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Short communication

Lipoxin A_4: Problems with its determination using reversed phase chromatography–tandem mass spectrometry and confirmation with chiral chromatography

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ABSTRACT

Lipoxins belong to the family of so-called pro-resolving endogenous lipid mediators which are derived from arachidonic acid and play a key role in the counter-regulation of inflammation. Arachidonic acid is also precursor of multiple pro-inflammatory lipid mediators, such as prostaglandins and leukotrienes, which are simultaneously present in biological compartments. The close structural relationship between several of these lipid mediators and the absence of blank matrix samples enormously complicates the unequivocal identification of these compounds.

The determination of lipoxin A_4 has been accomplished by chromatographic separation using a C18 reversed phase column and tandem mass spectrometry detection. Samples were liquid–liquid extracted with ethyl acetate before injection. Identification of the analyte was done based on three criteria: retention time, ratio of the *m/z* transitions and MS/MS spectrum. To avoid false positive results due to endogenous interferences, the extracted samples were re-injected into a chiral Lux Amylose-2 chromatographic column. The authors recommend the use of chiral chromatography in the determination of pro-resolving lipid mediators, together with transition area ratio and fragmentation spectra to improve selectivity for identification and quantitation purposes.

1. Introduction

Inflammation is a self-limiting, physiological response to a damaging stimulus, defined by an initial recruitment of polymorphonuclear granulocytes followed by monocytes, which differentiate locally into macrophages [1]. The acute inflammatory response is a physiological response which protects the organism against damage, but it can develop into a pathological condition if it is dysregulated.

The inflammation response starts with the synthesis of pro-inflammatory mediators, including eicosanoids, cytokines, chemokines, cell adhesion molecules, etc. After a few hours to days, resolution of inflammation is initiated with the synthesis of anti-inflammatory and resolving mediators. Finally, the organism returns to normal homeostasis [2]. If resolution mechanisms do not function properly, the inflammation persists and becomes chronic.

Lipoxins (LX) are lipid mediators formed in vivo which appear to play a unique role in anti-inflammation and as signals for the resolution phase of the acute inflammatory responses in human and mammalian systems [3,4]. They are trihydroxy tetraene-containing eicosanoids generated from arachidonic acid (AA) via the sequential action of lipoxygenases (LOs) [5] (see Fig. 1) that evoke stereospecific and selective actions [6]. Pro-inflammatory mediators, such as prostaglandin E_2, also promote resolution by inducing the production of key enzymes in the biosynthesis of lipoxins [7–9] or its own production by a negative feedback mechanism [10].

Three main routes of lipoxin biosynthesis have been identified and lead to two different epimers [11]. Two pathways are formed by the cooperation of different LO-isoforms leading to the formation of 5 (S), 6(R), 15(S)-lipoxin A_4 (LXA_4). The 5-/15-LO pathway can be initiated by 5- or 15-LO. In one case, 5-LO converts AA to leukotriene A_4, which is further metabolized by 15-LO to an intermediate and then enzymatically metabolized to lipoxin A_4 and B_4. In the other case, AA is converted by 15-LO into 15(S)-HpETE, which is reduced to

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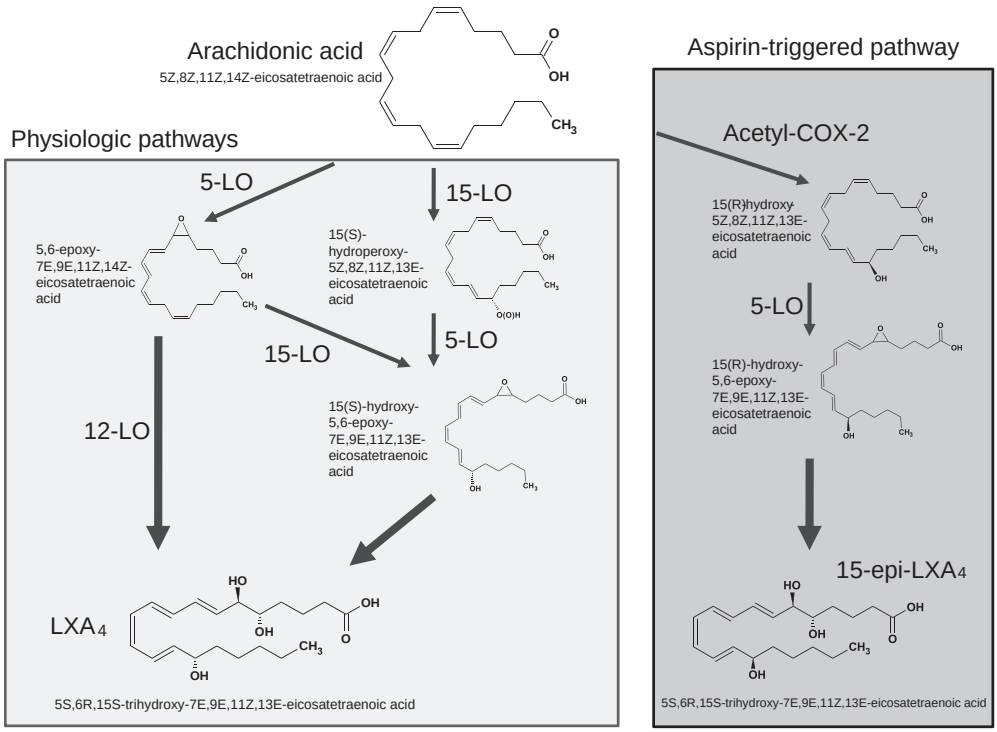

Fig. 1. Synthesis of lipoxin A_4 by physiologic and aspirin-triggered pathways. Modified from [11].

15(S)-HETE by a peroxidase. Both molecules are transformed by 5-LO into an intermediate and enzymatically metabolized to LXA_4 and lipoxin B_4 (LXB_4) [12]. In the 5-/12-LO pathway, 12-LO converts leukotriene A_4 (synthesized from AA via 5-LO) into LXA_4 and LXB_4. The third pathway generates 5(S), 6(R), 15(R)-lipoxin A_4 (15-epi-LXA_4) by interaction of acetylated cyclooxygenase-2 (COX-2) and 5-LO under the influence of acetylsalicylic acid [13].

We generated a trans-cellular system for the putative production of LXA_4. Therefore, human primary macrophages and cancer cells were co-cultured and the expression of 5-LO and 15-LO within one system was established. By stimulating this system with AA, a synthesis of LXA_4 was expected.

The pro-resolving activity of LX has been related to the activation of the peptide receptor FPR2, also known as ALX [7,9]. However, new studies suggest that FPR2/ALX is not activated by LXA_4 and that the molecular mechanism by which LXA_4 exerts its effects still needs to be identified [14].

The determination of lipoxins in biological samples is challenging due to their structural similarity to other AA derivatives and their short half-lives in vivo, since they undergo rapid metabolic inactivation by 15-hydroxyprostaglandin dehydrogenase [15]. The analysis of lipoxins has been carried out mainly using C18 reversed phase chromatographic separation in several matrices such as cell culture and tissue [16] or sputum [17], either alone [18] or in combination with other AA derivatives [19,20]. In some cases, no lipoxins were detected while in other ones, the presence of these molecules in the samples was assessed.

The determination of such molecules in biological matrices is very difficult, since the expected concentrations are very low, isomers and isobaric compounds are present in the samples (giving low selectivity during the extraction pretreatment) and no blank matrices are available. Several difficulties in the quantitation of LXA_4 are summarized in this work. As an example, we show a case in which the selectivity of liquid chromatography–tandem mass spectrometry is not adequate to discriminate LXA_4 from other endogenous compounds. To increase this selectivity and avoid false positive determinations of the analyte, a chiral chromatographic separation has been developed.

2. Materials and methods

2.1. Chemicals and solvents

The standards 5(S), 6(R), 15(S)-lipoxin A_4 (LXA_4); 5(S), 6(R), 15(R)-lipoxin A_4 (15-epi-LXA_4); 5(S), 6(S), 15(S)-lipoxin A_4 (6-epi-LXA_4) and deuterium labeled 5(S), 6(R), 15(S)-lipoxin A_4 (LXA_4-d_5) were purchased from Cayman Chemicals (Tallin, Estonia). Arachidonic acid (AA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) were also from Cayman Chemicals. Water, acetonitrile (ACN) and methanol, all LC–MS grade, were purchased from Carl Roth (Karlsruhe, Germany). Ethanol, LC grade, and formic acid (p.a.) were obtained from Merck (Darmstadt, Germany). Ethyl acetate was purchased from Sigma-Aldrich (Seelze, Germany). Dulbecco's Phosphate Buffered Saline (PBS) solution was obtained from PAA Laboratories (Pasching, Austria).

2.2. Standards preparation

Stock solutions of LXA_4 and 15-epi-LXA_4 were prepared in a concentration of 25 μg/mL in ethanol and LXA_4-d_5 was prepared at 10 μg/mL in methanol. All primary stock solutions were stored in amber vials at −80 °C. Standard sample solutions of the analytes were prepared freshly by dilution of primary stock solutions in methanol at concentrations of 0.1–100 ng/mL (0.1, 0.5, 1, 2.5, 5, 7.5, 10, 15, 50 and 100 ng/mL).

2.3. Instrumentation

During sample pre-treatment, a Biofuge 15 centrifuge (Heraeus, Hanau, Germany) was used.

The chromatographic separation was carried out on an Agilent 1200 LC system (Agilent Technologies, Waldbronn, Germany) consisting of a binary pump, a thermostated column compartment and a CTC PAL autosampler (CTC Analytics, Zwingen, Switzerland). Mass spectrometric detection was performed with a hybrid ion trap-triple quadrupole mass spectrometer, 5500 QTrap (AB Sciex,

Darmstadt, Germany) equipped with an electrospray source operated in negative mode. Nitrogen for the mass spectrometer was produced by a NGM 22-LC/MS generator (cmc Instruments, Eschborn, Germany). Peaks were quantified using Analyst Software Version 1.5 (AB Sciex, Darmstadt, Germany).

2.4. Chromatographic and mass spectrometric conditions

Chromatographic separation was achieved using a Synergi Hydro-RP 80 Å column (150 × 2 mm i.d., 4 μm particle size and 80 Å pore size) from Phenomenex (Aschaffenburg, Germany) and a precolumn from the same material. The eluent consisted of water (A) and acetonitrile (B). The complete chromatographic run time of each sample was 10 min. The analyte was eluted in gradient mode as follows: from $t=0$ to 0.5 min 60% A, flow rate 410 μL/min, from $t=1.5$ to 2.5 min 25% A, flow rate 425 μL/min, from $t=3.4$ to 4.0 min, flow rate varies from 425 μL/min to 410 μL/min followed by the constant gradient of 0% A for 1.5 min, flow rate 410 μL/min and then from $t=6.5$ to 10 min by the constant gradient of 60% A, flow rate 410 μL/min for the re-equilibration of the system. The column temperature was maintained at 40 °C and the temperature of the autosampler at 7 °C.

The mass spectrometer was operated using a Turboion Spray source in negative ionization mode at 550 °C with an electrospray voltage of −4500 kV. Curtain gas was 30 (instrument units, i.u.) and collision gas 9 i.u. Nebulizer gas and heater gas were set at 40 and 70 i.u., respectively. Analysis was performed in multiple reaction monitoring (MRM) mode with a dwell time of 40 ms.

For each analyte, 3 transitions were monitored: one for quantitation (m/z 351.1 → 115.0, collision energy (CE) −46 V) and two for identification purposes (m/z 351.1 → 58.9, CE −20 V; m/z 351.1 → 235.1, CE −18 V). For the internal standard, the transition m/z 356.2 → 115.0, CE −50 V was recorded. The fragmentation of the analytes has already been described elsewhere [21].

2.5. Chiral chromatographic conditions

To eliminate interferences due to the co-elution of endogenous compounds with the same retention time, same molecular m/z and several identical m/z fragments as LXA_4, a chiral separation was carried out. In this case, 5(S), 6(R), 15(S)-lipoxin A_4 (LXA_4), 5(S), 6(R), 15(R)-lipoxin A_4 (15-epi-LXA_4) and 5(S), 6(S), 15(S)-lipoxin A_4 (6-epi-LXA_4) were used as standard compounds. The internal standard was the same as for non-chiral chromatography.

For the chromatographic separation, a Lux Amylose-2 (150 × 2 mm ID, 3 μm particle size and 1000 Å pore size) column from Phenomenex (Aschaffenburg, Germany) was added to the system described above. The chromatographic conditions were slightly modified to allow the inclusion of the chiral column in the non-chiral separation system. Mobile phases consisted of water (A) and acetonitrile (B) eluted in gradient mode (flow rate 300 μL/min) as follows: from $t=0$ to 4 min isocratic 60% A, from $t=4$ to 4.5 min 40% A which was maintained for 7.5 min; from $t=12$ to 12.5 min, 10% A also maintained for 7.5 min; from 20 to 20.5 min 5% A for 3.5 min to wash the column and then returned to initial conditions (60% A) for 2.5 min to re-equilibrate the system. The complete chromatographic run time for each sample was 27 min. The column temperature was maintained at 40 °C and the temperature of the autosampler at 7 °C. The mass spectrometric conditions were the same as those used for the non-chiral analytical method.

Stock solutions of the three isomers described above were prepared in methanol as previously described. Standard sample solutions of the analytes were prepared at concentrations of 0.1–100 ng/mL.

2.6. Cell culture samples

Human macrophages (Mφ) were generated from buffy coats (DRK-Blutspendedienst, Baden-Würtemberg-Hessen). Briefly, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque gradients (PAA Laboratories). PBMCs were washed twice with PBS containing 2 mM EDTA. Cells were subsequently seeded on 6-well dishes in RPMI 1640 media supplemented with 100 U/mL penicillin and 100 g/mL streptomycin. After 1 h incubation, non-adherent cells were washed off with PBS and cells were cultured in Mφ-media (RPMI 1640 containing 5% human serum (DRK-Blutspendedienst)) for 7 days for differentiation. The density of Mφ was 2×10^5 cells per well.

MCF-7 breast carcinoma cells were cultured in RPMI 1640 supplemented with 100 U/mL penicillin, 100 g/mL streptomycin, 1 mM sodium pyruvate, 1 × non-essential amino acids, and 10% heat-inactivated fetal calf serum (FCS) (PAA Laboratories).

For co-culturing Mφ and MCF-7 cells, an established protocol was used [22]. Briefly, MCF-7 cells were harvested and seeded on top of Mφ in Mφ-media in a 1:1 ratio. Co-cultures were maintained for 5 days until complete apoptosis and phagocytosis of MCF-7 cells occurred. Subsequently, the remaining Mφ were co-cultured once more with MCF-7 cells in a 1:1 ratio for 2 days in Mφ-media.

Before harvesting the supernatants, samples were incubated with either AA, DHA or EPA (20 μM) for 24 h. Supernatants from control Mφ cultures, co-cultures (after 5 days) and with a second addition of cancer cells were snap frozen in liquid nitrogen and analyzed.

2.7. Liquid–liquid extraction (LLE) procedure

For extraction of the analytes, 200 μL of the sample (cell culture medium) were added to a 1.5 mL polypropylene extraction tube. After addition of 20 μL internal standard (250 ng/mL LXA_4-d_5) and 600 μL extraction solvent (ethyl acetate), the extraction tubes were vortexed for one minute and centrifuged at 16,000*g* for 5 min. The organic layer was removed and transferred to a glass autosampler vial and the extraction procedure was repeated with the addition of 600 μL ethyl acetate. After pooling the organic layers, they were evaporated to dryness at 45 °C under a stream of nitrogen. The samples were reconstituted in 50 μL water: acetonitrile (60:40, v/v) containing 0.1% formic acid and transferred to a conical glass insert. 10 μL were injected into the LC–MS/MS system for analysis.

Calibration samples were prepared by adding 20 μL of the appropriate working solutions to 200 μL PBS and were extracted as described above.

3. Results and discussion

3.1. Identification of LXA_4 using reversed phase chromatography

The medium samples were analyzed using reversed phase chromatography as described and compared with blank samples (culture medium) spiked with commercially available LXA_4. We also tested whether blank medium samples (not spiked with the standards) revealed interferences with the retention time of the analytes (data not shown).

Several cell culture medium samples were analyzed but only in cells incubated with AA, a match was obtained for the measured transitions at the same retention time of the standard LXA_4. Concentrations of lipoxins in biological samples are expected to be low, in the range of pg/mL [19], but in the case presented in Fig. 2, the concentration of the sample was suspiciously high, nearly 10 ng/mL. To confirm the presence or absence of interferences,

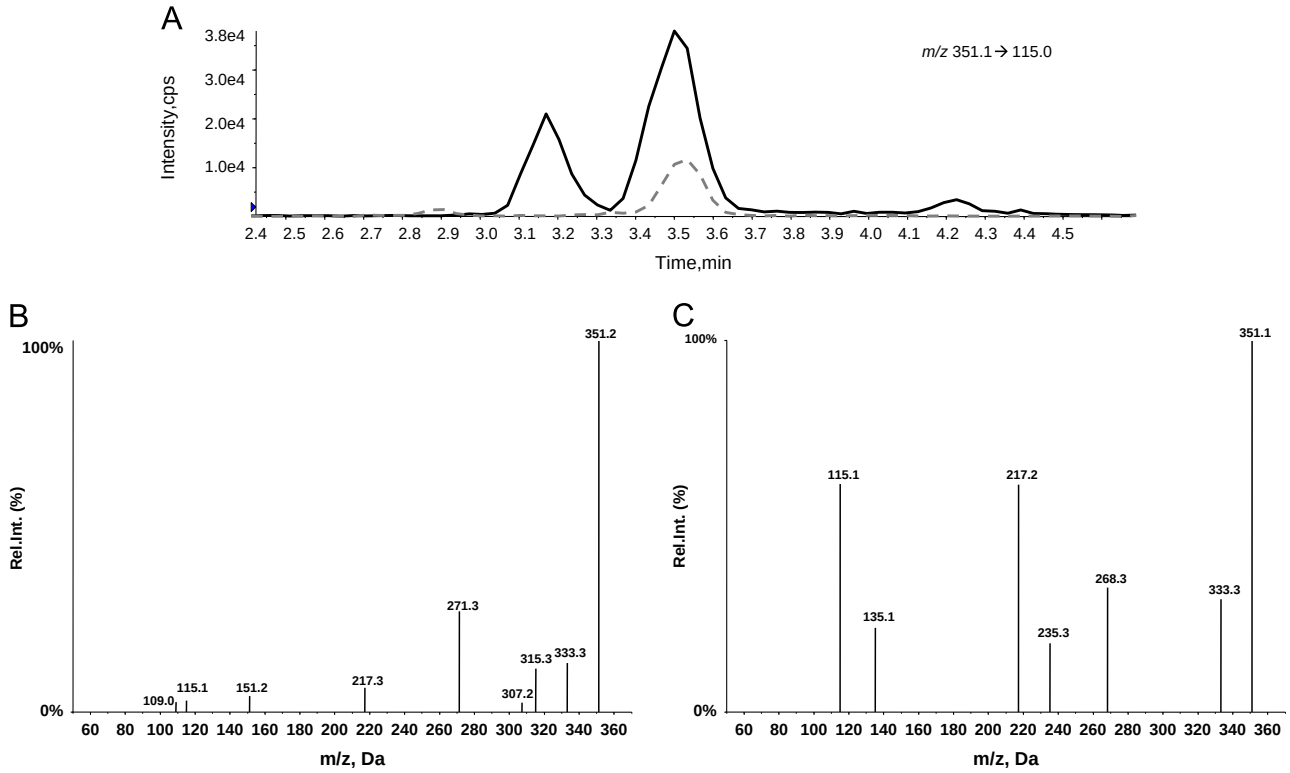

Fig. 2. (A) Chromatograms corresponding to an extracted cell culture medium sample (——) and to a 2.5 ng/mL calibration standard (·····). MS/MS spectra (*m*/*z* 351) of the chromatographic peak at the retention time corresponding to LXA_4 in cell culture medium (B) and in the standard sample (C). Collision energy −30 V and collision energy spread 20 V.

further examinations were carried out. These identification procedures are described as follows.

In most analytical procedures using LC–MS/MS, the first criterion to identify an analyte is the chromatographic peak at the analyzed mass to charge transition and the match with the retention time of the analyte and the deuterated internal standard (or the standard calibrator and if necessary, corrected by the internal standard). In this case, the match of both chromatographic peaks was exact (Fig. 2A).

The second criterion we used for identification purposes was the ratio "quantifying transition/qualifying transition 1" (*m*/*z* 351.1→115.0/ 351.1→58.9). It was 3.09 for the standard calibrator and 2.81 for the sample. The small difference observed in the calculated ratio could be due to the different matrices for the sample (in which cells had been incubated) and the standard (where no cells had been incubated) and a conclusive determination of whether or not LXA_4 was present in the analyzed cell culture medium was not possible. The qualifying transition 2 could not be used for this purpose due to interferences with matrix components (integration of this chromatographic peak was not possible).

For the final identification of LXA_4 in the analyzed sample, a third criterion had to be used: the MS/MS spectrum of the chromatographic peak corresponding to the *m*/*z* 351 at the retention time of the LXA_4 standard. The spectra obtained are shown in Fig. 2B and C. The comparison of both spectra indicates unequivocally, that the suspected chromatographic peak observed in the cell culture medium samples was not LXA_4 or at least, not only LXA_4.

3.2. *Identification of lipoxin A_4 using chiral separation*

The same sample extracts which were analyzed using reversed phase chromatography were injected into the chiral chromatographic system. The stability of the analytes was tested by comparing the calibration standards, injected into the reversed phase chromatographic column and the chiral chromatographic column. The extracts were stored at −80 °C for maximally three days between both measurements. No loss of LXA_4 was observed in this period of time.

The chromatograms corresponding to the cell culture medium sample in which LXA_4 was suspected and a standard sample are shown in Fig. 3. After the chiral separation, the absence of LXA_4 in the analyzed sample was verified.

This case is a clear example of the complexity of identifying endogenous compounds in biological samples. The authors recommend using very selective separations for the determination of such compounds, when concentrations in biological samples are very low, no blank matrix samples are available and when molecular fragmentation is not very specific. One option is chiral chromatography; however, it must be born in mind that this application has several disadvantages like broader chromatographic peaks, lower sensitivity and limited use of chromatographic conditions (mobile phase, temperature, etc.). The use of mass spectrometry is also recommended, combining information such as *m*/*z* transitions for quantitation and MS/MS spectra for identification purposes.

The data presented provide an example of the fact that in several cases, retention time and *m*/*z* transition ratios are insufficient to determine whether an endogenous compound is present in a complex sample. The use of these parameters and only reversed phase chromatography would lead, at least in some cases, to a false positive determination of LXA_4 in the samples. Comparison of the MS/MS spectra of samples and standards is a very useful tool for identification purposes. Moreover, the subsequent addition of the chiral column to the system allows the unequivocal identification of the presence of LXA_4 interferences in the samples. The identification of this interference or set of interferences is currently being studied.

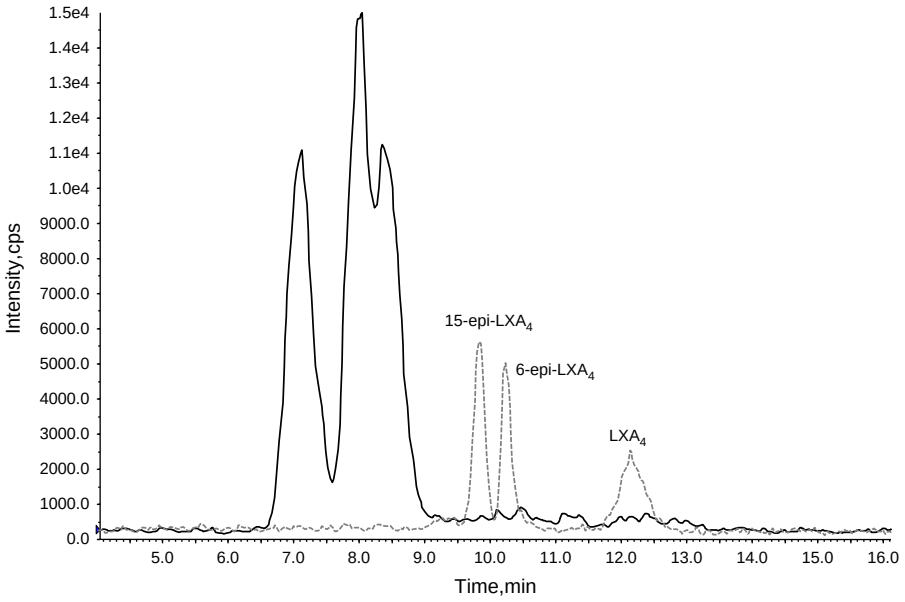

Fig. 3. Chromatograms (m/z 351.1 → 115.0) corresponding to an extracted cell culture medium sample (——) and to a 2.5 ng/mL calibration standard (·····) obtained using chiral separation. The absence of LXA_4 in the cell culture sample was demonstrated.

3.3. Quantitation of LXA_4 in biological samples

To assure the suitability of the developed method for the determination of LXA_4 in biological samples, the stability of the analyte under the cell culture conditions, storage time and analytical procedure was tested. For this purpose, four cell culture samples were prepared as indicated in the experimental section, but in these case, the samples were spiked with LXA_4 at a concentration of 0.5 ng/mL, instead of arachidonic acid. The spiked concentration of LXA_4 is much lower than the one described by Le Faouder et al. [16] in order to assure that the analytical method described in this manuscript has enough sensitivity for the determination of the analyte. After extraction and analysis, $91.5 \pm 8.2\%$ (mean ± standard deviation) of the spiked amount of LXA_4 could be recovered. This indicates that the analyte maintains stable during the sample preparation and analysis time and that the chiral separation carried out is adequate to determine LXA_4 even at very low concentrations.

Although the analyte of interest could be determined without problems in spiked samples, it could not be quantified in any of the biological samples.

To track the synthesis of LXA_4, the concentrations of 15-HETE (15-hydroxyeicosatetraenoic acid, the precursor of LXA_4) in the cell culture samples were monitored. This compound could be determined in all samples and it was observed, that its concentration was significantly increased in comparison with control samples (data not shown) but LXA_4 could not be determined in any of the biological samples.

However, when cell culture samples were directly spiked with 15-HETE and incubated under different conditions, the presence of LXA_4 could be determined (data not shown). This fact indicates that the lack of positive determination of LXA_4 in the cell culture samples in this manuscript is related to the synthesis of this molecule in the biological conditions assayed and not due to the capability of the analytical procedure to quantify the analyte.

4. Conclusion

A rapid and sensitive LC–MS/MS method for the quantitation of LXA_4 has been developed using reversed phase chromatography.

For identification purposes, three criteria were used: retention time, transitions ratio and MS/MS spectra of the analytes.

Possible interactions with co-eluting compounds were tested by adding a chiral chromatographic column to the analytical system. This chiral method allowed unequivocal confirmation that LXA_4 was not present in any of the analyzed samples.

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