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Journal of Chromatography B, 855 (2007) 173–179
JOURNAL OF CHROMATOGRAPHY B
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Liquid chromatography–tandem mass spectrometric determination of a new antibacterial agent (AVE6971) in human white blood cells

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Received 30 November 2006; accepted 26 April 2007
Available online 10 May 2007

Abstract

An LC–MS/MS assay for the quantitative determination of a new antibacterial agent (AVE6971) has been developed and validated in human white blood cells (WBC). The assay involved a lysing procedure of white blood cells and ultra centrifugation of the extracts. Chromatography was performed on a Supelcosil ABZ+ C_{18} (2.1 mm × 50 mm, 5 μm) column using a mobile phase consisting of methanol/acetonitrile/10 mM ammonium formate mixture (10:30:60, v/v/v) at a flow rate of 0.2 ml/min. The linearity was within the range of 10–10000 ng/ml of extracts, corresponding to 0.5–500 ng of AVE6971 in WBC pellets tubes. The validated lower limit of quantification was 10 ng/ml. The inter- and intra-run coefficients of variation (CV) for the assay were <12.9% and the accuracy were from −9.0 to −1.2%. AVE6971 was stable in WBC for at least 1 month at −75 °C. This assay proved to be suitable for the determination of AVE6971 in WBC from clinical studies.

Keywords: Antibacterial agents; Leukocytes; Mass spectrometry; Liquid chromatography

1. Introduction

Antibiotics are used for treatment or prevention of bacterial infection. The introduction and increasing use of antibacterial agents such as β-lactams, macrolides, vancomycin or quinolones for antibacterial therapy has resulted in the emergence of multi-resistant pathogens, especially in Gram-positive bacteria [1,2]. Gram-positive infections have become a major problem, particularly in the hospital setting, creating a need for new antibacterial drugs effective against bacteria resistant to multiple antibiotics [3]. The choice of a new antibiotic should be based on the likely causative microorganism and the drug's pharmacokinetics, pharmacodynamics, and pharmaceutical properties. Biological effectiveness is also an important parameter in determining optimal dosages of molecular compound. As an example, the interactions of antibiotics with phagocytes, and the influence of these interactions on the fate of intra phagocyticbacteria, may be of therapeutic importance. Obviously, penetration of antibiotics into phagocytic cells is essential for activity against intracellular organisms. The use of therapeutic agents, which are able to penetrate phagocytic cells and demonstrate intracellular activity, is especially desirable in therapy of infections caused by facultative intracellular organisms. Pharmacokinetics of intracellular drugs have interest in the field of antibacterial therapy [4–6]. So, it is important to evaluate antimicrobial agents penetration. The ability to detect and quantitate the antibacterial agents in human WBC cells will lead to a better understanding of the pharmacokinetic and pharmacodynamic characteristics of these agents.

A novel class of antibacterial agents, the quinolyl-propyl-3-piperidinecarboxylic acid series [7] was intented to be tested in the treatment of nosocomial Gram-positive infections and also moderate to severe community acquired pneumoni, including those at hospital. To determine concentration–effect relationships, robust and quantitative biological assays are prerequisite.

Consequently, we developed and validated a simple and selective assay for the determination of a quinolyl-propyl-3-piperidinecarboxylic acid derivative (AVE6971) in human

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doi:10.1016/j.jchromb.2007.04.034

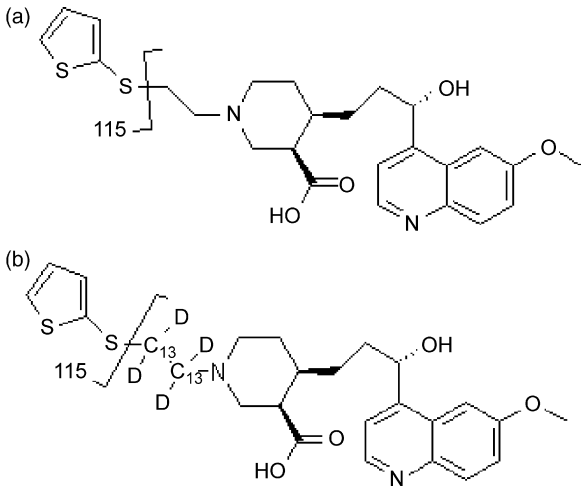

Fig. 1. Structures of AVE6971 (a) and the internal standard (AVE6971[$^{13}C_2$-D_4]) (b).

WBC using high performance liquid chromatography tandem mass spectrometry with ionspray ionisation. In anticipation of clinical, method was designed with specific attentions to the handling, preparation and analysis of WBC samples. The analytical method was developed to allow a fast analysis for numerous WBC samples from clinical studies.

2. Experimental

2.1. Chemicals

AVE6971 (purity = 99.1%, Mw = 486.2 g/mol) and the internal standard (purity = 99%; Mw = 492.1 g/mol) were obtained from Aventis Pharma (Vitry sur Seine, France). The structures of AVE6971 and the labelled internal standard (I.S.) (AVE6971 [$^{13}C_2$-D_4]) used in the assay are shown in Fig. 1. HPLC-grade methanol and acetonitrile were purchased from Carlo Erba (Val de Rueil, France) and J.T. Baker Co. (Phillipsburg, NJ, USA), respectively. Acetic acid and ammonium formate were purchased from Merck (Darmstat, Germany). De-ionized water was purified in a Millipore system Milli Q (Molsheim, France). Dextran sulfate sodium salt, Phosphate Saline Buffer (PBS) and NaCl were purchased from Sigma (St. Louis, MO, USA).

2.2. Chromatography

The HPLC system consisted of a Perkin-Elmer (Norwalk, CT, USA) 200 series pump and a Perkin-Elmer 200 series auto-sampler. Chromatographic separations were performed on a 5 μm Supelcosil LC-ABZ + column, 50 mm × 2.1 mm I.D. (Supelco, Saint-Quentin-Fallavier, France), operated at ambient temperature at a flow rate of 0.2 ml/min. An isocratic elution mode was used. The mobile phase was a mixture of methanol/acetonitrile/ammonium formate buffer 10 mM (10:30:60, v/v/v). The sample injection volume was 10 μl and the run time of the assay was 3 min.

2.3. Mass spectrometry

A Sciex API 365 triple quadrupole mass spectrometer (MDS Sciex, Toronto, Canada) was coupled to the HPLC system through a Sciex TurboIonspray Source (TIS) operated in positive mode. Instrument control, data acquisition and processing were performed using Analyst 1.1 software. The mass spectrometer was initially calibrated using polypropylene glycol as standard (Applied Biosystems, Foster City, CA, USA), setting the resolution, as peak width at half height, in the range 0.7 ± 0.1 a.m.u.

The nebulizer and the curtain gas flows (nitrogen) were set at 10 units (discrete value). The TIS was operated at 450 °C with the auxiliary gas flow (nitrogen) set at 8 l/min. The TIS voltage was set at 4900 V and the orifice voltage and ring voltages at 36 and 220 V, respectively. Selected reaction monitoring (SRM) experiments in the positive ionization mode were performed to detect ion transitions at m/z 487.2/115.1 $[M+H]^+$ for (AVE6971) and 493.1/115.1 for I.S. with a dwell time of 500 ms per transition. Product ions used for monitoring were selected based on their significance in the MS/MS spectra. The collision energy was optimized for each analyte, using the auto tune feature of the software (45 eV).

2.4. Preparation of WBC pellets

There are different methods available for preparation of WBC from whole blood, e.g., preparation of leucocytes by dextran sedimentation or preparation of leucocytes by differential lysis of erythrocytes. Either method is acceptable and merely depends on the availability of expertise, resources and equipment. According to our experience, WBC preparation by differential sedimentation in dextran/saline solution is easier to perform and was retained. WBC preparation is derived from the Boyum method [8]. Twenty millilitre of blood for WBC preparation were collected in tubes containing lithium heparinate (10–30 IU/ml blood. A mixture solution of 2 ml of 9% Dextran and 0.9% NaCl was added to the whole blood to allow sedimentation of the red blood cells. The tubes were placed vertically for 40–120 min at 37 °C. After sedimentation the upper layer of plasma containing the WBC was transferred into preweighed tubes. Aliquots of 1 ml were taken for cell counting by flow cytometry including WBC and residual red blood cells. The tubes containing remaining upper layer were centrifuged at 1000 × g and at 4 °C for 10 min. After decantation and discarding the supernatant plasma, the WBC pellets were washed with 2 ml of phosphate buffer saline (PBS) and then frozen at −75 °C. The pellets were white or at most just slightly pink in colour. In this case, red blood cells interference in WBC isolation was small enough to be neglected and no differences in AVE6971 concentration were observed between quality control samples prepared from white or just slightly pink WBC pellets.

2.5. Preparation of stock solutions

Two stock solutions of analyte were prepared independently in methanol at a concentration of 1 mg/ml. One solution was

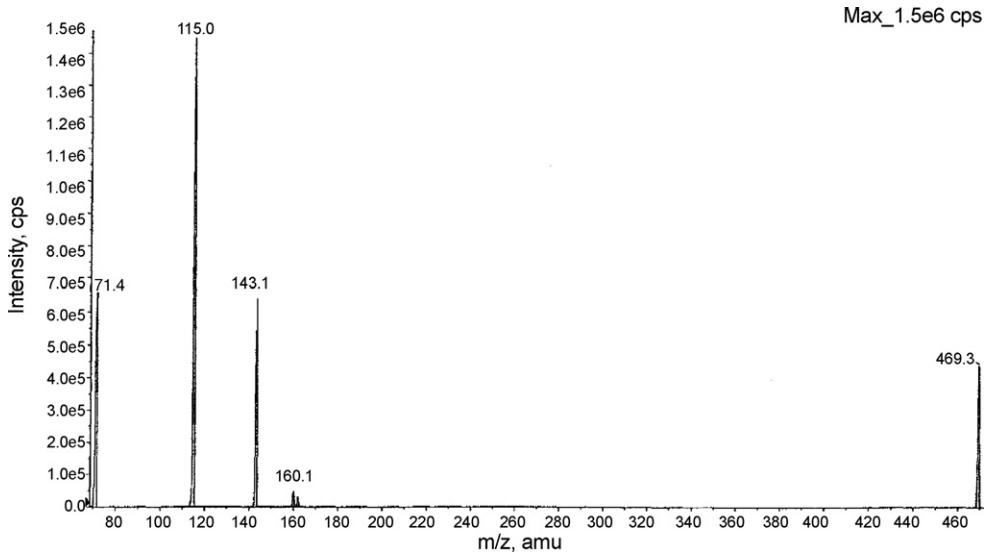

Fig. 2. Product ions scan of AVE6971.

used to prepare the calibration standard samples; the quality control (QC) samples were made from the other solution. A stock solution of internal standard was also prepared in methanol at a concentration of 0.1 mg/ml. Stocks solutions were kept at 5 °C protected from light. The stock solutions were stable for at least 4 months.

2.6. Preparation of calibration standards and quality control samples

The white blood cells matrix is not easy to obtain as compared to blank plasma. Consequently calibration standards were prepared in methanol without WBC and only a minimum of QC samples contain the WBC to mimic real study samples.

Calibration standards were prepared from aqueous working solutions of AVE6971 at concentrations of 10 000, 8000, 3000, 1000, 250, 50, 20 and 10 ng/ml. These were prepared by serial dilutions of the methanolic stock solution. Similarly, QC samples were prepared from separate working solutions at 7500, 500, 30 and 10 ng/ml. The internal standard stock solution was diluted in methanol to a working concentration of 625 ng/ml. These working solutions were stored at +4 °C until use. A standard curve was prepared each day by adding 50 μl of each standard working solution to 200 μl of internal working solution to make eight levels of calibration: 10, 20, 50, 250, 1000, 3000, 8000 and 10 ѕ000 ng/ml corresponding to 0.5, 1.0, 2.5, 12.5, 50.0, 150, 400 and 500 ng of AVE6971 in each tube.

QCs samples were prepared individually as follows: 50 μl of QC samples working solutions were added to each prepared WBC pellets sample and then evaporated to dryness under a gentle stream of nitrogen in order to obtain final amounts of 0.5, 1.5, 25 and 375 ng of AVE6971 per tube containing around 3–7 × 10^6 WBC and corresponding to 10, 30, 500 and 7500 ng/ml. QCs were stored with study samples at −75 °C until used.

2.7. Cell lysis and sample extraction

In the present assay, 50 μl of methanol and 200 μl of internal standard working solution in methanol were added to the prepared clinical samples and QCs. Samples were vortexed for a few seconds and cells were lysed by sonication for 15 min. Then, 200 μl of an acetic acid solution of 0.02% was added (for chromatographic reason) and the cell suspension solution was centrifuged (14000 × g for 15 min at +5 °C). The supernatants were immediately transferred into autosampler injection vials.

2.8. Validation procedure

Given the difficulty of supply of blank WBC matrix, the present LC–MS/MS assay was partially validated for the linearity, limit of quantification, recovery, dilution, stability of extracts, selectivity, long term stability storage as well as intra- and inter-day accuracy and precision. The accuracy and precision of the assay were assessed by replicate analysis ($n = 6$) of the QC samples. The concentrations were determined using the calibration equations resulting from calibration standards analysis.

3. Results and discussion

3.1. Method development

AVE6971 was determined using SRM detection. To find appropriate SRM conditions, full-scan mass spectra and product ion spectra for AVE6971 and internal standard were carried out. Positive-ion electrospray MS/MS product-ion spectra (Q3) of AVE6971 is shown in Fig. 2. For each compound, the $[M + H]^+$ was the predominant ion in Q1 spectrum (data not shown), which was used as the precursor ion for obtaining MS/MS product-ion spectrum. The proposed fragments for the major product ions are shown in Fig. 1. The settings on the mass spectrometer were

optimized for the production of the ions that are monitored. According to the best specificity and signal intensity (S/N), the ideal precursor/product ions pairs chosen for the SRM detection were determined to be *m/z* 487.2/115.1 for AVE6971 and *m/z* 493.1/115.1 for internal standard. The chromatographic conditions were designed to achieve maximum response (peak area), minimum baseline noise, along with the shortest possible run time. During method development, various analytical columns of different lengths and bonded phases and mobile phases compositions were evaluated. The short analytical column (50 mm × 2.1 mm I.D.) Supelcosil LC-ABZ+ and an isocratic mobile phase composed of methanol/acetonitrile/10 mM ammonium formate (10:30:60, v/v/v) provided fast elution time far from void volume (retention factor of 1.8) with good peak shape.

Extraction of the analyte from the intracellular medium should ensure complete release of the analyte, keeps the analyte stable and should be done in a medium that does not interfere with the assay. Several lysis techniques have been employed including freeze–drying, freezing/thawing cycles, and sonication procedure [9]. Whatever the procedure used, the effect on the drug response must be studied. Furthermore, the cell medium contains small amount of proteins. It is possible to inject the cell extract directly into the chromatographic system without further purification. If necessary, an extraction can also be performed to improve sample cleanup. In the developed LC–MS/MS assay cell lysis is performed by sonification using methanol and cell debris are removed by centrifugation. To ensure precision, LC–MS/MS assays require the use of an internal standard, which can be added directly to the cell lysis solvent or to the cell extract just before sample treatment. The choice is related to long-term sample storage, either in the frozen cells or in cell extract. The lysing solution is expected to completely solve AVE6971 contained within the cells. We chose 100% methanol because AVE6971 has been shown to be very soluble and stable in methanol. Acetonitrile gave similar results but was not retained, as internal standard stock solution was prepared in methanol. Methanol is also known to denature proteins and while doing so it releases AVE6971 from the cells. The use of 60% methanol in water as lysing solution has been reported in the literature [10,11] but to the best of our knowledge, there was no discussion as to why this particular medium was chosen.

Table 1
Parameters of calibration curves

	Slope (*a*)	Intercept (*b*)	Correlation coefficient (*r*)
Day 1	0.00040453	0.00043407	0.998499
Day 2	0.00038471	0.00062589	0.995052
Day 3	0.00039613	0.00023502	0.996480
Day 4	0.00038421	−0.000016849	0.998778
Mean	0.00039240	0.00031953	0.997202
SD	0.0000097854	N/C	N/C
Precision[a]	2.49	N/C	N/C

$y = ax + b$, where y is the peak area ratio (AVE6971/internal standard) and x the nominal concentration; weighting: $1/x^2$.
N/C: not calculated.
[a] Expressed as %RSD: (SD/mean) × 100.

3.2. Linearity

The linearity of the method was evaluated during validation on 4 separate days. The peak area ratios of AVE6971 to the internal standard were used as the assay variable. They were plotted against nominal concentrations. To determine the best weighting factor, concentrations were back calculated and the residual plot examined. The model with the lowest bias and the most constant variance across the concentration range was considered the best suited. Calibration curves were obtained from weighted ($1/x^2$) least-squares linear regression analysis of the data ($y = ax + b$), where y is the peak area ratio and x the nominal concentrations. The calibration curve was linear within the range of 10–10 000 ng/ml corresponding to 0.5–500 ng of AVE6971 in tubes. The correlation coefficients ranged from 0.995052 to 0.998778. The calibration parameters are shown in Table 1. The accuracy of back-calculated concentrations ranged from −1.90 to 5.50% (data not shown).

3.3. Precision and accuracy

Intra-day accuracy (mean determined conc./nominal conc. × 100) and precision (SD/mean conc. × 100) ($n = 6$ for each level) were evaluated by analysis of QC samples on the same day. Inter-day accuracy and precision were determined by repeated analysis over 3 consecutive days (each QC in duplicate). The concentration of each sample was determined

Table 2
Intra- and inter-day precision and accuracy

Nominal concentration (ng/ml)	Intra-day variation			Inter-day variation		
	Determined concentration (Mean ± SD)[a]	Precision (%)[b]	Accuracy (%)[c]	Determined concentration (Mean ± SD)[d]	Precision (%)[b]	Accuracy (%)[b]
10	9.99 ± 0.928	9.29	−0.10	10.9 ± 0.473	4.34	9.00
30	30.7 ± 3.77	12.28	2.33	31.6 ± 4.07	12.88	5.33
50	5061 ± 25.1	4.96	1.20	503 ± 29.3	5.83	0.60
7500	7700 ± 368	4.78	2.67	7410 ± 619	8.35	−1.20

[a] $n = 6$.
[b] Expressed as RSD: (SD/mean) × 100.
[c] Expressed as %difference:[(concentration found − concentration added)/concentration added] × 100.
[d] $n = 3$ days with two replicates per day.

using calibration standards freshly prepared in methanol. The intra- and inter-day precision and accuracy are shown in Table 2. The intra-day accuracy ranged from −0.10 to 2.67% while precision ranged from 4.78 to 12.28%. The inter-day accuracy ranged from −1.20 to 9.00% and precision from 4.34 to 12.88%. These results satisfied validation criteria, since the accuracy and precision were within 15%, except for the lower limit of quantification (LLOQ), at which the accuracy and precision were within 20% acceptance limits.

3.4. Lower limit of quantification

According to a signal to noise ratio of ten, the lower limit of quantification (LLOQ) was set at 10 ng/ml in human WBC (corresponding to 0.5 ng of AVE6971) and at this level the intra- and inter-batch precisions were 9.29% and 4.34%, respectively, while intra- and inter-batch accuracies were −0.1% and 9.0%, respectively.

3.5. Selectivity and matrix effect

The sample extraction and chromatographic analysis were developed to produce a selective assay for the analyte. The specificity of the method was determined by analyzing human WBC samples prepared and extracted without addition of AVE6971 or internal standard. Six different batches of WBC were analyzed and checked for interference in the assay. Inspection of SRM chromatograms showed no interference from WBC at the retention time of AVE6971 and in the ion channel of either AVE6971 or I.S. Fig. 3 shows typical SRM chromatograms. The accurate quantitation of AVE6971 QC extracted samples against calibration standard prepared in methanol solution indicates that matrix effect from WBC, if present, does not adversely affect accuracy and precision of the method. The use of stable isotope labelled AVE6971 as an I.S. is though to have contributed to the satisfactory analytical performance.

3.6. Recovery

The extraction recovery of AVE6971 was determined at two concentrations: the low (30° ng/ml) and high (7 500 ng/ml) validation QC levels. The extraction recovery of the internal standard was determined at its working concentration. Extraction recoveries of AVE6971 and its labelled IS were determined to be greater than 94% for tested concentrations with a precision, expressed as %RSD, lower than 7%.

3.7. Dilution for re-assay

Re-analysis of WBC is not as simple as for plasma or urine. With plasma or urine, only an aliquot is removed from the tube and the remaining plasma or urine can be frozen again. In the case of WBC samples, the thawed cells cannot be re-frozen, and only the extract can be re-assayed because all WBC pellets were lysed with methanol. There is only one possibility in the case of re-assay in the extract as the procedure includes the addition of internal standard during cell lysis; to freeze extracts samples and to re-assay if needed. The IS stability in stored cell extract has been checked. In case of re-assay due to high concentration of samples, an aliquot of the extract should be diluted into methanol containing an appropriate amount of internal standard. In our case, a 1/10 dilution was validated. QCs were prepared ($n = 6$) at a concentration above the upper limit of quantification (3000 ng/ml). Forty-five microlitre of extracted sample were mixed with 100 μl of internal standard solution at 1125 ng/ml and 305 μl of methanol/acetic acid solution 0.02% (55/45, v/v). The precision and accuracy of the method were within pre-set acceptance limits, at 5.86% and −6.67%, respectively.

3.8. Long-term storage at −75 °C

The stability of AVE6971 in WBC tube was evaluated at −75 °C over a 3 months period using WBC spiked with 30 ng/ml and 7 500 ng/ml AVE6971 solutions. After 1 and 3 months, the samples were analyzed against a freshly prepared standard curve. As per FDA guidelines, the compound is deemed "stable" if the concentration of the compound through the stability experiment does not deviate by more than 15% from the control (time 0) concentration. The results in Table 3 indicate that AVE6971 in human WBC is stable only for at least 1 month frozen at −75 °C.

3.9. Autosampler stability

Autosampler stability was investigated as part of regular validation run. Freshly spiked standards and QCs were extracted and injected for precision and accuracy. The QCs were left in the autosampler at room temperature for 66 h and then injected again with a new validation run. These were analyzed against the original curve. The results in Table 3 indicate that AVE6971 is stable for at least 66 h at room temperature.

Table 3
Stability under a variety of conditions

Statistical variable	Theoretical concentration (ng ml^{-1})	
	30	7500
Extract stability (66 h at room temperature)		
Mean ± SD[a]	29.0 ± 3.76	7150 ± 346
Precision (%)[b]	12.97	4.84
Accuracy (%)[c]	−3.33	−4.67
Long-term stability (−75 °C for 1 month)		
Mean ± SD[a]	30.6 ± 2.41	7790 ± 338
Precision (%)[b]	7.88	4.34
Accuracy (%)[c]	2.00	3.87
% Diff. *vs.* time 0	−3.16	1.30
Long-term stability (−75°C for 3 month)		
Mean ± SD[a]	25.7 ± 1.72	6580 ± 265
Precision (%)[b]	6.69	4.03
Accuracy (%)[c]	−14.33	−12.27
% Diff. *vs.* time 0	1.30	−14.4

[a] $n = 6$.
[b] Expressed as RSD: (SD/mean) × 100.
[c] Expressed as %difference:[(concentration found − concentration added)/concentration added] × 100.

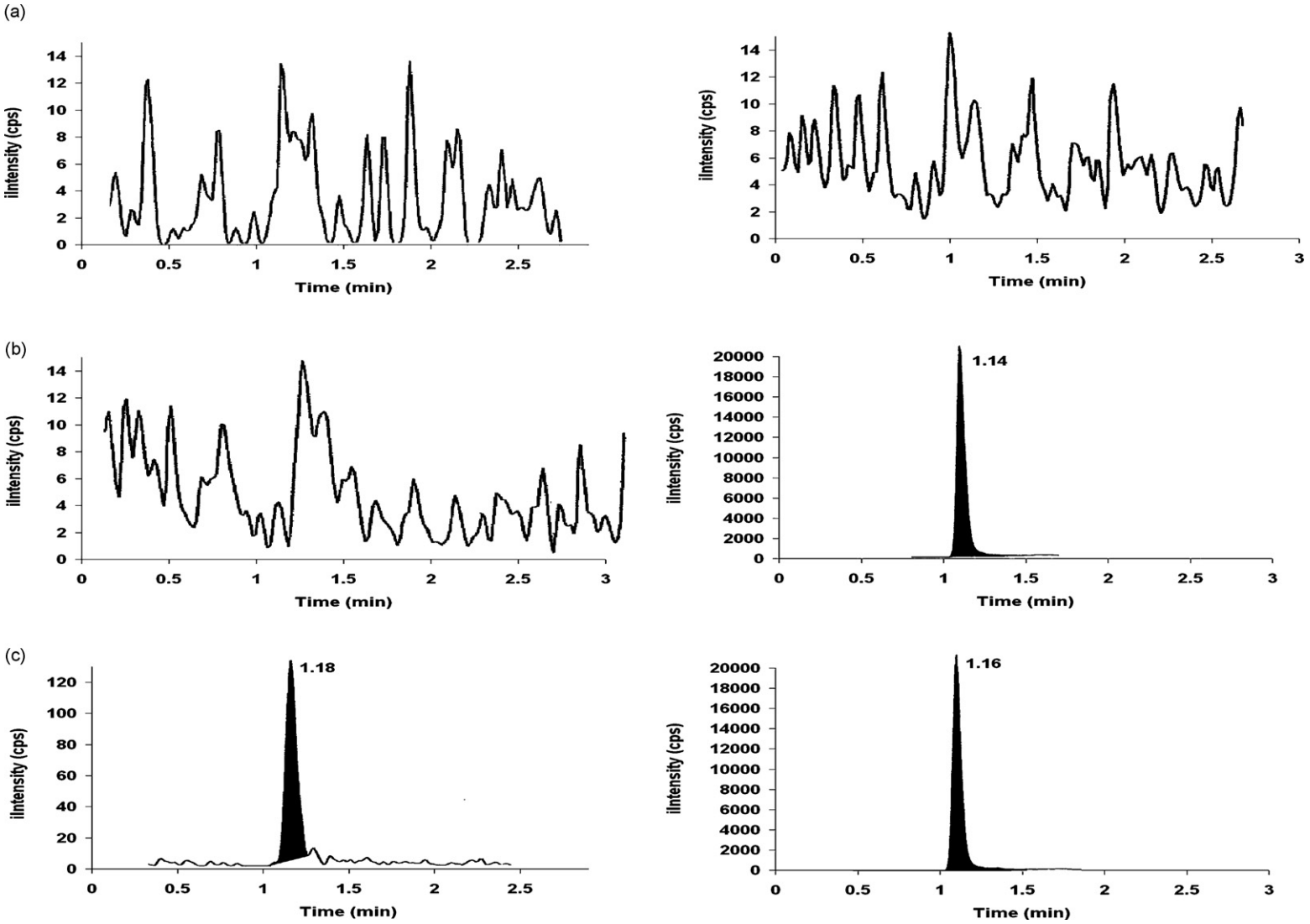

Fig. 3. Chromatograms of: (a) a blank white blood cells extract; (b) a control blank white blood cells extract (625 ng/ml IS); (c) spiked white blood cells extract sample at LLOQ (10 ng/ml WBC and 625 ng/ml IS) (AVE6971 on the left and IS on the right panels).

Table 4
Concentration *vs*. time following administration of 100 mg or 500 mg single intravenous dose of AVE6971

Time (h)	Subject A 100 mg			Subject B 500 mg		
	Concentration calculated (ng/ml)	Number of leucocytes per ml	AVE6971 content (ng/million of leucocytes)[a]	Concentration calculated (ng/ml)	Number of leucocytes per ml	AVE6971 content (ng/million of leucocytes)
−2 (predose)	BLQ	52,122,000	NR	BLQ	60,780,000	NR
1	72.7	52,581,000	0.0691	911	89,622,000	0.506
6	12.6	24,508,000	0.0257	60.7	64,209,000	0.0473
24	BLQ	32,592,000	NR	11.6	70,644,000	0.00821

BLQ: below limit of quantitation.
NR: not reported.
[a] 0.05 × conc./WBC count (million).

3.10. Application to clinical samples

The suitability of the developed method for the clinical use was demonstrated by the determination of AVE6971 in human WBC obtained from two healthy volunteers dosed with AVE6971 at intravenous dose levels of 100 mg and 500 mg (Table 4). Samples were collected at the following time points: before administration, then 1, 6 and 24 h after administration.

The AVE6971 content in test samples is reported as following: Level AVE6971 (ng/million of leucocytes) = (0.05 × conc.)/WBC count (million). In which 0.05 is the volume (ml) of calibration standards and conc. is the determined concentration (ng/ml).

4. Conclusion

A validated LC–MS/MS method for the determination of AVE6971 in human WBC has been developed. The extraction procedure was employed to extract compound from WBC with good recovery. Considering its sensitivity and selectivity, this proved to be capable of quantifying AVE6971 in WBC for the purpose of clinical studies. The method was shown to meet the current bio-analytical requirements, providing adequate accuracy and precision.

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