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Development and validation of a high-performance liquid chromatography–tandem mass spectrometry assay for the determination of sanfetrinem in human plasma

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Abstract

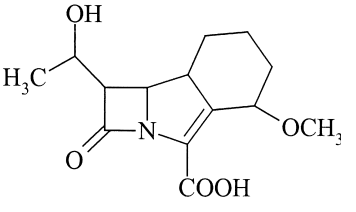
A rapid, selective and accurate high-performance liquid chromatography–tandem mass spectrometry assay for the quantification of sanfetrinem in human plasma has been developed and validated. The performance of manual and automated sample preparation was assessed; 50 μl of plasma sample was deproteinized with acetonitrile, followed by dilution with water and injection onto the LC system. Chromatographic separation was achieved on a Phenomenex Luna C_{18}(2), 50×2.0 (5 μm) column with a mobile phase consisting of water–acetonitrile with 0.1% formic acid followed by detection with a Perkin-Elmer API3000 mass spectrometer in multiple reaction monitoring mode. The lower limit of quantification was improved by five times compared to the UV method previously reported. A range of concentration from 10 ng/ml to 5 μg/ml was covered. The method was applied to the quantification of sanfetrinem in human plasma samples from healthy volunteers participating in a clinical study.

Keywords: Sanfetrinem

1. Introduction

Sanfetrinem (Fig. 1) is the first member of the new class of trinem antibacterial agents [1] with a broad spectrum of activity against Gram-positives [2] including penicillin-resistant Streptococcus pneumoniae [3], Gram-negatives and anaerobes [4] with high potency and high stability to β-lactamase enzymes [5].

A fully automated method has been previously developed and validated in our laboratory [6]. This method involves dilution of plasma with buffer and



Sanfetrinem

Fig. 1. The structure of sanfetrinem.

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automated on-line solid-phase extraction (SPE) followed by reversed-phase high-performance liquid chromatography (HPLC) separation with ultraviolet (UV) detection. This method was validated over the range of 50–2000 ng/ml and has a productivity of 60 samples per batch.

Another column-switching HPLC method for the quantification of sanfetrinem in dog and rat plasma has been recently published by the authors [7]. The method is based on dilution of samples with buffer and direct injection onto the system; after clean-up in an enrichment column, the analyte is back-flushed to the analytical column for quantification with a UV detector. A washing phase of the enrichment column and the alternation of injections between two enrichment columns ensure an increase in sample batch size. Validated over a range of concentration from 100 to 2000 ng/ml, the method has a productivity of 70 samples per batch.

The need of a more productive and more sensitive analytical method to assay plasma samples produced during clinical studies required the development of a new analytical method.

The use of liquid chromatography coupled with tandem mass spectrometry (LC–MS–MS), drastically reducing the chromatographic run time, enabled the authors to develop and validate a high-throughput, selective and sensitive method for the quantification of sanfetrinem in clinical plasma samples [8,9].

Plasma samples were prepared for analysis by manual or automated protein precipitation. Plasma protein precipitation with organic solvents is widely used as a sample clean-up procedure for the analysis of plasma samples by LC–MS–MS [8]. Compared with liquid–liquid extraction (LLE) and SPE, protein precipitation is made attractive by its simplicity, its speed, the opportunity to be applied to almost any analyte and its suitability for automation using the 96-well format [10].

2. Experimental

2.1. Chemicals

Sanfetrinem and $^{13}C_2$-sanfetrinem (internal standard, I.S.) were supplied by the Department of Pharmaceutical Development, Glaxo Wellcome (Verona, Italy) as natrium salts. Acetonitrile (HPLC grade) was purchased from Carlo Erba (Rodano, Italy). Formic acid (Analar grade) was obtained from Fluka (Milan, Italy). Water was purified by a Milli-Q system from Waters Millipore (Vimodrone, Italy). The structure of sanfetrinem is reported in Fig. 1.

2.2. Apparatus

The Robotic Sample Processor Genesis RSP 200 for the automated pre-treatment of samples was supplied by Tecan (Geneva, Switzerland). 96-Well Whatman filter plates were supplied by Merck Eurolabo (Milan, Italy).

The HPLC system Model 1100 from Agilent Technologies (Cernusco sul Naviglio, Italy) was connected to a CTC-Pal autosampler purchased by Alfatech (Genoa, Italy). Detection was performed by a Perkin-Elmer Sciex API3000 detector (Ontario, Canada) using TurboIonSpray ionization (TIS) for ion production.

2.3. Preparation of calibration standards and validation control samples

Sanfetrinem stock solutions were prepared in water at a concentration of 100 μg/ml; further dilution in water was performed to obtain analytical working solutions for both calibration standards (CSs) and validation control (VC) samples, immediately used to spike plasma and discarded thereafter. CSs and VCs prepared in bulk were divided into aliquots in polypropylene test tubes and stored at −80°C.

Internal standard stock solution was prepared in water at a concentration of 500 μg/ml; a final solution (500 ng/ml) in water was obtained for dilution of this primary stock solution.

2.4. Sample preparation

2.4.1. Manual procedure

A 50-μl volume of plasma sample was thoroughly mixed with 100 μl of acetonitrile in a polypropylene test tube. A 100-μl volume of 500 ng/ml internal standard solution was added and mixed thoroughly. Deproteinized samples were centrifuged for 10 min

at 3000 *g* at 4°C. The supernatant phase was directly injected (40 μl) onto the HPLC system.

2.4.2. Automated pre-treatment

The automated method involves the simultaneous application of plasma and acetonitrile (same volumes as the manual procedure) to 96-well filter plates. The RSP sequentially aspirates acetonitrile and plasma and then transfers them to individual channels of the 96-well filter plate; the dispensing speed is such that they are effectively mixed without the need of shaking. Deproteinized samples are then automatically filtered by vacuum application and extracts are collected in a 96 deep well plate, diluted with the internal standard solution (100 μl). Aliquots of 40 μl were injected onto the chromatographic apparatus.

2.5. LC–MS–MS conditions

LC separation was performed using a Phenomenex Luna $C_{18}(2)$, 50×2.0 (5 μm) column supplied by Chemtek (Milan, Italy). A six-port Rheodyne valve (LabPRO Model 700-100, Gilson, Italy) was used as a gate valve between the HPLC system and the mass spectrometer.

Gradient HPLC was used with a mobile phase A consisting of 0.1% formic acid in water and a mobile phase B consisting of 0.1% of formic acid in acetonitrile. The elution timetable was set as follows: 1% of mobile phase B isocratically for 0.3 min, then up to 100% of mobile phase B from 0.3 to 2.0 min and finally from 2.0 to 2.5 min isocratic at 100% of mobile phase B; at 2.6 min the percentage of mobile phase B was set again at 1%. The flow-rate was 0.8 ml/min with a split ratio entering the source of 250 in 800.

TurboIonSpray ionization was performed in the positive ion mode with purified air as nebulizing and auxiliary gas; nitrogen was used as curtain gas. The ionization source was heated at 350°C and a voltage of 5 kV was applied to the capillary.

Detection was performed using the tandem mass spectrometer. Nitrogen was used as collision gas. Acquisition was achieved in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ions (*m*/*z* 282.0 and 284.0) to the product ions (*m*/*z* 196.0 and 198.0) for sanfetrinem and its internal standard, respectively. Dwell times of 500 and 200 ms were used for sanfetrinem and the internal standard, respectively. The total acquisition time was 4.5 min.

The instrument was interfaced to an Apple Macintosh computer. All data were acquired and processed by Perkin-Elmer MassChrom 1.1 software with Sample Control and MacQuan, respectively.

2.6. Validation

The method was validated according to Divisional Policy and specificity, linearity, accuracy, precision, limits of quantification and stability were assessed.

Two calibration curves, one at the beginning and one at the end, were included in each batch assayed, consisting of nine calibration standards spanning the concentration range (10 to 5000 ng/ml). The method was validated by analyzing VC samples at five different concentrations (10, 20, 500, 4000 and 5000 ng/ml) in replicates of six. Blank plasma samples were bracketed amongst VC samples to reach the validation batch size (96 samples). A standard curve without the zero concentration was constructed using the sanfetrinem/internal standard peak area ratios.

Four different runs on separate days on two different instruments were performed to evaluate linearity, intra- and inter-day accuracy and precision. An additional run was performed to validate the use of a Robotic Sample Processor for the preparation of samples.

The specificity of the method was evaluated by comparison of LC–MS–MS chromatograms of sanfetrinem at the lower limit of quantification (LLOQ) and blank plasma samples from six individual volunteers.

3. Results and discussion

3.1. LC–MS–MS optimization

The instrument response was optimized by infusing solutions of sanfetrinem and of $^{13}C_2$-sanfetrinem into the stream of mobile phase eluting to the analytical column.

The influence of formic acid and ammonium acetate as mobile phases on MS response was studied. Higher MS response was obtained using

0.1% formic acid compared to 0.01% formic acid and to 5 m*M* ammonium acetate. Different HPLC gradients were taken into account trying to minimize the matrix effect observed comparing the MS response of the analyte in the absence or presence of matrix extract.

Fast gradient chromatography has been successfully used prior to MS–MS detection. This technique involves the use of short columns (5 cm) with conventional particle size (5 μm) and eluent linear velocities of four times the optimal flow-rate (800 μl/min) and enables chromatography to be performed with very short run times whilst maintaining the chromatographic performance. Minimal LC method development and the possibility to inject samples containing organic solvent at higher concentrations than those tolerated by conventional LC, allowing the application of minimal sample pretreatment techniques, are other remarkable advantages of this approach. The high separating power of the gradient elution, coupled with the well-known selectivity of MS–MS detection allowed to develop a method with very short run time (4.5 min), with a retention time of 1.6 min for both the analyte and the internal standard.

3.2. Specificity

The full scan mass spectra of sanfetrinem and its mass-labelled internal standard are shown in Figs. 2 and 3 and confirm their molecular masses at 282.0 and 284.0, respectively. No gross impurities were observed in an examination of the LC–MS total ion current chromatogram. The corresponding product ion spectra shown in Figs. 2 and 3 are also concordant with the assigned structures.

Visual examination and comparison of the LC–MS–MS chromatograms of blank and VC samples obtained during the validation experiments, including those of control human plasma from individual volunteers, showed the method has good specificity for sanfetrinem. The analyte has good chromatographic peak shape and no significant interferences from endogenous material at the retention time of sanfetrinem were observed. A representative chromatogram of a blank sample in comparison with a plasma sample at the LLOQ is shown in Fig. 4.

3.3. Linearity

Calibration curves were interpolated by least-squares regression analysis using the formula $y = ax + b$ where y is the peak area ratio, x is sanfetrinem concentration and a and b are the slope and the intercept, respectively. The linear regression equation was weighted $1/x^2$. Intercept, slope and correlation coefficients R^2 are reported in Table 1. A good agreement between nominal and back-calculated concentration for calibration samples has been observed, with a % bias between −4.7 and 11.2% (see Table 2).

3.4. Accuracy and precision

The intra- and inter-assay precision and accuracy of the method, evaluated as relative standard deviation (RSD) for all VC concentrations, are reported in Table 3 for both manual and automated sample preparation. The maximum intra-assay precisions observed for full and abbreviated validation (manual and automated procedure) were 1.3 and 5.4%, respectively; the maximum inter-assay precision was 4.6%. The maximum % bias range recorded was −11.8÷9.1%.

3.5. Stability

The stability of sanfetrinem in biological matrix and in processed extracts of spiked human plasma samples was assessed at one concentration prior to validation; data were analyzed by two one-sided *t*-tests using a macro within Excel (Microsoft). Sanfetrinem was found to be stable in human plasma stored at nominally ambient temperature and in processed extracts of spiked human plasma at nominally 4°C for at least 2 and 24 h, respectively

3.6. Recovery and limits of quantification

The recovery of sanfetrinem was determined by comparison of the analyte peak area after extraction with that obtained by addition of the same concentration of sanfetrinem to extracted blank plasma. The registered recovery was higher than 95%. As defined by the lower and upper validation control concentrations possessing acceptable accuracy and

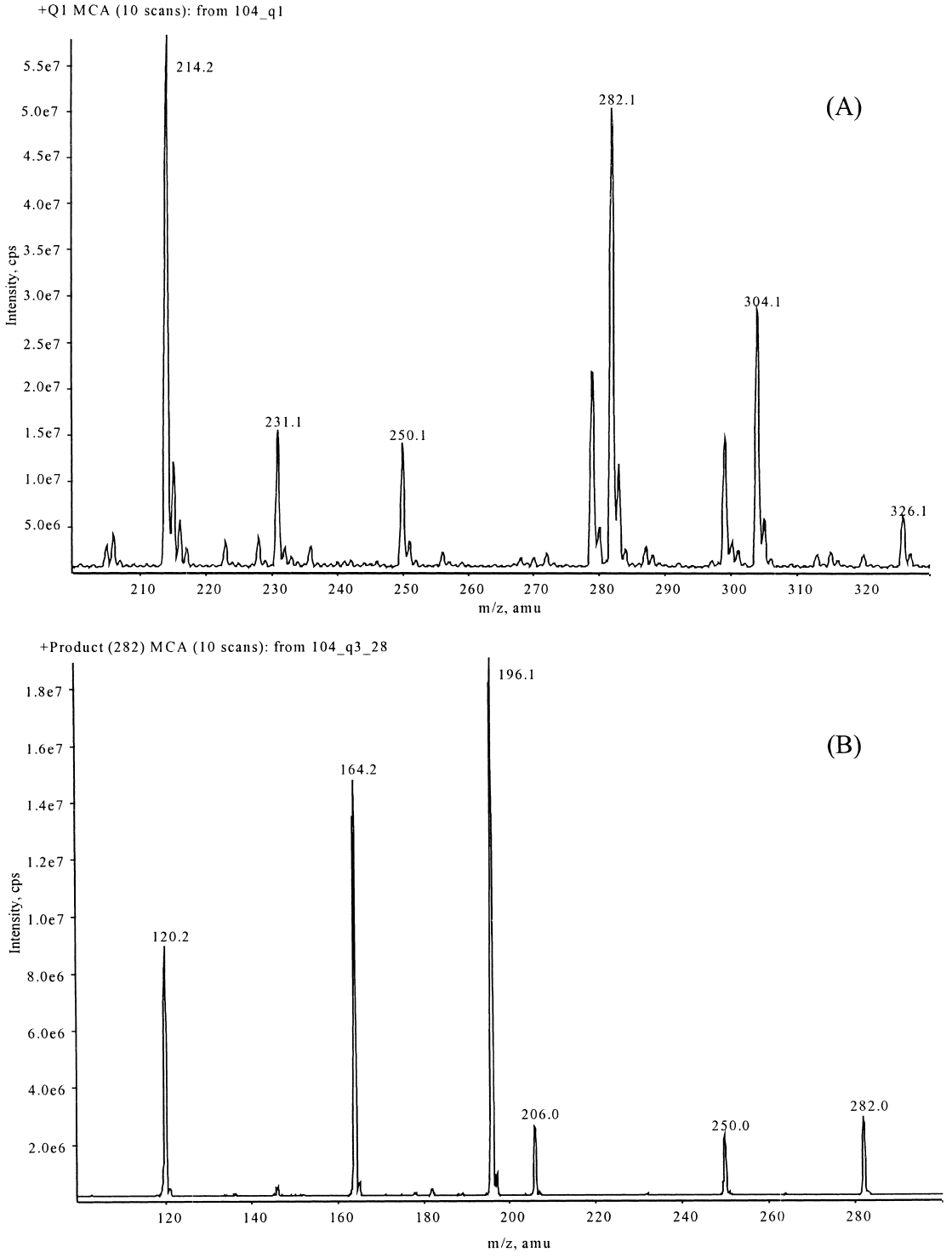

Fig. 2. (A) Q1 full scan of precursor ion of sanfetrinem; (B) MS–MS full scan of product ions of sanfetrinem.

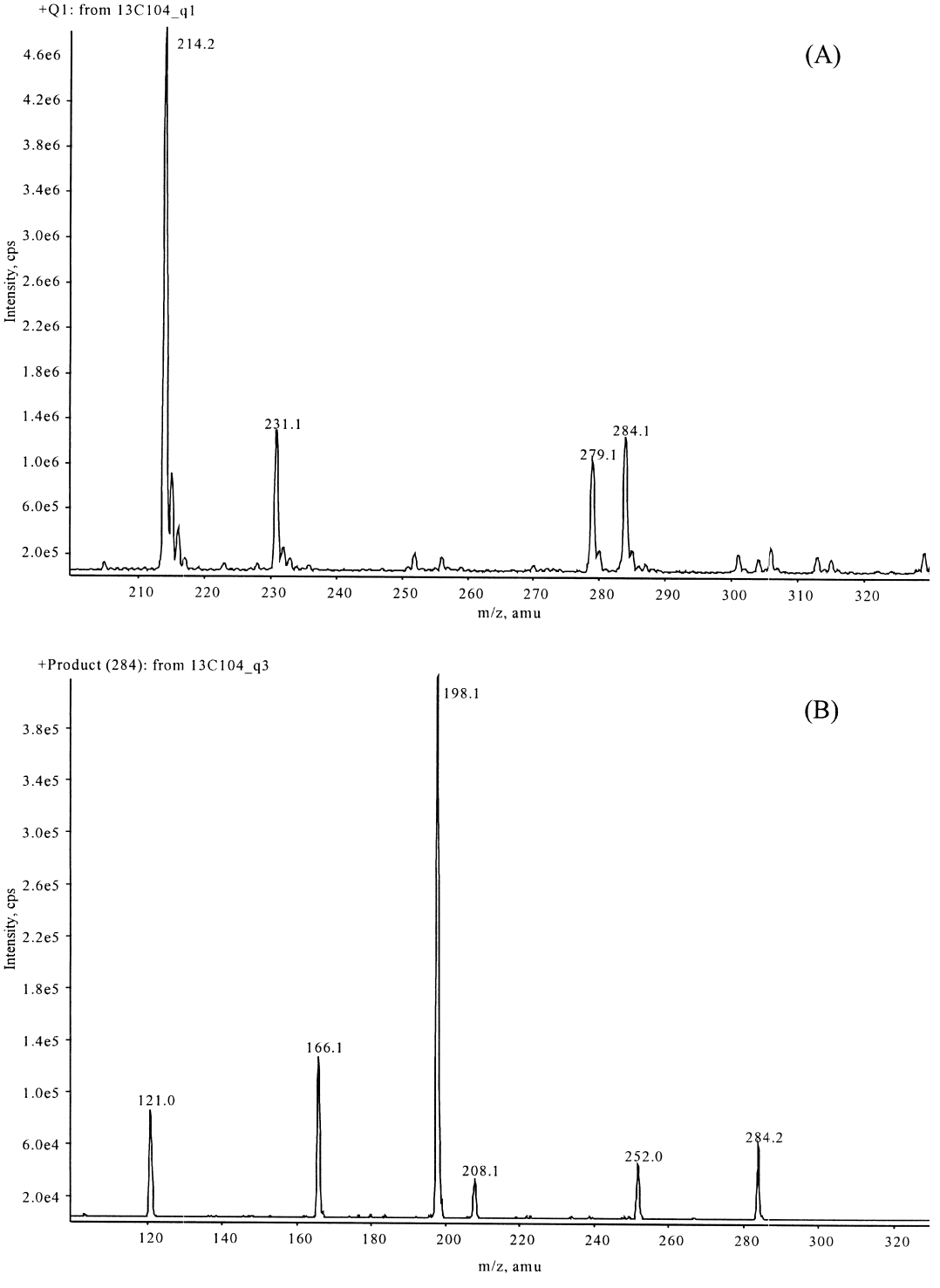

Fig. 3. (A) Q1 full scan of precursor ion of internal standard; (B) MS–MS full scan of product ions of internal standard.

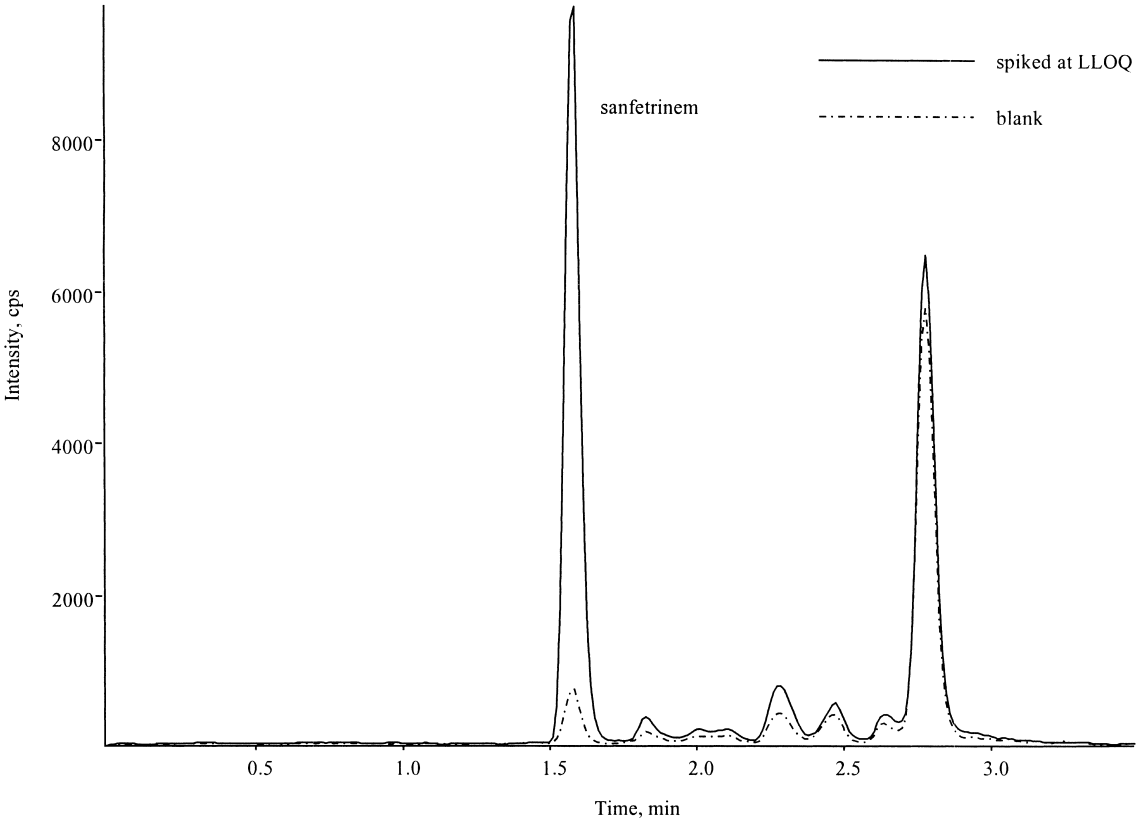

Fig. 4. Representative chromatogram of a blank sample compared with spiked plasma at LLOQ.

precision (±15% of nominal value), the limits of quantification of the method for sanfetrinem are 10 to 5000 ng/ml.

3.7. Application of the method

The LC–MS–MS method was applied to the quantification of sanfetrinem in plasma samples of volunteers from a clinical study after single oral administration of sanfetrinem cilexetil, its prodrug.

A typical chromatogram of a real patient sample is shown in Fig. 5. About 3000 samples were injected

Table 1
Regression parameters for the calibration curves of sanfetrinem in human plasma

Batch	Slope (a)	Intercept (b)	R^2
1	0.9076	−0.0043	0.9982
2	0.7417	−0.0027	0.9988
3	0.9008	−0.0039	0.9981
4	0.8978	−0.0041	0.9984
5	0.7187	−0.0029	0.9961

Table 2
Back-calculated values of the calibration samples of sanfetrinem in human plasma

Concentration (ng/ml)	Mean concentration (n=10)	RSD (%)	Accuracy (%)
10	9.53	2.2	−4.7
20[a]	22.25	4.1	11.2
50	49.09	3.4	−1.8
100	99.98	3.3	0.0
200	200.58	4.3	0.3
500	517.76	3.2	3.6
1000	980.33	2.9	−2.0
2000	1976.11	2.4	−1.2
5000	4789.62	3.2	−4.2

[a] n=5.

Table 3
Intra- and inter-assay precision and accuracy for sanfetrinem in human plasma – clinical validation

Concentration (ng/ml)	Intra-assay precision				Inter-assay precision		Mean accuracy (%, $n=30$)
	Manual		Automated		Mean ($n=30$) (ng/ml)	RSD (%)	
	Mean ($n=6$) (ng/ml)	RSD (%)	Mean ($n=6$) (ng/ml)	RSD (%)			
10.0	11.3	1.3	10.5	2.6	10.9	4.6	9.1
20.0	17.8	1.2	17.2[a]	4.2	17.6[b]	3.2	−11.8[b]
500	503.7	1.0	477.9	5.4	500.5	3.3	0.1
4000	3870.5	0.6	3685.2	3.6	3819.8	2.8	−4.5
5000	4838.9	0.9	4524.1[a]	3.7	4761.2[b]	3.4	−4.8[b]

[a] $n=5$.
[b] $n=29$.

during application of the method, including 600 calibration standards and 200 quality control samples; only one calibration standard and three quality control samples were rejected during the whole study being out of acceptance limits. The maximum precisions recorded were 4.9 and 5.4% for calibration standards and quality control samples, respectively. The maximum % bias ranges were $-5.1 \div 2.6$ and $-4.1 \div 5.2\%$ for calibration standards and quality control samples, respectively. Some typical profiles

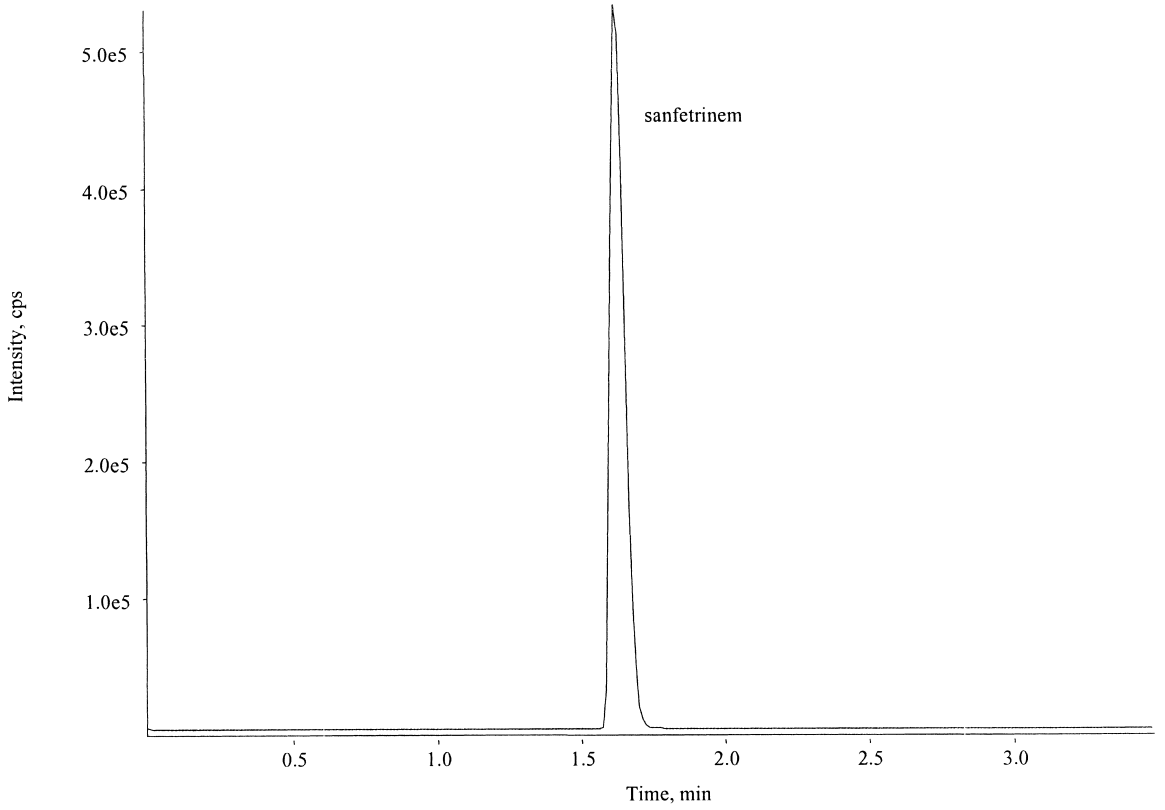

Fig. 5. Representative chromatogram of a real patient sample (30 min after administration).

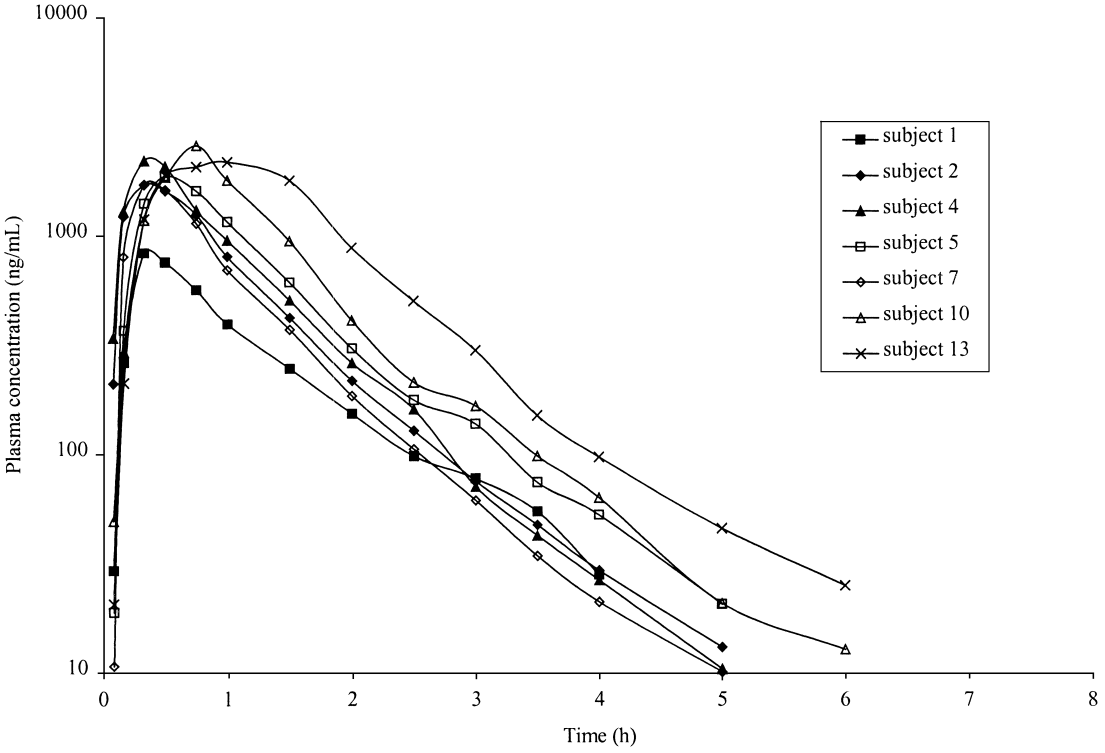

Fig. 6. Plasma concentrations vs. time of sanfetrinem in human volunteers after 250 mg oral dosing of its prodrug.

of the drug up to 6 h after 250 mg oral administration of the prodrug are shown in Fig. 6.

4. Conclusions

The LC–MS–MS method reported above was validated according to internationally accepted criteria. The method is sensitive, precise and accurate and was applied to the quantification of sanfetrinem in clinical plasma samples. The procedure has a good productivity as a combination of automated sample pre-treatment, with 96-well samples ready for analysis in 20 min and a reduced assay run time, with 12 samples assayed per hour. In comparison with the method previously published [7], the automated procedure has reduced to a minimum all problems related to manual handling during the sample pre-treatment phase and remarkably decreased total time of analysis from sample to result.

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