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Development and validation of a column-switching high-performance liquid chromatographic method for the determination of sanfetrinem in rat and dog plasma by direct injection

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Abstract

A direct injection column-switching HPLC method was developed and validated for quantification of sanfetrinem in rat and dog plasma. Following dilution with buffer, samples were directly injected onto the system. The analyte was retained in an enrichment column while endogenous plasma components were eluted to waste. Sanfetrinem was then back-flushed to the analytical column for separation and quantification with an ultraviolet detector. Sample batch size was increased by adding a washing phase of the enrichment column and by alternating the injections between two enrichment columns. The method is very simple and sample preparation is minimal. The method has been fully validated and shown to be specific, accurate and reproducible.

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 including penicillin-resistant Streptococcus pneumoniae, Gram-negatives and anaerobes with high potency and high stability to β-lactamase enzymes.

An analytical method was developed and validated in our laboratory using simple plasma deproteinization, reduction to dryness, reconstitution with appropriate buffer and injection onto a high-performance liquid chromatographic (HPLC) system [2]. The work described in this paper was undertaken to develop an alternative simpler analytical method to assay sanfetrinem in rat and dog plasma samples.

Several HPLC methods are described in literature for the quantification of beta-lactam antibiotics in

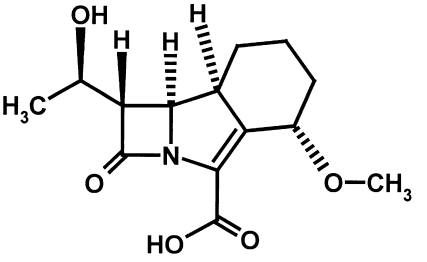

Fig. 1. Structure of sanfetrinem.

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various biological matrices based on direct injection of the samples without sample clean up onto a column switching system [3–6]. Column switching assays have the advantages of avoiding off-line procedures, minimizing drug loss that might occur during sample clean-up procedures and generally of a higher throughput.

2. Experimental

2.1. Apparatus

The HPLC apparatus consisted of three model 305, 306 and 307 pumps (Gilson, Milan, Italy), a model 232 bio automatic injection system (Gilson) with a water bath sample cooler (Gilson) and equipped with two Rheodyne injection valves, a further automated Rheodyne 6-port switching valve (Gilson), a column oven (Gilson) and a model 975 UV detector (Jasco, Milan, Italy).

The Access*Chrom computer program (PE Nelson, Monza, Italy) was used for chromatographic data acquisition and elaboration.

2.2. Materials

Sanfetrinem was supplied by Pharmacy Department, GlaxoWellcome S.p.A. (Verona, Italy). Deionised water was obtained by passage through a MilliQ water purification system (Waters, Vimodrone, Italy). All the other reagents were of analytical or HPLC grade.

2.3. Preparation of solutions

Two amounts of about 10 mg of sanfetrinem sodium salt (conversion factor $w_{acid}/w_{salt}=0.927$) were accurately weighed into amber volumetric flasks and diluted to 10 mL volume with water to obtain A and B stock solutions. Stock solution A was intended for the preparation of calibration standards (CS), B for the preparation of validation control samples (VC). The concentrations of analytical working solutions of sanfetrinem to be used for the preparation of CSs were: 2, 4, 10, 20, 40, 100, 200 and 400 μg/mL. They were obtained from serial dilution of stock solution A with water. The concentrations of analytical working solutions of sanfetrinem to be used for the preparation of VCs were: 2, 4, 40, 320 and 400 μg/mL. They were obtained from serial dilution of stock solution B with water. CSs and VCs were all prepared from a 1:20 dilution of related working solution with rat or dog plasma. Final CS concentrations were 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 μg/mL. Final VC concentrations were 0.1, 0.2, 2.0, 16.0 and 20.0 μg/mL.

2.4. Sample preparation

Plasma samples (CSs, VCs and blanks) were centrifuged for 10 min at 3000 *g* at 4°C; 50 μL of plasma was then transferred into an autosampler vial and 150 μL of 0.05 *M* 3-[*N*-Morpholino]propanesulfonic acid (MOPS) was added.

2.5. Chromatographic conditions

Fig. 2 shows a schematic diagram of the automated system. The two enrichment columns were H5-ODS, 10×3 mm ID, 5 μm, HiChrom (CPS Analitica, Milan, Italy) and the analytical column was a Hypersil ODS, 100×4.6 mm ID, 5 μm, Thermoquest (CPS Analitica). The mobile phase (Pump 3) was acetonitrile–100 m*M* ammonium acetate–glacial acetic acid (10/89.9/0.1 v/v/v). The enrichment phase (Pump 1) was 20 m*M* ammonium acetate and the washing phase (Pump 2) was acetonitrile. The analytical column was maintained at 40°C while the two enrichment columns operated at ambient temperature. The flow rate for the analytical column was 1 mL/min while the two enrichment columns were flushed at 0.5 mL/min either for the enrichment or washing phases. The autosampler was set with an injection volume of 100 μL, a rack temperature of 4°C and a run time of 10 min. The absorbance wavelength on the detector was set at 268 nm.

Following injection of the sample, the enrichment column was washed with enrichment phase for 2 min to remove plasma proteins and other endogenous interferences. For the following 0.5 min, the Rheodyne 2 changed the flow path of the mobile phase to backflush the analyte from the enrichment column into the analytical column with the mobile phase. Afterwards, the mobile phase flow path was returned

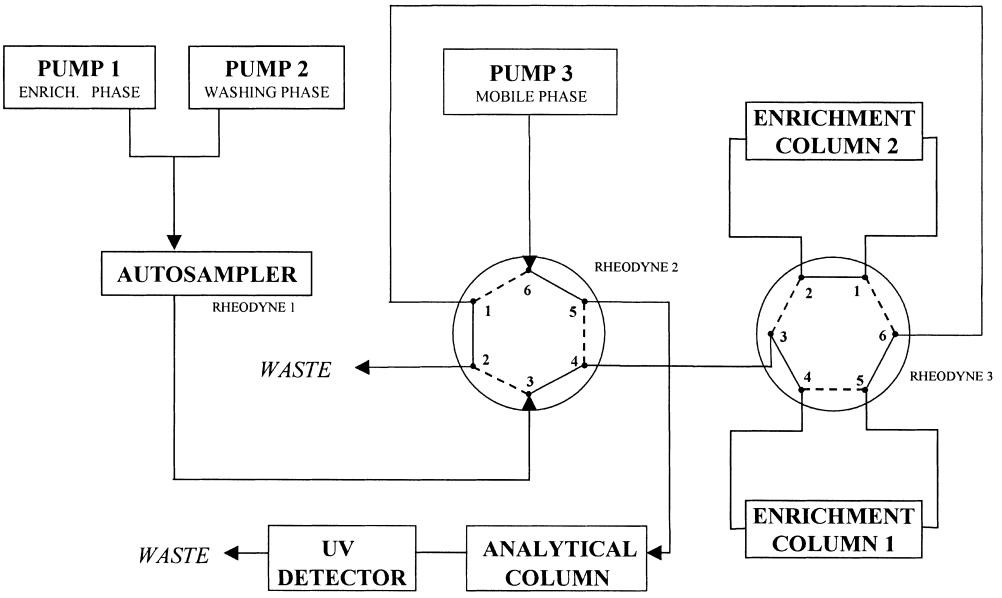

Fig. 2. Schematic diagram of the column switching system. Flow path of mobile phase was changed using two-positions six-port switching valve. Plasma was injected into an enrichment column and endogenous components washed to waste. Enrichment column was then put on-line with the analytical column to transfer the analyte. After each sample, Rheodyne 3 was switched to alternate the use of the enrichment columns. The solid and dotted lines in Rheodyne 2 and 3 indicates valve position 0 and 1.

to initial condition to separate the analyte in the analytical column from co-eluted endogenous compounds. At the same time the enrichment column was washed by a gradient which reached 50% of acetonitrile for 1.5 min. At the end of the washing phase, Rheodyne 3 was switched in order to alternate the use of the two enrichment columns.

2.6. Validation study

The method was validated prior to the analyses of study samples according to internal guidelines and the most recent reviews published in the literature [7,8]. The following parameters were assessed.

The specificity of the method was assessed by the inclusion of blank samples prepared from pooled control rat and dog plasma in validation assays. Uniquely identified samples of control rat and dog plasma from at least six individual animals were also analyzed.

The accuracy of the method (% bias) was determined by assessing the agreement between the measured and nominal concentrations of VC samples. The measured concentration is the mean of the concentrations obtained during the precision assessment, in which spiked VC samples containing sanfetrinem at 0.1, 0.2, 2.0, 16.0 and 20.0 μg/mL, were analyzed in replicates of six, on one occasion.

The precision of the method (% CV) was determined by assessing the agreement between replicate measurements of VC samples as described above.

The lower (LLOQ) and upper (ULOQ) limits of quantification were defined by the VC sample concentrations possessing acceptable accuracy and precision.

The stability of sanfetrinem in spiked rat and dog plasma samples stored at nominally ambient temperature and at −80°C was assessed at 16 μg/mL in replicates of six by comparing the peak areas against those of freshly prepared spiked samples.

The stability of sanfetrinem in spiked rat and dog plasma samples after 3 freeze–thaw cycles from nominally −80°C to ambient temperature was assessed at 16 μg/mL in replicates of six by comparing the peak areas against those of freshly prepared spiked samples.

The stability of sanfetrinem in processed extracts

of spiked rat and dog plasma samples stored in the autosampler rack at 4°C was assessed at 10 μg/mL in replicates of six by comparing the peak areas against those of freshly prepared extracts after 16, 20 and 24 h.

3. Results

3.1. Chromatography

The major issue in the development of a column-switching method for sanfetrinem was, considering the very high polarity of sanfetrinem, the retention of the compound into the enrichment column. Three different factors were taken into account to optimize this parameter: enrichment column with high efficiency stationary phases, enrichment mobile phases at different pHs and with different percentage of organic solvent. Sanfetrinem was insufficiently retained in common cartridges used for column-switching assays and only cartridges with high efficiency stationary phase, commonly used as guard column, allowed higher retention and quantitative recoveries even if easier clogging of cartridges was found. The use of enrichment phases with acidic pH increased the retention of the compound but caused a dramatic decrease of cartridges duration.

The column switching system finally developed provided adequate clean up of plasma as shown by the absence of interfering peaks in blank plasma samples. The reduced and focused transferring time also limited the transfer of unwanted late eluting interfering compounds from the enrichment to the analytical column. This phenomenon was also prevented by inserting a washing procedure of the enrichment column with a mobile phase with a percentage of solvent that should allow the elution of all endogenous compounds still retained after the transfer of the analyte. The washing phase proved

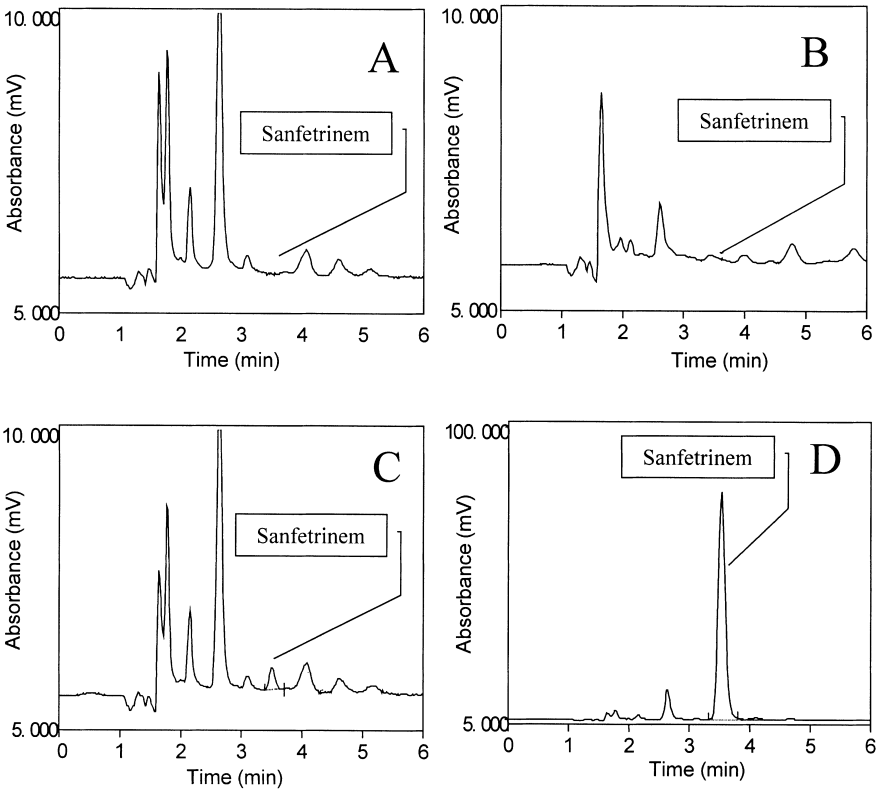

Fig. 3. Representative chromatograms of rat and dog plasma samples. Shown are blank rat plasma (A), blank dog plasma (B), rat plasma containing 0.1 μg/mL (C) and 20 μg/mL (D) of sanfetrinem.

Table 1
Accuracy and precision for the determination of sanfetrinem in rat and dog plasma[a]

Sanfetrinem plasma concentration (μg/mL)	Rat plasma		Dog plasma	
	Accuracy Bias%	Precision CV%	Accuracy Bias%	Precision CV%
0.10	−0.8	9.3	0.7	5.7
0.20	−4.3	3.1	4.7	2.6
2.00	−3.1	2.6	3.3	1.6
16.0	−4.9	3.5	4.4	1.5
20.0	−2.7	1.5	4.6	0.4

[a] Number of replicates=6.

also to be beneficial for the lifetime of the enrichment columns.

Recovery of sanfetrinem, calculated comparing the response factor of a plasma samples processed throw the column switching system with the response factor of a standard in water directly injected onto the column, was quantitative.

Enrichment column allowed the injection of about 30–40 plasma samples before backpressure, probably due to the irreversible adsorption of endogenous plasma components, increased over the acceptable limit. The alternate use of two enrichment columns allowed running batches with sizes of 70–80 samples, limiting the risk of losing samples because of system blockage due to high pressure.

In the chosen experimental conditions, more than 500 samples have been assayed with the same analytical column without significant loss in peak shape and separation.

3.2. Specificity

Visual examination of the HPLC–UV chromatograms of blank samples obtained during the validation experiments, including those of control rat and dog plasma from individual sources, indicated high specificity. The analytes were chromatographically resolved and no significant interferences from endogenous material were observed. A representative chromatogram of a blank sample is shown in Fig. 3.

3.3. Sensitivity and linearity

As defined by the lower and upper validation control concentrations possessing acceptable accuracy and precision, the limits of quantification of the

Table 2
Stability of sanfetrinem in rat and dog plasma in different conditions[a]

Temperature/ Condition	Time interval	Rat plasma			Dog plasma		
		Difference (%)	Confidence interval[b]		Difference (%)	Confidence Interval[b]	
			lower	upper		lower	upper
Plasma ambient temp.	1 h	nt	–	–	−5.0	−5.2	−4.7
	2 h	−3.0	−4.6	−1.3	−10.2	−11.2	−9.2
Plasma −80°C	7 days	−1.0	−1.3	−0.7	−3.7	−6.6	−0.7
	10 days	−2.2	−2.6	−1.9	−0.4	−1.4	0.7
	30 days	−7.2	−7.5	−7.0	−9.2	−9.3	−9.0
	66 days	2.4	1.3	3.5	4.3	4.0	4.6
Freeze-Thaw	cycle 1	−11.0	−13.0	−8.9	−6.5	−6.6	−6.3
	cycle 1	−14.5	−17.0	−11.0	−9.4	−9.6	−9.1
	cycle 1	−10.2	−13	−7	−9.5	−10	−9.2
Processed samples stored at 4°C in the autosampler rack	16 h	−1.0	−1.5	−0.4	−3.9	−4.7	−3.2
	20 h	−2.1	−2.7	−1.7	−7.2	−7.5	−6.9
	24 h	−4.0	−4.6	−3.4	−8.8	−9.2	−8.4

[a] nt=not tested.
[b] 95% confidence interval.

method for sanfetrinem were 0.1 to 20 μg/mL. Representative chromatograms of validation controls at the LLOQ and ULOQ are shown in Fig. 3.

3.4. Accuracy and precision

The accuracy (% bias) of the method for sanfetrinem is detailed in Table 1. At all VC concentrations examined the mean % bias is less than ±15% and therefore acceptable. The maximum % bias recorded was −4.9 and 4.7% for rat and dog respectively.

Estimates of the intra-assay precision (% CV) of the method for sanfetrinem are presented in Table 1. At all VC concentrations examined, the intra-assay precision are less than 15%, and therefore accept-

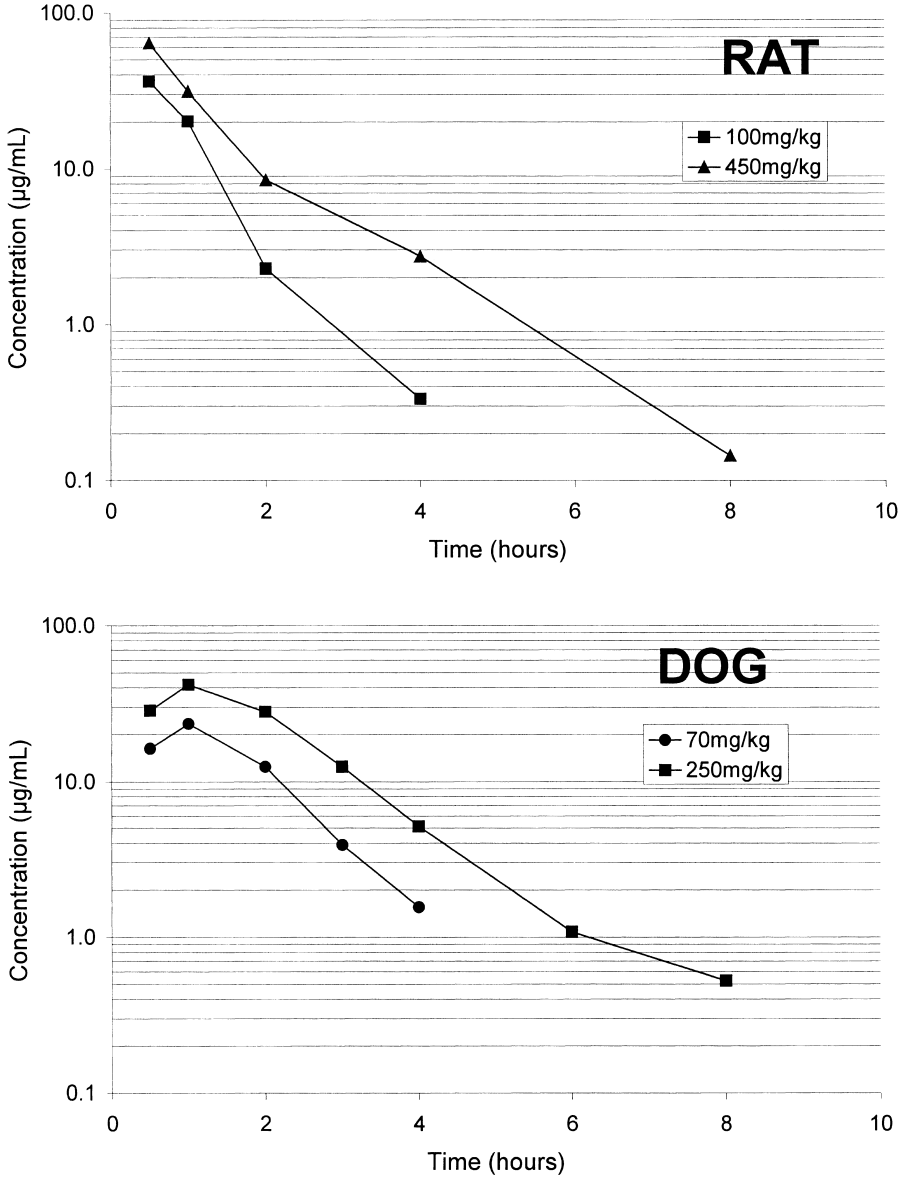

Fig. 4. Representative plasma concentration profiles of sanfetrinem from toxicokinetic studies in rat and dog.

able. The maximum intra-assay CV% observed for sanfetrinem was 9.3 and 5.7% for rat and dog respectively.

3.5. Stability

The beta-lactam moiety is chemically and enzymatically vulnerable to hydrolysis to the open-lactam form and thus the stability of sanfetrinem was carefully studied under different conditions.

Both in rat and dog plasma, sanfetrinem results were stable (difference within ±15%) at nominally ambient temperature and at −80°C for at least 2 h and 66 days, respectively. Thus plasma samples can be handled and processed without implementing procedures to further enhance plasma stability and plasma specimens can be stored at −80°C for at least 2 months prior to analysis (Table 2).

Freeze-thawing seems to affect stability of sanfetrinem but not to an unacceptable degree (Table 2).

Processed samples (plasma diluted 1:4 with 0.05 *M* MOPS buffer) placed in the autosampler rack were stable over 24 h, sufficient to permit overnight unattended analysis (Table 2).

3.6. Method application

The validated method has been used to support several pre-clinical and toxicokinetic studies. Fig. 4 shows representative plasma concentration profiles obtained during toxicokinetic studies in rat and dog. In the assay of samples, when the concentrations of the drug were higher than the upper calibration standard, the samples were appropriately diluted with the corresponding matrices and a re-assay performed.

The method presented in this paper was capable of characterizing the pharmacokinetic parameters in all the pre-clinical studies conducted up to now.

4. Conclusion

A direct injection column-switching HPLC method was developed for quantification of sanfetrinem in rat and dog plasma. The method is very simple and sample preparation is minimal. The method has been fully validated and shown to be specific, accurate and reproducible.

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