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Rapid determination of gemifloxacin in human plasma by high-performance liquid chromatography–tandem mass spectrometry

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Abstract

A method was developed for the determination of gemifloxacin (**I**) in human plasma using high-performance liquid chromatography–tandem mass spectrometry. Prior to analysis, the protein in plasma samples was precipitated with acetonitrile containing [$^{13}C^{2}H_{3}$] gemifloxacin (**II**) to act as an internal standard. The supernatant was injected onto a PLRP-S column without any further clean-up. The mass spectrometer was operated in positive ion mode, employing a heat assisted nebulisation, electrospray interface. Ions were detected in multiple reaction monitoring (MRM) mode. The assay requires 50 μl of plasma and is precise and accurate within the range 10–5000 ng/ml. The average within-run and between-run coefficients of variation were <11% at 10 ng/ml and greater concentrations. The average accuracy of validation standards was generally within ±7% of the nominal concentration. There was no evidence of instability of **I** in human plasma following three complete freeze–thaw cycles and samples can safely be stored for at least 6 months at −20°C. The method proved very robust and was successfully applied to the analysis of clinical samples from patients dosed with gemifloxacin.

Keywords: Gemifloxacin

1. Introduction

Gemifloxacin, (*R,S*)-7-(3-aminomethyl-4-(*syn*)-methoxyimino - 1 - pyrrolidinyl) - 1 - cyclopropyl - 6 - fluoro-1, 4-dihydro-4-oxo-1, 8-naphthyridine-3-carboxylic acid methanesulfonate (**I**; Fig. 1), is a new, broad-spectrum quinolone antibacterial with activity against both Gram-negative and Gram-positive organisms [1–3].

In order to follow the pharmacokinetics of absorption and elimination during clinical efficacy trials, a high-performance liquid chromatography–tandem mass spectrometry method (LC–MS–MS) was developed for the determination of **I** in human plasma. This paper represents the first detailed description of that method which consists of the precipitation of protein in plasma with acetonitrile and injection of the centrifuged supernatant for isocratic reversed-phase chromatography with MS–MS detection. Consequently the analytical procedure is simple and the method has a high sample throughput. This uncomplicated approach is possible because of the selectivity of LC–MS–MS which allows accurate measurement at levels as low as 10 ng/ml with minimal sample clean-up. The method proved very

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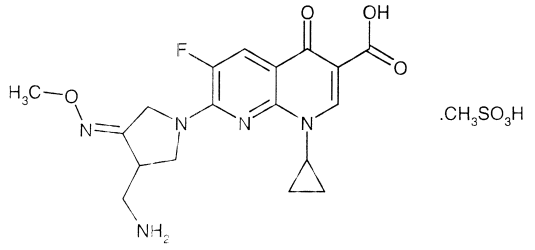
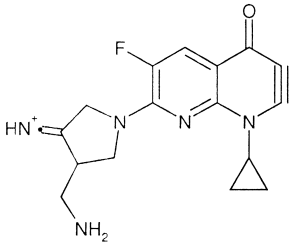


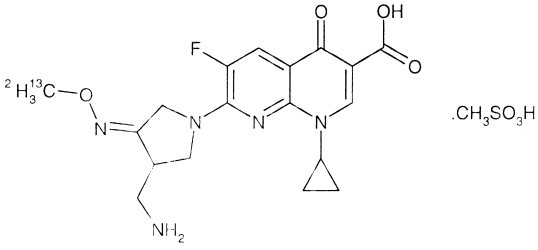
I

$C_{18}H_{20}FN_5O_4.CH_3SO_3H$, MW = 485.1



III

$C_{16}H_{16}FN_5O$, MW = 313.1



II

$^{13}CC_{17}{}^{2}H_3H_{17}FN_5O_4.CH_3SO_3H$, MW = 489.1

Fig. 1. Structures of gemifloxacin **I**, [$^{13}C^{2}H_3$] gemifloxacin (**II**, I.S.) and the major ion-radical observed in the product spectrum of both **I** and **II** at m/z 313 (**III**).

robust and was successfully applied to the analysis of clinical samples from patients dosed with **I** [4,5].

2. Experimental

2.1. Materials and reagents

All chemicals used were at least reagent grade and solvents of HPLC grade. They were purchased from Aldrich Chemical Co. Ltd. (Dorset, UK), Hayman Ltd. (Essex, UK), Romil Ltd. (Cambridge, UK) and Pierce (Illinois, USA). Pure HPLC grade water was obtained using an Elgastat MAXIMA (Elga Ltd., High Wycome, UK) and gases were high purity obtained from BOC (Luton, UK).

(*R,S*)-7-(3-aminomethyl-4-(*syn*)-methoxyimino-1-pyrrolidinyl)-1-cyclopropyl-6-fluoro-1, 4-dihydro-4-oxo-1, 8-naphthyridine-3-carboxylic acid methanesulfonate (**I**; Fig. 1) was supplied by LG Chemical Limited (Taejon, South Korea) and [$^{13}C^{2}H_3$] (*R,S*)-7-(3-aminomethyl-4-(*syn*)-methoxyimino-1-pyrrolidinyl)-1-cyclopropyl-6-fluoro-1, 4-dihydro-4-oxo-1, 8-naphthyridine-3-carboxylic acid methanesulfonate (**II**; Fig. 1) was synthesised by SmithKline

Beecham Pharmaceuticals (PA, USA) and were greater than 98.6% pure as the salt form.

2.2. Preparation of drug standard solutions

I and **II** were stored away from light at all times and allowed to reach ambient temperatures before opening and weighing. Approximately 2 mg of **I** was weighed out and dissolved in ultra pure water–ethanol (1:1, v/v) to give a solution containing 1 mg/ml **I**. The solution was stable for at least 1 month when stored at 4°C and protected from light. The 1 mg/ml stock solution was serially diluted with ethanol to give working stock solution containing 100 μg/ml, 10 μg/ml, 1 μg/ml and 0.1 μg/ml **I**. All working solutions were protected from light and used on the day of preparation. Separate weighings were made for preparing the standard curves and for the estimation of precision.

Approximately 2 mg of **II** was weighed out and dissolved in ethanol to give a solution containing 1 mg/ml **II**. This solution was protected from light and stored at 4°C. The solution was stable for at least 1 month when stored under these conditions. On the day of assay this solution was diluted to 0.25 μg/ml with acetonitrile, and used to precipitate plasma proteins in samples and standards.

2.3. Preparation of plasma standards and validation samples

Blood samples were taken from human subjects who had not received any medication in the previous 48 h, into EDTA tubes, mixed and centrifuged. The plasma was then frozen immediately and stored at −20°C until required for assay. Calibration standards were prepared over the range 10–5000 ng/ml by adding appropriate amounts of **I** as a standard solution. Ethanol was added to the plasma standard to maintain a constant plasma–ethanol ratio of 1:10. Validation standards were prepared at concentrations of 10, 20, 1000 and 5000 ng/ml in plasma by adding appropriate amounts of **I**. Standards used for the estimation of precision were prepared in the same way as those used for calibration, but from a different stock solution.

2.4. Sample preparation

Samples and standards were prepared for direct injection onto the HPLC column after precipitation of the plasma proteins with acetonitrile containing internal standard. Plasma (50 μl) and internal standard working solution (250 μl) were pipetted into a polypropylene tube (1.5 ml). The tube was capped and contents mixed on a vortex mixer for about 10 s, then shaken for approximately 30 min and left to stand at room temperature for 10 min to reach equilibrium. The sample was then centrifuged at approximately 14 000 *g* for 15 min. The supernatant was then carefully pipetted into a silanised 1.1 ml amber tapered autosampler vial containing ammonium acetate buffer (200 μl; 0.01 *M*, pH 2.5 with trifluoroacetic acid); capped and vortex mixed. Finally, 10 μl of the extract were injected onto the HPLC column.

2.5. Instrumentation and chromatographic conditions

We used a Hewlett-Packard 1100 binary liquid chromatograph with integral column oven (Hewlett-Packard, Bracknell, UK) coupled to a PE SCIEX API 300 tandem mass spectrometer (Perkin-Elmer Sciex Instr., Onteria, Canada) using a heat assisted nebulisation, electrospray interface. A PLRP-S column (100 A, 5 μm, 500×4.6 mm I.D., Polymer Laboratories Ltd., Shropshire, UK) was maintained at a constant 40°C and the mobile phase (70% ammonium acetate buffer (0.01 *M*, pH 2.5 with trifluoroacetic acid) and 30% acetonitrile) flow-rate was 1 ml/min. The tandem mass spectrometer was operated in positive ion mode with Multiple Reaction Monitoring (MRM) and quantification was achieved by comparison of the chromatographic peak areas for gemifloxacin (nominal positive ion 390 and nominal product ion 313) and internal standard (nominal positive ion 394 and nominal product ion 313). A dummy mass transition of m/z 400–10, 10 ms dwell time, was inserted between those for gemifloxacin and internal standard to minimise cross-talk. Major operating parameters were typically nebuliser gas flow = 12 (60 p.s.i.); curtain gas flow = 11 (40 p.s.i.); collision gas thickness = 4; auxiliary gas flow

=7.0 l/min; dwell time =400 ms; pause time =5 ms.

2.6. Method validation

A calibration curve containing eleven points and a blank which was not included in the calculation were prepared in human plasma. **I** and **II** area ratios were determined and plotted against concentration of **I** to construct the line. The slope and intercept were determined by a weighted ($1/y^2$) least-squares linear regression. This weighting factor was chosen because it gave the best individual residual values.

Four pools of human plasma containing 10, 20, 1000 and 5000 ng/ml were prepared from a fresh stock solution of **I** as described above. Six replicate standards from each pool were extracted and analysed in each of three separate analytical runs. Concentrations were determined by comparison with a calibration curve prepared on the day of analysis. The within-run precision was estimated using the mean and the standard deviation of the six replicate results. The between-run precision was estimated by calculating the ratio of the standard deviation of the within-run means to the average of the within-run means, and expressed as a percentage. Accuracy was estimated as the ratio of the mean concentration by analysis to the nominal concentration, and expressed as a percentage. Overall accuracy was reported as the average of the individual run accuracy estimations.

2.7. Stability

Standards at concentrations of 20, 5000 ng/ml in plasma were prepared in the same way as those used for the estimation of precision and accuracy (Section 2.3) and used for the estimation of freeze–thaw stability. Six replicate standards at each concentration were put through three complete freeze–thaw cycles, freezing to −20°C, and analysed for **I** with six replicate samples for each concentration prepared in the same way freshly on the day of analysis.

Long term stability was estimated using plasma samples from 3 healthy subjects given a single oral dose of 320 mg gemifloxacin. The plasma samples from these subjects were assayed before and after storage at −20°C for a period of 6 months.

3. Results and discussion

3.1. Method development

Under the LC–MS–MS conditions described above, the positive ion spectrum for **I** consisted predominately of a single ion at m/z 390, which corresponds to the protonated molecular (parent) ion. By selecting m/z 390, in the first mass analyser, and inducing fragmentation, the major ion observed in the product spectrum was detected at m/z 313. This ion (**III**; Fig. 1)) gave a signal intensity greater than twice that of any other ion observed in the spectrum and was therefore selected for sensitive quantification of **I**. This fragment is consistent with the loss of a methoxy radical from the imine moiety plus loss of methanoic acid. The positive ion and product spectra for II were very similar to those for **I** except that the parent ion at m/z 394 (**III**; Fig. 1) shows the presence of the stable labels, and the major fragment ion-radical at m/z 313 shows the loss of the methoxy radical which contained the stable labels, plus loss of methanoic acid.

Since the method was required to support clinical pharmacokinetic studies with **I**, the assay had to be precise to a coefficient of variation within 20% at the lower limit of quantification and within 15% throughout the required concentration range. Accuracy (±15% bias) and linearity had also to be demonstrated throughout the required range. In addition, the method had to have a high rate of sample throughput and be rugged enough to be able to support a large and very intensive development programme. These conditions were met because of the simplistic approach to sample preparation that yielded an extract which was clean enough for subsequent quantitative LC–MS–MS analysis. The recovery of **I** and **II** was not estimated but it is the authors experience that recovery is essentially complete using acetonitrile protein precipitation and that **II** serves as an almost ideal internal standard.

3.2. Separation and specificity

No significant interfering peaks that co-eluted with **I** or **II** were observed. These results were good evidence that the assay was selective for **I** over

endogenous compounds. The *R* and *S* forms of **I** both eluted together with a retention time of under 1.5 min and their MS–MS spectra were identical. No known metabolites have been observed to interfere with this assay. A typical chromatogram of human control plasma with **II** added at the lower limit of quantification (10 ng/ml) is shown in (Fig. 2).

3.3. Linearity, precision and accuracy

Mean back-calculated concentrations for three representative calibration curves for each of three analytical runs are within 6% of the nominal concentration (Table 1). Linearity was demonstrated over the range 10–5000 ng/ml for gemifloxacin extracted from 50 μl of plasma. This is an appropriate range over which to study the kinetics of **I** following repeated single daily doses between 20 and 800 mg to healthy subjects and to study the kinetics of I in patients (Fig. 3).

The within-run and between-run precision values for the assay were better than 11% at all concentrations studied (Table 2). Mean accuracy [calculated as the mean over 3 runs of the individual run

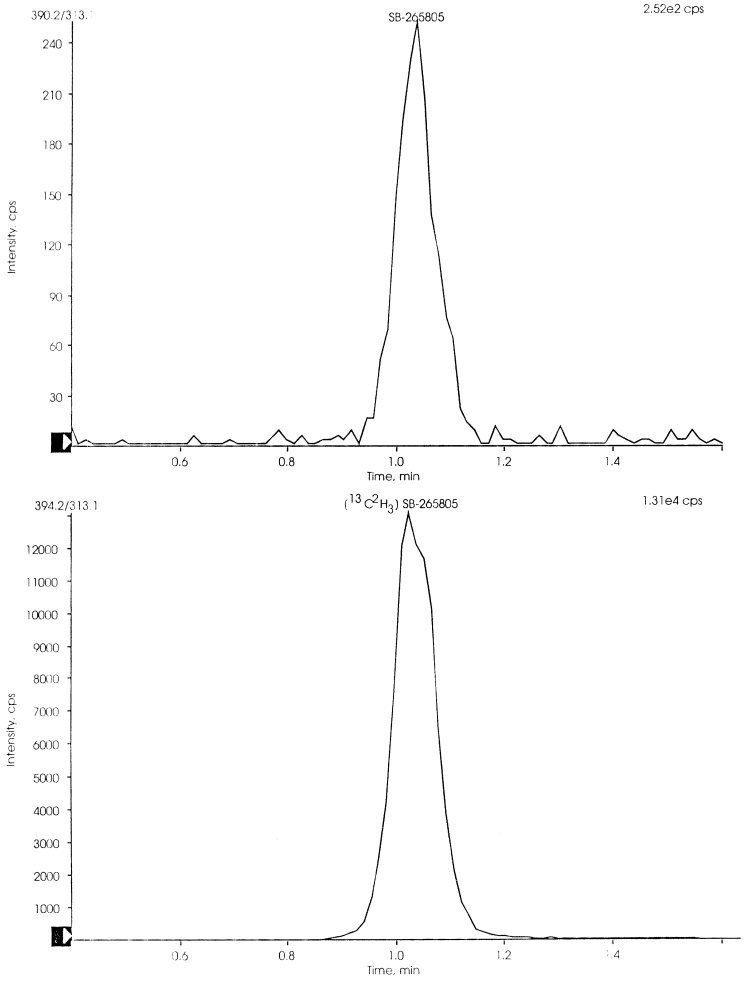


Fig. 2. Representative MRM chromatogram of **I** in human plasma at the LLQ of the assay (10 ng/ml).

Table 1
Back-calculated standard curve data for **I** in in human plasma[a]

Nominal	10	20	50	100	200	500	1000	2000	3000	4000	5000
Run 1	10.7	18.5	55.4	87.4	204.1	495.4	965.8	2055.2	3127.2	3957.8	5426.8
Run 2	9.8	20.9	48	102.2	219.2	490.5	1006.7	2061	2887.9	3875.9	4901.4
Run 3	9.9	20.6	49.6	94.2	201.4	501.4	978	2087.6	2895.6	4087.1	5223.9
Mean	10.14	19.98	51.01	94.57	208.22	495.79	983.47	2067.92	2970.24	3973.61	5184.06
SD	0.47	1.32	3.92	7.38	9.58	5.45	20.98	17.27	135.99	106.49	264.95
CV%	4.65	6.61	7.68	7.8	4.6	1.1	2.13	0.83	4.58	2.68	5.11
% of Nominal	101.43	99.92	102.02	94.57	104.11	99.16	98.35	103.4	99.01	99.34	103.68

[a] Concentrations of **I** (ng/ml).

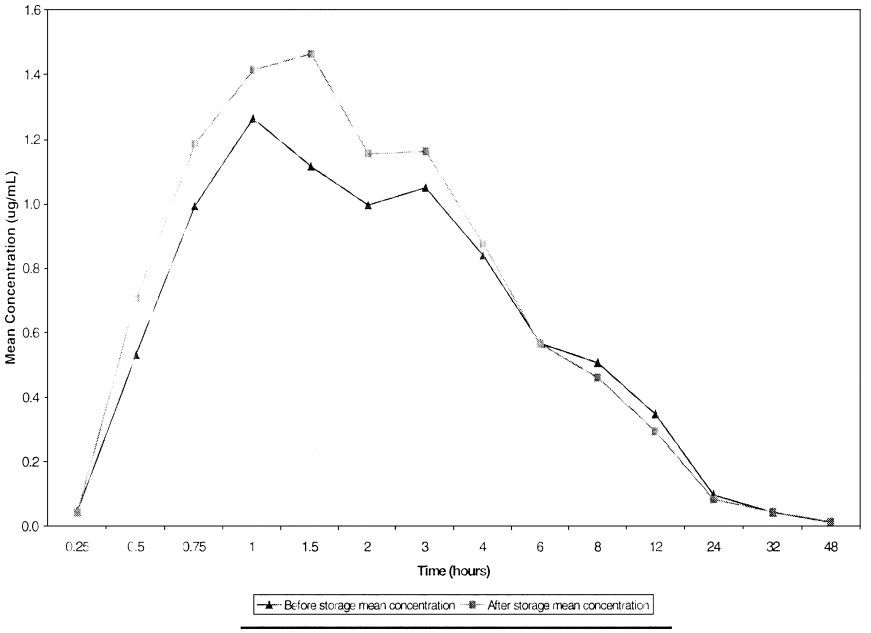


Fig. 3. Plasma concentrations of **I** in healthy human subjects after single administration of 320 mg **I**, measured (a) before and (b) after storage at −20°C for 6 months.

mean concentration by analysis to nominal concentration, and expressed as a percentage] was generally within ±7% of nominal at the concentrations studied (Table 2). The method was therefore shown to be sufficiently accurate and precise to support clinical pharmacokinetic studies with **I**.

Table 2
Precision and bias for **I** in human plasma

Nominal **I** (ng/ml)	10	20	1000	5000
Average within-run precision (%)	10.5	5.8	4.1	3.1
Between-run precision (%)	9.5	8	3.6	5.5
Average bias (%)	−0.8	5	7.3	6

3.4. Stability

Results obtained for six replicate validation samples containing **I** at each of two concentrations (20 ng/ml and 5000 ng/ml) which were put through three complete freeze–thaw cycles and those for six replicate samples prepared freshly at the same con-

Table 3
Freeze–thaw stability of **I**

Concentration **I** (ng/ml)	Fresh plasma concentration (ng/ml)	Freeze–thaw plasma concentration (ng/ml)	% Difference[a]
20	19.5	22.7	
	20.9	22	
	22.5	22.5	
	20.8	20.5	
	19.2	21.9	
	22.8	21.9	
Mean	21	21.9	4.6
5000	4950.7	5398.9	
	4950	5211.4	
	5103.3	4905	
	4853.6	5277	
	5025.9	5138.9	
	4984.6	5346.1	
Mean	4978	5212.9	4.7

[a] % Difference = ((Freeze–Thaw concentration − Fresh concentration)/Fresh concentration)*100.

centrations are shown in Table 3. The results for both sets of samples differed by less than 8.5%. Hence there was no evidence of instability of **I** in human plasma following three complete freeze–thaw cycles.

The analysis of post-dose samples stored at −20°C for up to 6 months showed no significant deterioration in the performance of the assay in that plasma concentration time profiles before and after storage were similar (Table 4, Fig. 3). Hence there was no evidence of instability of **I** in human plasma when stored at −20°C for 6 months.

4. Conclusion

An accurate and precise LC–ESI-MS–MS assay using protein precipitation sample pretreatment was developed for the assay of **I** in human plasma. There was no evidence of instability of **I** in human plasma following three complete freeze–thaw cycles and samples can safely be stored for 6 months at −20°C. The method proved very robust and was successfully applied to the analysis of clinical samples from patients dosed with **I**.

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Table 4
Plasma concentrations of **I** (μg/ml) in healthy human subjects after single administration of 320 mg **I**, measured (a) before and (b) after storage at −20°C for 6 months

Time (h):	0	0.25	0.5	0.75	1	1.5	2	3	4	6	8	12	24	32	48
Before storage															
Subject 1	NQ	NQ	0.1606	0.2745	0.8295	0.7129	0.9717	0.4343	0.5118	0.4044	0.3066	0.2678	0.0886	0.0320	NQ
Subject 2	NQ	0.0349	0.5940	1.0237	1.0988	1.3449	0.9895	1.8632	1.3477	0.8230	0.8670	0.5342	0.1487	0.0692	0.0116
Subject 4	NQ	0.0548	0.8362	1.6756	1.8596	1.2829	1.0227	0.8449	0.6528	0.4650	0.3418	0.2345	0.0493	0.0243	NQ
Mean 1	–	0.0449	0.5303	0.9913	1.2626	1.1136	0.9946	1.0475	0.8374	0.5641	0.5051	0.3455	0.0955	0.0418	0.0116
After storage															
Subject 1	NQ	0.0101	0.1363	0.2878	0.7074	1.0744	0.8881	0.5903	0.5916	0.3696	0.3111	0.2233	0.0701	0.0386	0.0102
Subject 2	NQ	0.0527	0.9849	1.3227	1.5487	1.9341	1.3971	1.8384	1.2670	0.7950	0.6840	0.3907	0.1188	0.0618	0.0150
Subject 4	NQ	0.0602	0.9932	1.9402	1.9809	1.3766	1.1718	1.0507	0.7636	0.5226	0.3823	0.2640	0.0574	0.0283	0.0124
Mean 2	–	0.0410	0.7048	1.1836	1.4123	1.4617	1.1523	1.1598	0.8741	0.5624	0.4591	0.2927	0.0821	0.0429	0.0125
Mean 2 − Mean 1	–	−0.0039	0.1745	0.1923	0.1497	0.3481	0.1577	0.1123	0.0366	−0.0017	−0.0460	−0.0528	−0.0134	0.0011	0.0009
% Difference[a]	–	−8.58	32.91	19.40	11.86	31.26	15.86	10.72	4.37	0.31	−9.11	−15.29	−14.06	2.55	8.05

[a] %Difference = ((Mean 2 − Mean 1)/Mean 1)*100.

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