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Development and validation of a semi-automated assay for the highly sensitive quantification of Biolimus A9 in human whole blood using high-performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

Drug-eluting stents are sustained-release intra-coronary devices that are usually coated with a few hundred micrograms of drug. Measuring the drugs that are released over weeks in order to assess human pharmacokinetics is a challenge that requires assays with high sensitivity. We developed and validated a semi-automated LC–MS/MS assay for the quantification of Biolimus A9, a proliferation signal inhibitor that was specifically developed for coating on drug-eluting stents in human EDTA blood. The only manual step was the addition of a zinc sulfate/methanol protein precipitation solution which included the internal standard. Samples were injected into the HPLC and extracted online. The assay had the following performance characteristics: range of reliable response 0.01–100 ng/mL ($r^2 > 0.99$), inter-day accuracy (0.033 ng/mL): 111.7%, and inter-day precision: 8.6%. There was no ion suppression, matrix interferences or carry-over. Extracted samples were stable in the autosampler at +4 °C for at least 24 h and could undergo three freeze–thaw cycles. The assay, with a lower limit of detection of 333 fg/mL and a lower limit of quantitation of 10 pg/mL, was sufficiently sensitive and robust for quantifying Biolimus A9 in clinical trials after IV injection and after stent implantation.

1. Introduction

Drug-eluting stents reduce in-stent restenosis after percutaneous coronary interventions as a treatment for *de novo* stenosis and reduce the need for repeat revascularization compared with bare-metal stents [1–3]. Although drug-eluting stents are still associated with clinical risks that will require further development such as late stent thrombosis, in many countries drug-eluting stents have replaced bare-metal stents as the clinical standard of care. Nevertheless, understanding the pharmacokinetics/pharmacodynamics of drugs eluted from stents is critical for developing new drug-eluting stent technologies, concepts and strategies with improved risk/benefit ratios.

Sirolimus and its derivate everolimus were developed primarily as immunosuppressants to be used in organ transplantation. Following the discovery of their antiproliferative effects on coronary smooth muscle cells, they were coated and tested on drug-eluting stents [4]. In contrast, Biolimus A9 is a novel sirolimus derivative that was specifically designed and developed for coating coronary artery stents [5,6]. Like sirolimus and everolimus, Biolimus A9 inhibits cell growth via formation of a complex with FK-binding proteins. The Biolimus A9/FK-binding protein complex then inhibits the mammalian target of rapamycin (mTOR).

The chemical structure of Biolimus A9 ($C_{55}H_{87}NO_{14}$, molecular weight 986.29 Da) consists of a 31-membered triene macrolide lactone that preserves the core sirolimus ring structure. The only modification is an ethoxyethyl group in position C(40) instead of the hydroxy group of the sirolimus molecule in the same position (Fig. 1). This addition increases lipophilicity and seems to facilitate Biolimus A9 uptake by the coronary vessel wall. This, the fact that the BioMatrix stent (Biosensors International, Newport Beach, CA) is coated with Biolimus A9 on the outside only, and that it is in direct contact with the vessel wall lead to relatively low systemic concentrations after stent implantation. Biolimus A9 has

Abbreviations: EDTA, ethylene diamino tetra acetylic acid; LLOQ, lower limit of quantitation; QC, quality control.

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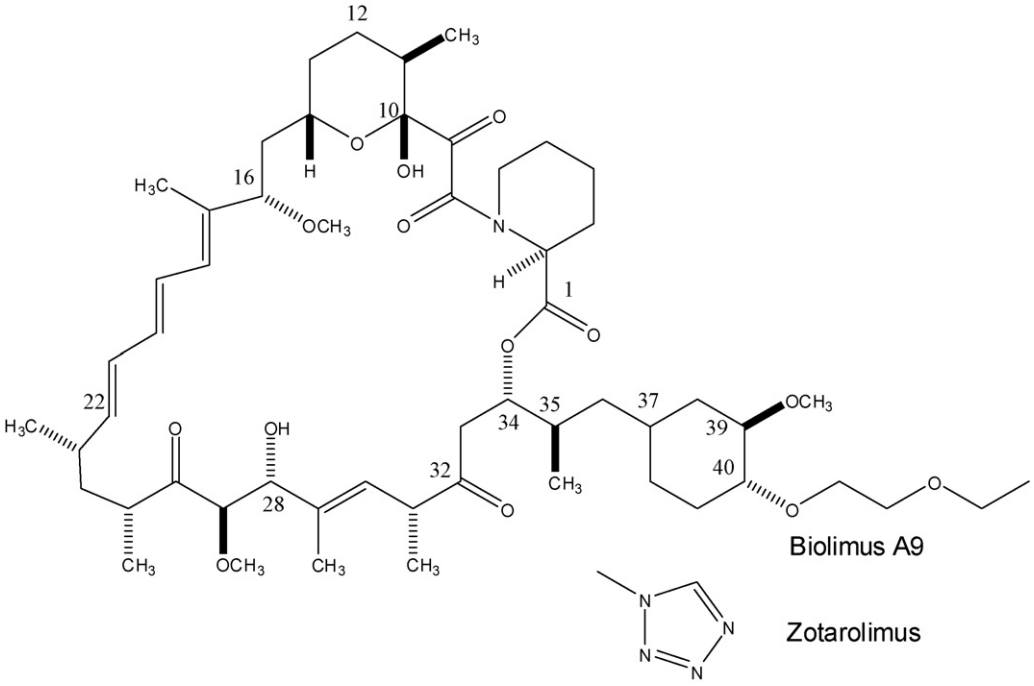

Fig. 1. Structures of Biolimus A9 and the internal standard zotarolimus. The numbering follows the IUPAC nomenclature.

been shown to be highly effective in reducing late lumen loss after coronary interventions in clinical trials [7–10]. Biolimus A9 coated stents are currently marketed in various countries in Europe and Asia.

A sufficiently sensitive assay for the quantification of Biolimus A9 is critical for the assessment of its pharmacokinetics and pharmacodynamics in clinical trials.

Here we report the development and validation of a specific and highly sensitive assay for the quantitation of Biolimus A9 in human EDTA blood using high-performance liquid chromatography–tandem mass spectrometry. This method has a lower limit of quantitation of 10 pg/mL that was sensitive enough to study the pharmacokinetics of Biolimus A9 in phase I clinical trials and after elution from drug-coated stents.

2. Materials and methods

2.1. Chemicals and reagents

Solvents and reagents (HPLC grade acetonitrile, methanol and water, formic acid 88%, zinc sulfate) used for sample preparation and as mobile phases were purchased from Fisher Scientific (Fair Lawn, NJ) and used without further purification. Biolimus A9 including valid certificate of analysis were supplied by Biosensors International, USA (Dr. R. Betts, Newport Beach, CA) and the internal standard zotarolimus was obtained from LC Laboratories (Woburn, MA). The structures of Biolimus A9 and the internal standard are shown in Fig. 1.

2.2. Calibrators and quality control samples

Each stock solution was based on three independent weightings of each compound. Stock solutions (1 mg/mL) of all compounds were prepared in acetonitrile and stored at −80 °C. Working solutions for quality control samples and standard curves were prepared by dilution of the stock solutions with EDTA blood. Blood samples used for assay development and validation purposes were obtained from healthy volunteers. Collection of human blood samples from outdated blood bank blood for assay validation and quality control was considered exempt by the local Internal Review Board (Colorado Multi-Institutional Review Board, Denver, CO).

The zotarolimus internal standard solution was prepared, resulting in a final concentration of 0.1 μg/mL. Due to instability of the internal standard, protein precipitation solution could only be used for 6 h and was discarded hereafter. Calibration standards and quality control samples were prepared by spiking blank human blood with Biolimus A9. In addition to blank and zero samples, the calibration curve had the following concentrations of Biolimus A9: 0.005 ng/mL, 0.01 ng/mL, 0.025 ng/mL, 0.05 ng/mL, 0.1 ng/mL, 0.25 ng/mL, 0.5 ng/mL, 1 ng/mL, 2.5 ng/mL, 5 ng/mL, 10 ng/mL, 25 ng/mL, 50 ng/mL, and 100 ng/mL. Quality control samples had the following concentrations: 0.033 ng/mL, 0.33 ng/mL, 3.33 ng/mL and 33.3 ng/mL.

2.3. Sample extraction

The extraction procedure consisted of two steps: a protein precipitation and subsequent online column extraction. The only manual step during blood sample extraction was protein precipitation. Eight hundred microliter protein precipitation solution (methanol/0.2 M $ZnSO_4$, 7:3, v/v) was added to 200 μL blood. After vortexing (5 min) and centrifugation (4 °C, 13000 × *g*, 10 min), the supernatant was transferred into a glass HPLC vial.

2.4. Equipment

The supernatants were analyzed using an LC–MS/MS system in combination with online extraction (LC/LC–MS/MS). The system consisted of the following components: two G1312A binary pumps, two G1322A vacuum degassers, a G1329A/G1330A thermostatted autosampler and a G1316A thermostatted column compartment (all Agilent 1100 series, Agilent Technologies, Santa Clara, CA) in combination with a CTC/PAL thermostatted autosampler (adjusted to +4 °C, Zwingen, Switzerland) and an integrated 6-port Rheodyne column-switching valve (Rheodyne, Cotati, CA). The connections of the switching valve are shown in Fig. 2. A Sciex API 5000 triple-stage

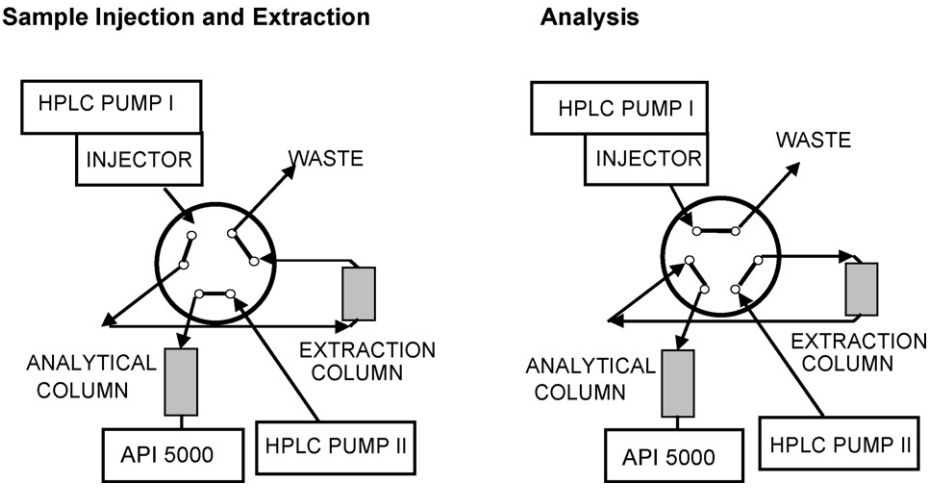

Fig. 2. Connections and positions of the column-switching valve.

quadrupole mass spectrometer was used as a detector (Applied Biosystems, Foster City, CA). The HPLC, the switching valve and the mass spectrometer were controlled by Analyst software (version 1.4.2).

2.5. LC/LC–MS/MS analysis

One hundred microliter of the samples were injected onto a 12.5 mm × 4.6 mm extraction column C8, 5 μm particle size (Agilent Technologies, Palo Alto, CA). Samples were washed with a mobile phase of 55% acetonitrile and 45% of 0.01% formic acid. The flow was 5 mL/min. After 1 min, the switching valve was activated and the analytes were eluted in the backflush mode from the extraction column onto a 150 mm × 4.6 mm C8, 5 μm analytical column (Zorbax XDB C8, Agilent Technologies, Santa Clara, CA). The mobile phase consisted of acetonitrile and 0.01% formic acid, run isocratically at 90% acetonitrile. The flow rate was adjusted to 1 mL/min and the column temperature of extraction and analytical columns were 65 °C. The HPLC system was interfaced with the mass spectrometer using a turbo electrospray source. The MS was run in the positive mode. The following parameters were used (nomenclature as used in the Analyst software). Compound dependent: collision-activated dissociation (CAD), 8.00 arbitrary units; declustering potential (DP), 80 V; entrance potential (EP), 9.00 V; collision energy (CE), 79.00 V; cell exit potential (CXP), 4.20 V. Source dependent: curtain gas (CUR), 25.00; ion source gas 1 (GS1), 25.00; ion source gas 2 (GS2), 25; temperature (TEM), 600.00 °C; ihe, ON; ion spray voltage (IS), 5.000 V.

For Biolimus A9, the following ion pair was detected: $m/z = 1008.9\ [M+Na]^+ \rightarrow 417.5$. Zotarolimus, the internal standard, was detected using the transition $m/z = 988.7\ [M+Na]^+ \rightarrow 369.5$.

After the analysis was completed, peaks were integrated and the results were printed. Biolimus A9 concentrations were corrected based on the internal standard and quantified using the calibration curves that were included in each batch. All calculations were carried out by the Applied Biosystems Analyst Software (version 1.4.2).

2.6. Validation procedures

The assay was validated using spiked human K2 EDTA blood samples from healthy volunteers following the guidelines for bioanalytical method validation as issued by the FDA Center for Drug Evaluation and Research (CDER) [11].

2.6.1. Predefined acceptance criteria

The performance of the assay was considered acceptable if precision at each concentration was ≤15% for intra-day (coefficient of variance, CV%) and inter-day variability (residual standard deviation in % as estimated using one-way analysis of variance and "day" as grouping variable). Intra- and inter-day accuracy had to be within ±15% of the nominal values. The only exception was the lower limit of quantitation (*vide infra*). The calibration curve had to have a correlation coefficient r^2 of 0.99 or better.

2.6.2. Lower limit of detection, lower limit of quantitation (LLOQ) and linearity

Linearity and the range of reliable response were assessed using the blank, zero and calibrator samples ($n = 6$). The lower limit of detection was the lowest Biolimus A9 concentration in blood that resulted in a peak-to-noise ratio of 4:1. The lower limit of quantitation (LLOQ) was the lowest Biolimus A9 concentration in EDTA that consistently resulted in accuracy ≤±20% of the nominal concentration and intra- and inter-day precisions ≤20%.

2.6.3. Precision and accuracy

Intra-day precision and accuracy were determined by analysis of the QC samples (0.033 ng/mL, 0.33 ng/mL, 3.33 ng/mL and 33.3 ng/mL) Biolimus A9 ($n = 6$/concentration). Determination of inter-day precision and accuracy was based on the QC samples. Samples were extracted and analyzed on three different days ($n = 6$/concentration and day).

2.6.4. Absolute recoveries

The recoveries were determined by comparing the signals for Biolimus A9 after extraction of blood samples enriched with 0.1 ng/mL, 1 ng/mL and 10 ng/mL ($n = 4$/concentration) with the signals of extracted blank matrix spiked with the respective concentrations of Biolimus A9 after the extraction procedure.

2.6.5. Matrix interferences, ion suppression and carry-over effect

To detect changes in ionization efficiency by co-eluting matrix substances, blank human EDTA blood samples from 10 different healthy individuals were used. Protein precipitation samples were extracted online and back-flushed onto the analytical column as described above. Biolimus A9 or zotarolimus (10 μg/mL dissolved in 0.1 formic acid/methanol, 1:1, v/v) were infused post-column *via* T-piece at 1 mL/min using a syringe pump (Harvard Apparatus, Holliston, MA). The extent of ion suppression was established by monitoring the intensity of the ion currents

in MRM-mode (Biolimus A9 $m/z = 1008.9 \rightarrow 417.5$, zotarolimus $m/z = 988.7 \rightarrow 369.5$) at the retention times of analyte and internal standard after injection of blank extracted blood samples into the LC/LC–MS/MS system [12].

Potential carry-over was assessed by alternately analyzing blood samples spiked with concentrations of Biolimus A9 at the upper limit of quantitation (100 ng/mL, $n = 3$ each) followed by blank methanol samples.

2.6.6. Stability studies

QC samples were freshly prepared and one set ($n = 6$/concentration level) was extracted and analyzed immediately (baseline). The remaining samples were used to establish stability in blood during three freeze–thaw cycles ($n = 6$/concentration level and cycle). Samples were kept frozen at −80 °C and thawed at room temperature. Benchtop and storage stability were also tested using QC samples. Samples were kept at either −80 °C, −20 °C, +4 °C or at room temperature. After 4 h, 8 h, 12 h, 24 h, 48 h and 168 h (for −20 °C and −80 °C also 1, 3 and 6 months) samples ($n = 3$ per temperature group and time point) were extracted, analyzed and compared with freshly prepared samples. Extracted sample/autosampler stability was tested by placing extracted QC samples ($n = 6$/concentration) into the thermostatted autosampler that was adjusted to +4 °C. Samples were injected immediately (baseline) and after 12 h, 24 h and 48 h. In all cases, samples were considered stable when the results were within ±15% of the baseline values.

2.6.7. Dilution integrity

Dilution integrity was established using freshly prepared EDTA blood samples spiked with 40 ng/mL Biolimus A9. Dilutions (1:1, 1:10, 1:100 and 1:400, $n = 3$) were made using fresh blank EDTA blood. Deviations from the nominal concentrations after dilution were calculated. Results that fell within the range of 85–115% of the nominal concentrations were considered acceptable.

3. Results

As a first step, MS and MS/MS spectra were recorded after direct infusion of Biolimus A9 and zotarolimus into the electrospray source *via* a syringe pump (Harvard Scientific). Both compounds were dissolved at a concentration of 10 μg/mL in methanol/0.1% formic acid 80/20, v/v, and were delivered at a rate of 1 mL/min. Fig. 3 shows the product ion scan spectra of Biolimus A9 and zotarolimus. The sodium adducts of Biolimus A9 ($[M+Na]^+$, $m/z = 1008.9$) and zotarolimus ($[M+Na]^+$, $m/z = 988.7$) were the predominant Q1 ions. All fragments detected were the product of α-cleavage and were detected as sodium adducts. The product ions at $m/z = 417.5$ (Biolimus A9) and $m/z = 369.5$ (zotarolimus) were most abundant and thus the transitions $m/z = 1008.9 \rightarrow 417.5$ and $m/z = 988.7 \rightarrow 369.5$ were selected for the quantification of Biolimus A9 and the internal standard.

No ion suppression was detected, as per the recommendations by Müller et al. [12], in any of the human EDTA blood samples tested from the 10 different individuals. Ion suppression was present at the time of the injection peak but not when Biolimus A9 and the internal standard eluted. Fig. 4 shows a representative experiment indicating lack of ion suppression for Biolimus A9.

The mean absolute recoveries of Biolimus A9 after protein precipitation of human blood were 73.3% (range 68.2–82.9%) for the 0.1 ng/mL samples, 76.0% (range 69.4–81.1%) for the 1 ng/mL samples and 80.0% (range 75.1–88.8%) for the 10 ng/mL samples (all $n = 4$/concentration).

A potential carry-over effect was assessed by analyzing extracted blank human EDTA blood samples after the highest calibrators (100 ng/mL). No carry-over was found.

Table 1
Intra- and inter-day precisions of the LC–MS/MS analysis of Biolimus A9 in human whole blood.

Concentration [ng/mL]	Precision R.S.D.%	Accuracy % of nominal
Intra-day		
0.03	11.2	111.2
0.33	2.1	102.7
3.33	5.3	100.1
33.3	4.5	100.6
Inter-day		
0.03	8.6	111.7
0.33	7.6	101.7
3.33	1.7	97.4
33.3	5.1	103.8

Intra-day precision and accuracy: $n = 6$/concentration; inter-day precision and accuracy: $n = 6$/concentration/day measured over 5 days. Inter-day precision is reported as relative standard deviation in % (R.S.D.%, as estimated by analysis of variance).

Several fitting algorithms and weighting methods were tested. Fitting calibration curves to a quadratic equation gave the best results in terms of accuracy and precision. In human EDTA blood, the lower limit of detection was 0.333 pg/mL and the lower limit of quantitation (LLOQ) was 3.3 pg/mL (Fig. 5). However, this lower limit of quantitation could only be achieved under ideal conditions such as the time immediately following thorough cleaning and tuning of the API5000. A LLOQ of 10 pg/mL could be achieved consistently even during analysis of larger batches of study samples. Thus, the LLOQ was set to 10 pg/mL (accuracy 114.2% and precision 14.5%). The range of reliable response was 0.01–100 ng/mL ($r^2 = 0.993$, $n = 6$). Assay accuracy and precision were determined by using four different concentrations of Biolimus A9 in human EDTA blood. At the tested concentration levels of 0.033 ng/mL, 0.33 ng/mL, 3.33 ng/mL and 33.3 ng/mL, intra-day accuracies in human EDTA blood were 111.2%, 102.7%, 100.1%, and 100.6%, respectively, and intra-day precisions were 11.2%, 2.1%, 5.3% and 4.5%. Inter-day accuracies were 111.7%, 101.7%, 97.4% and 103.8%, and inter-day precisions 8.6%, 7.6%, 7.1% and 5.1%, respectively (Table 1). Validity of sample dilution for the measurement of Biolimus A9 was also established. Accuracy was not affected by dilution of blood samples with blank matrix. The mean accuracies for dilutions of 1:1, 1:10, 1:100 and 1:400 in samples spiked with 40 ng/mL in blood were between 100.2% and 104.1% for the tested dilutions (all $n = 3$).

Biolimus A9 was stable in human EDTA blood over at least 3 freeze-thaw cycles. In human EDTA blood, Biolimus A9 stored on the bench at room temperature was stable for at least 12 h. Extracted samples were stable for only 8 h at room temperature and for at least 24 h at +4 °C. Biolimus A9 blood samples were stable if stored at +4 °C for 1 week, −20 °C for 3 months and −80 °C for at least 6 months. Our assay has proven to be reliable, sensitive, and accurate during the analysis of samples from multiple pharmacokinetics studies (e.g. Ref. [6]). An ion chromatogram representative of a clinical study sample is shown in Fig. 6.

4. Discussion

Drug-eluting stents have been shown to reduce coronary restenosis rates and the need for repeat revascularization after percutaneous coronary interventions (PCI) compared with bare-metal stents in patients with *de novo* stenosis [1–3]. In a recent cohort study, 7217 patients were included in a comparison between drug-eluting stents and bare-metal stents after acute myocardial infarction. After 2 years, patients treated with drug-eluting stents showed decreased mortality rates and reduction of repeat revas-

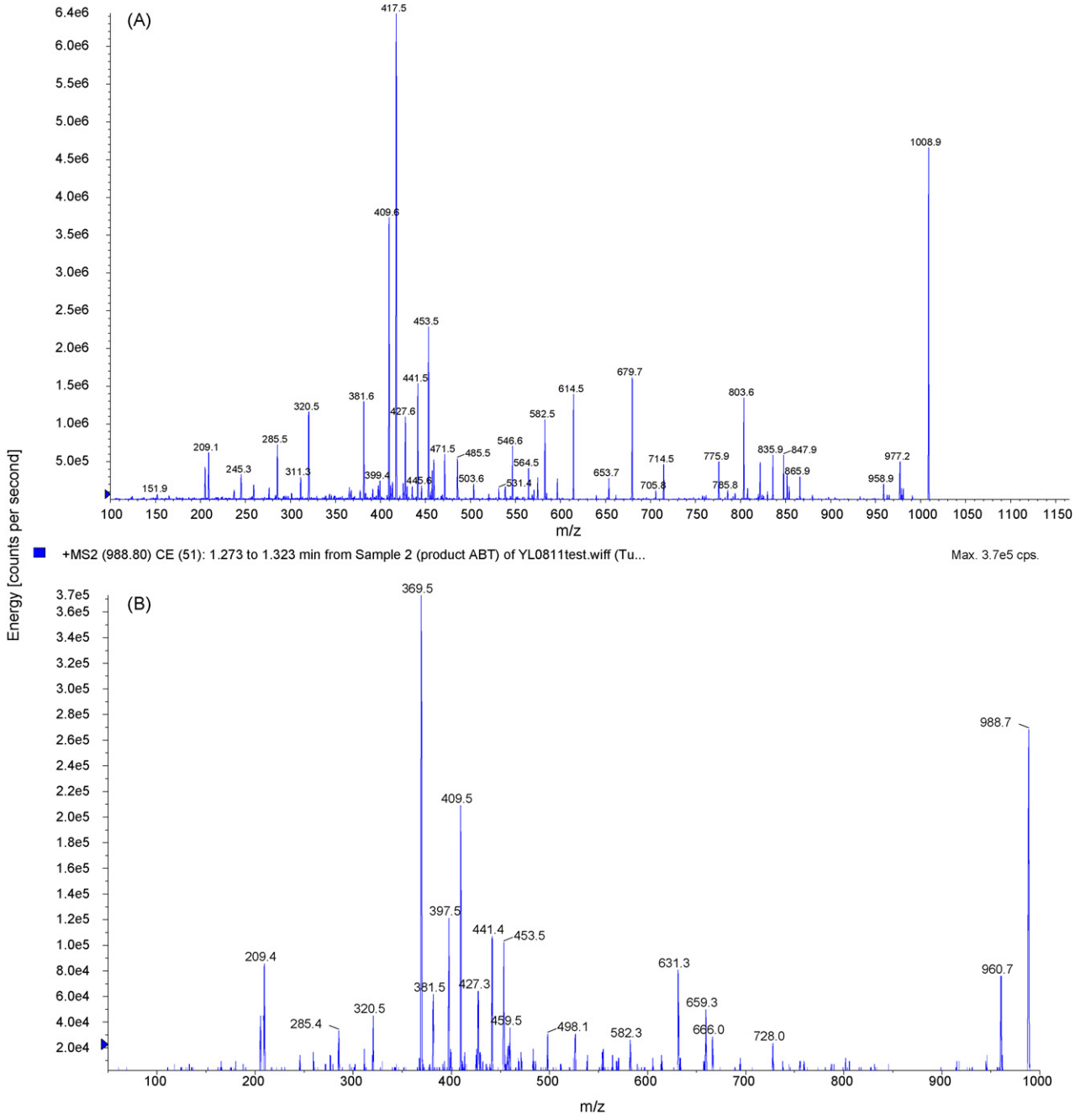

Fig. 3. MS/MS spectra of Biolimus A9 (A) and the internal standard zotarolimus (B). For Biolimus A9 the MS/MS spectrum was scanned from m/z = 100–1200, for zotarolimus from m/z = 100–1000. Please note that therefore the spectra in A and B do not line up.

cularization procedures as compared with patients treated with bare-metal stents [13].

Evaluation of the pharmacokinetics of drugs eluted from stents is an analytical challenge since typically only 100–400 μg of drug is coated on a stent, they are sustained-release devices, and the drug is released into the systemic circulation over weeks. Biolimus A9 coated stents are even more of a challenge since the drug distributes mostly into the target coronary tissues and concentrations in blood are very low [6]. While for other stents, such as the sirolimus eluting Cypher stent (Cordis, Warren, NJ), assays with LLOQs of 100 pg/mL are sufficient for the evaluation of pharmacokinetics after stent implantation [14], the Biolimus A9 pharmacokinetics require assays with much better sensitivity [6].

Here, we developed and validated a semi-automated LC/LC–MS/MS assay that allow for the detection of Biolimus A9 at concentrations as low as 333 fg/mL, and with a lower limit of quantitation of 10 pg/mL. This sensitivity is due to the online extraction step on the extraction column and the use of a Sciex API 5000 tandem quadrupole mass spectrometer. A major advantage of online column extraction was the facilitation of large injection volumes without negative effect on the analytical column and its chromatographic performance. After extraction, analytes were back-flushed from the extraction column. This volume-less injection strategy resulted in sharper peaks, providing a more reliable identification of the peak by the software and required less frequent manual integration of samples with concentrations close to the lower limit of quantitation. Reduction of the sample volume

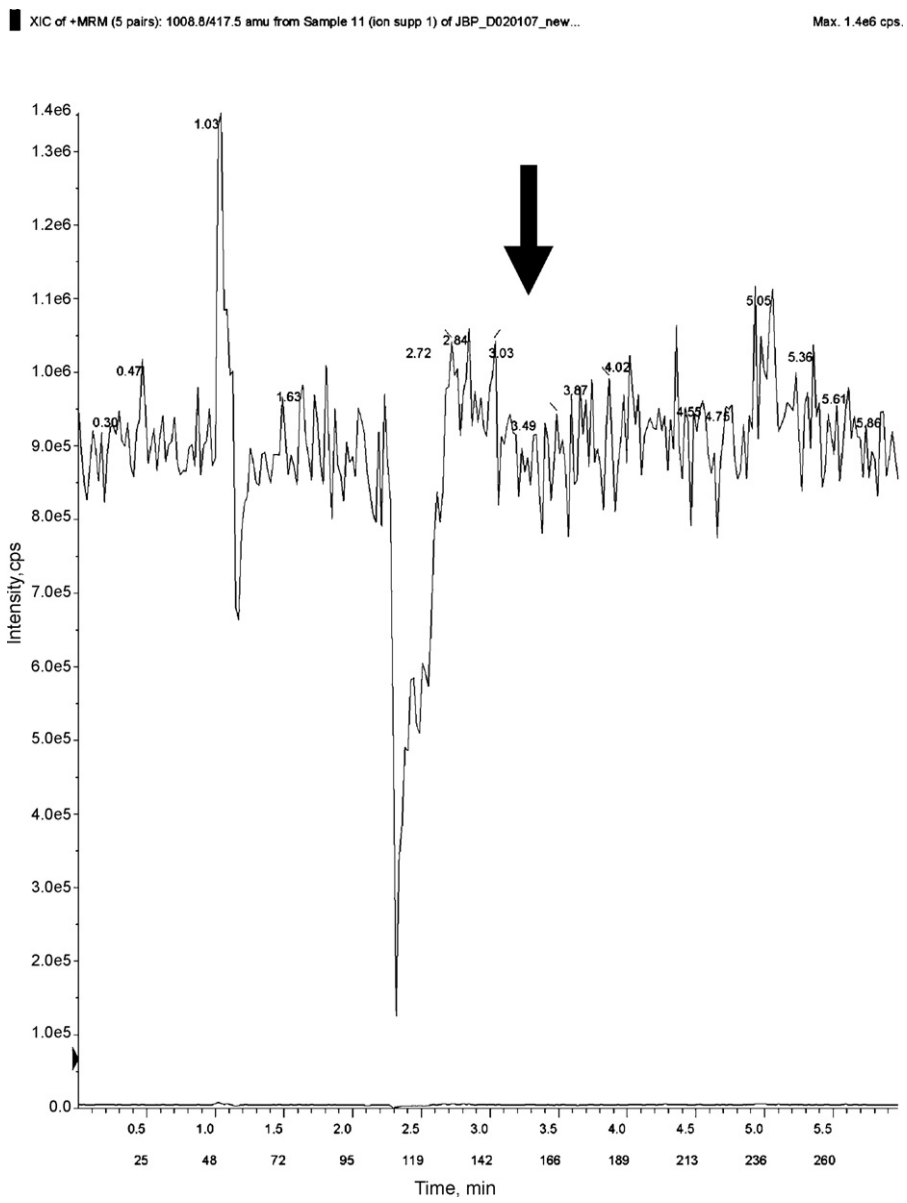

Fig. 4. Lack of ion suppression. Blank human EDTA blood samples from 10 different healthy volunteers were extracted and injected into the LC/LC–MS/MS system. A representative experiment is shown. Biolimus A9 (10 μg/mL dissolved in 0.1 formic acid/methanol, 1:1, v/v) was infused post-column *via* T-piece at 1 mL/min using a syringe pump (Harvard Apparatus, Holliston, MA). The extent of ion suppression was established by monitoring the intensity of the ion currents in MRM-mode ($m/z = 1008.9 \rightarrow 417.5$) at the retention times of the analyte (marked by arrow) after injection of blank extracted blood samples into the LC/LC–MS/MS system [12]. Ion suppression would have caused a "dip" in the Biolimus A9 signal produced by constant infusion.

before injection is a more common strategy as it allows for most of the extract to be injected without challenging the analytical column with a large injection volume. This was not an option since, like other sirolimus derivatives, Biolimus A9 has stability problems when dissolved in organic solvents and material is lost during evaporation partially because of the material sticking to glass and plastic walls [15,16]. Also, volume reduction would have added another manual step and it is reasonable to expect that this would have a negative impact on precision. In addition to being less labor-intense, the online extraction used in our assay also has better reproducibility, is less prone to random errors and allows for a complete automated documentation of the extraction of each individual sample using the Analyst software.

One of the major concerns during the extraction and analysis of sirolimus derivatives such as Biolimus A9 is degradation. The following measures helped decreasing the risk for degradation: (1) avoidance of an evaporation step during extraction, (2) a pH between 3 and 5 in Biolimus A9 solutions, (3) storage of extracted samples in the autosampler at +4 °C, and (4) avoidance of heat during extraction (e.g. all centrifugation steps were carried out at 4 °C). No detectable degradation could be attributed to the extraction processes.

Since it is known that sirolimus and derivatives mostly distribute into the cellular blood components (>90%), blood was used as matrix for analysis of Biolimus A9. For sirolimus and its derivatives, EDTA anti-coagulated blood is the generally accepted analytical matrix of choice [17,18].

Deuterated internal standards are usually considered ideal and it has been discussed that the choice of the correct internal standard for the analysis of sirolimus and derivatives has a significant impact on assay performance [19]. Unfortunately, deuterated Biolimus A9 is not available. We decided to use zotarolimus due to its struc-

tural similarity to Biolimus A9 with exactly the same macrolide ring structure, its molecular weight being only 20 Dalton different from Biolimus A9 and its easy commercial availability. Sirolimus and everolimus would have been potential alternatives. However, they are possible metabolites and degradation products of Biolimus A9 (personal communication, Biosensors International, USA, data on file), ruling them out as a viable option.

The problem with long-term stability of samples stored at −20 °C was not degradation of Biolimus A9, but rather degradation of the matrix. The critical step during extraction was methanol/zinc

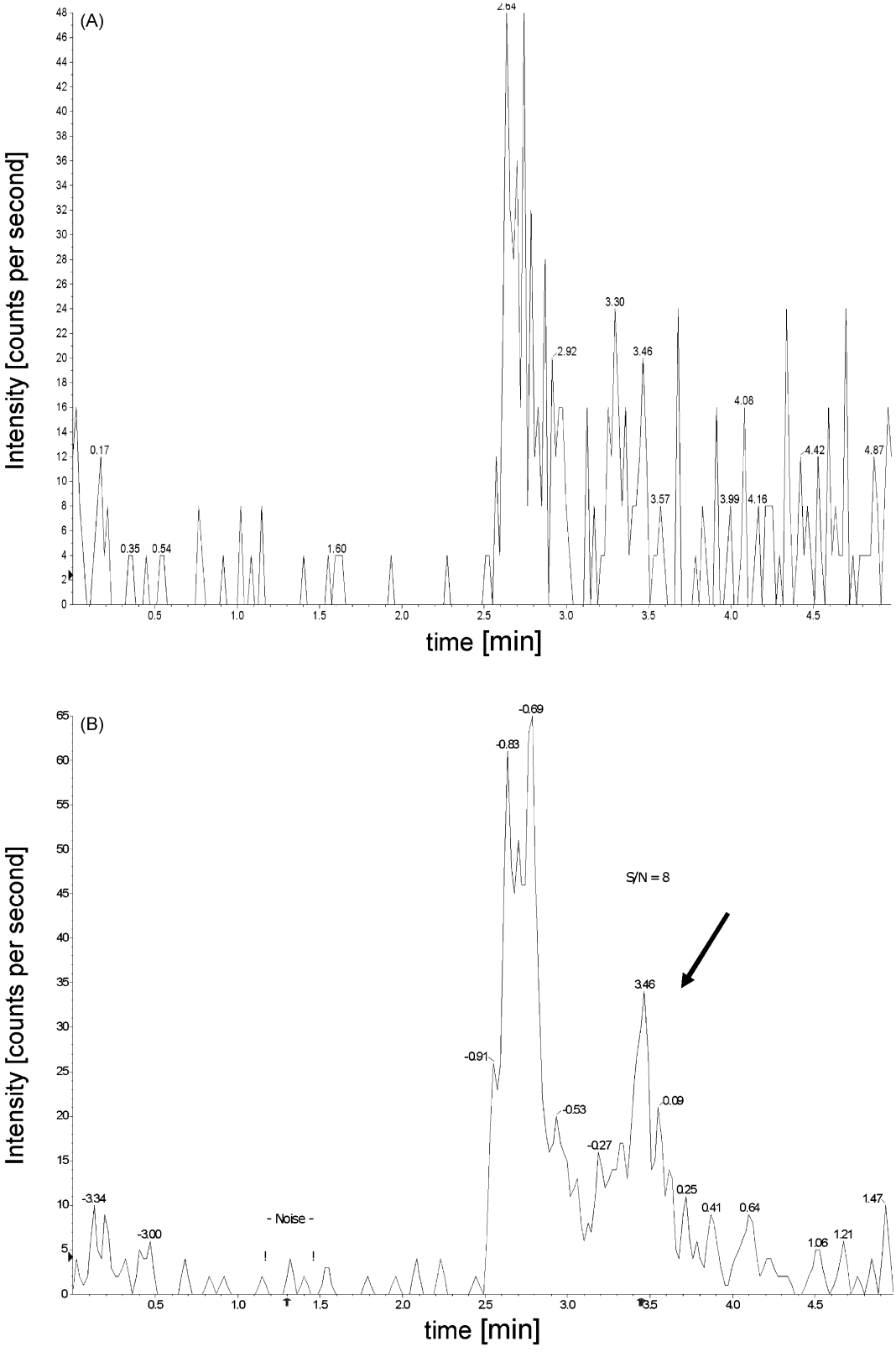

Fig. 5. Representative ion chromatograms of a blank blood sample (A) and blank blood samples enriched with Biolimus A9 at the lower limit of detection (0.333 pg/mL) (B) and Biolimus A9 enriched with Biolimus A9 at the lower limit of quantitation (3.3 pg/mL) (C).

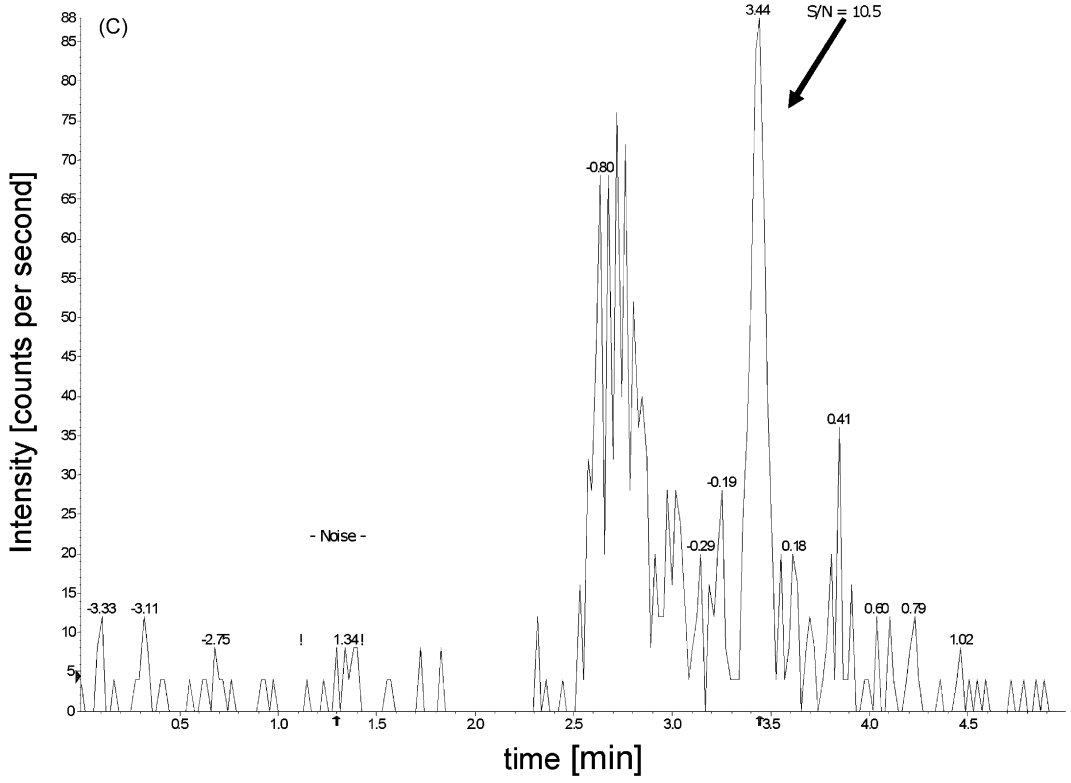

Fig. 5. (*Continued*).

sulfate protein precipitation. When older samples were tested that had not been stored at −80 °C, extraction recovery of Biolimus A9 was low (10% and below). Of even greater concern was that the extraction recovery of the internal standard (between 50% and 70%) in several of the samples was different from that of Biolimus A9 which led to falsely low results. A possible explanation is that the methanol/zinc sulfate solution failed to release Biolimus A9 from its binding proteins in the older samples due to cross-linking of blood protein during coagulation or related processes. The methanol/zinc sulfate extraction step was critical to ensure relatively clean supernatants. This was probably the major reason why so far no sample has been lost due to a plugged online extraction column after injection of the relatively high volume of extracts.

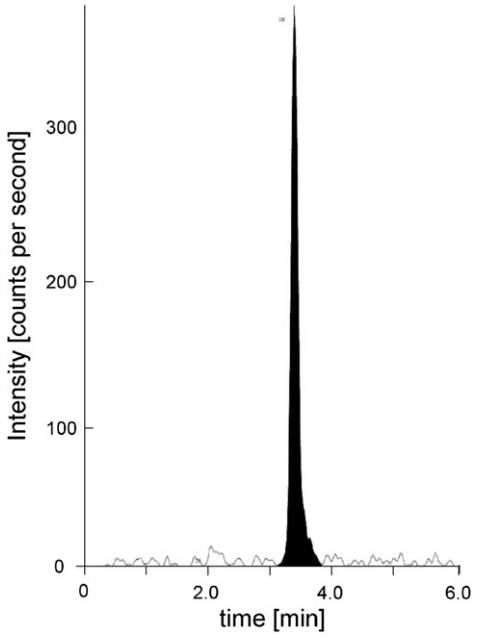

Fig. 6. Representative ion chromatogram of a blood sample from clinical trials. The study was approved by an internal review board and all patients had given their informed written consent. Study conduct followed the principles of good clinical practice and all applicable international guidances. Ion chromatogram is from a patient 45 min after implantation of a Biolimus A9 eluting stent. The Biolimus A9 concentration was 0.12 ng/mL.

In our study, sodium adducts gave the best signals. It was interesting to note that even all fragments were sodium adducts. This has been observed for other sirolimus derivatives, such as everolimus, as well [20,21]. Addition of sodium to the mobile phase (1 μmol/L sodium formiate) did not affect assay performance indicating that the sodium present in the LC/LC–MS/MS system was already sufficient to cause maximum ion formation.

As of today, our assay has been used to measure thousands of Biolimus samples. The online extraction column was exchanged every 300 and the analytical column every 1000 injections. However, this was just a preventive measure. As already mentioned above, no sample has been lost because of analytical column failure and it is likely that the columns may have lasted longer. This assay may provide a platform strategy that can be used for the highly sensitive quantification of other sirolimus derivatives coated on drug-eluting stents.

Acknowledgments

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