CSF samples. Studies on CSF stability of theobromine, SPARCL-1, BACE1, DOPAC, and
5-MTHF have not been previously reported.
The CSF proteins and compounds tested in this study vary with regard to their chemical characteristics. For instance, masses varied from 200 Da – 459 kDa, protein classes varied from membrane protein to enzyme to secreted protein, to enzyme, and configurations based on atomic structure varied from monoamines to proteins containing disulphide bonds, alpha-helices, beta-strands, or glycosylated side chains. No general patterns could be derived from this study, for example relating instability of specific chemical structures to specific pre-analytical conditions. Nevertheless, since effective instability was only observed for MHPG, this supports the claim that CSF molecules in general seem stable in vitro. Previous studies showed no effect of long-term (2-14 years) biobank storage on CSF biomarker concentrations, neither did evaporation occur after >4 years of biobank storage. Adsorption to lab plastics, however, significantly decreased levels of CSF Aβ42, emphasising that the effect of pre-analytical conditions is crucial to consider during biomarker development. We encourage the use of the currently presented standardised approach for testing the effect of pre-analytical processing conditions on levels of novel biomarkers, to serve as a solid basis for future biomarker validation tests.

The major strength of this study is the standardized approach of sample preparation that was used. To validate the sample preparation according to the SOP at VUmc, the laboratories at the University of Antwerp and the University Miguel Hernández also prepared and tested their own CSF stability samples (n=3 at each centre). These samples gave similar results as the sets prepared at the VU University Medical Center, indicating that the SOP can be successfully applied in other laboratories.

This study has nonetheless a few limitations. First, to prepare the stability sets according to the SOP, CSF pools were formed from biobanked CSF. This required one freeze/thaw cycle before actual exposure to the experimental storage conditions. Therefore, protein changes that might have occurred during the first freeze/thawing of the CSF could thus not be assessed. Second, the protein stabilities described in this study are limited to the specific (bio)chemistry of the used assays and cannot be extrapolated to other measurement platforms. Stability testing may thus still be required for novel methods, even if the protein had already shown stability with other techniques. Third, stability testing results depend on the characteristics of the assay, i.e., low detection levels in CSF and high variation coefficients decrease the power of stability testing. Some stability results had to be excluded from this study for that reason.

In conclusion, levels of potential novel CSF biomarkers appeared resistant to common experimental storage and freeze/thaw conditions, except for persistent – but slight – decreases of MHPG. It is, therefore, recommended to always store CSF samples for MHPG analyses almost instantaneously following lumbar puncture at -80°C or in liquid nitrogen, and reduce the number of pre-analytical sample preparation steps (at 4°C) before analysis. The influence of experimental storage and freeze/thaw conditions is crucial to consider during biomarker development. We encourage the use of the currently presented standardised approach for testing the effect of pre-analytical processing conditions on levels of novel biomarkers, to serve as a solid basis for future biomarker validation tests.

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the tested different pre-analytical storage procedures in biomarker studies that use historical cohorts or cohorts in multicentre studies is thus expected to be trivial.

Acknowledgements

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References


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Supplementary material

Supplementary material 1: Validation of sample preparation and exposure according to the SOP

Supplementary figure 1. A) storage stability and B) freeze/thaw stability of P75 truncated neurogranin prepared and measured at University of Antwerp, C) storage stability and D) freeze/thaw stability of BACE1 prepared and measured at University of Antwerp, E) storage stability and F) freeze/thaw stability of AChE activity prepared and measured at University Miguel Hernández. Circles and solid lines show RT results, squares and broken lines show 4°C results, and triangles show -20°C results. Dotted lines represent the reference line at 100%. Symbols represent the mean of 3 measurements normalised to the reference value. Error bars show the 95% confidence interval. RT= room temperature.

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Supplementary material 2: LC-MS methodology

Chemicals and reagents

Theobromine solution (Cerilliant) and Theobromine-d6 (98%, Aldrich) were purchased from Sigma. Acetonitrile, methanol and water were LC-MS grade and were from Fisher. Formic acid was LC-MS grade and was from Amresco.

Instrumentation

Samples were analyzed on an LC Nexera system (Shimadzu) coupled to an hybrid triple quadrupole/ linear ion-trap 4000 QTrap mass spectrometer operated by Analyst 1.6.1 (Sciex). The injector was a CTC-xt (PAL System). The chromatographic separation was performed using the 3 μm Gemini C18 column (50 x 2.0 mm, 110Å, Phenomenex) with a 4 x 2.0 mm C18 guard-column (Phenomenex). The flow rate was set to 250 μL/min and mobile phase A and B were 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. The LC program consisted in: 2% of B (0 - 0.3 min), 2 - 10% of B (0.3 - 5.0 min), 10 – 90% of B (5.0 - 6.0 min), 90% of B (6.0 - 7.0 min), 90 – 2% of B (7.0 – 7.1 min) and 2% of B (7.1 - 9.0 min).

The ionization source (ESI Turbo V) was operated in the positive mode set to an ion spray voltage of 5500 V, 35 psi for nebulizer gas 1 (GS1), 40 psi for the nebulizer gas 2 (GS2), 30 psi for the curtain gas (CUR), and the temperature was 600 °C. Theobromine and the internal standard were analyzed by Multiple Reaction Monitoring (MRM) where Q1 and Q3 were at unit resolution, the entrance potential (EP) was 10 eV, the collision cell exit potential (CXP) was 15 eV and the collision gas (CAD) was set to 8 psi. The MRM transitions for each compound and the parameters used are shown in Table 1.

Supplementary table 1. MRM transitions used for theobromine (THEOB) and theobromine-d6 (THEOB-d6) with the respective compound dependent parameters collision energy (CE), collision cell exit potential (CXP) and declustering potential (DP).

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<th>Q1</th>
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<th>CE</th>
<th>CXP</th>
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</table>
CSF samples preparation
Fifty microliters of each CSF plasma sample were spiked with 50 µL of the internal standard (Theobromine-d6) and the protein precipitation step was performed using methanol (400 µL), followed by centrifugation at 14,000 g for 20 minutes. The supernatant was collected, evaporated in a speedvac and samples were resuspended in 100 µL of 2% ACN + 0.1% FA. The volume of injection was 10 µL.

Calibration curve preparation
Successive dilutions of a theobromine solution were prepared in solvent (2% ACN + 0.1% FA) and the calibration curve (8 points) was prepared by mixing 50 µL of each dilution (0.08 - 10 µM) with 50 µL of the IS solution (4 µM). The final concentrations for each calibrator point, as for the IS, were reduced by a factor of 2 and the volume of injection was 10 µL. Peak areas were integrated using MultiQuant software (version 2.1.1, Sciex).