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Determination of BAY 12-8039, a new 8-methoxyquinolone, in human body fluids by high-performance liquid chromatography with fluorescence detection using on-column focusing

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Abstract

A reversed-phase (RP) high-performance liquid chromatographic (HPLC) method with fluorescence detection allowing the sensitive and specific quantification of BAY 12-8039, a new antimicrobially active 8-methoxyquinolone, in biological fluids is described. The method is compared to a microbiological assay (bioassay) based on *B. subtilis* test strain with a limit of quantification of approximately 60 μg/l. Following dilution and centrifugation, plasma, saliva or urine supernatant is directly injected onto the HPLC system. Concentrations down to a limit of quantification of 2.5 μg/l can be quantified in plasma, saliva and urine. Data on recovery, accuracy and precision of the method throughout the whole working range as well as results on stability of the analyte are presented. The concentration data are correlated with results from the bioassay. BAY 12-8039 is stable in plasma after repeated freeze–thaw cycles and following storage at −20°C for at least 12 months. The results of HPLC measurements excellently agree with bioassay data indicating the relevance of the method as a tool in clinical development to answer pharmacokinetic questions related to antimicrobial activity. The method was applied to human plasma, saliva and urine from subjects after a single oral dose of 400 mg of BAY 12-8039.

Keywords: BAY 12-8039; 8-Methoxyquinolones

1. Introduction

BAY 12-8039, 1-cyclopropyl-7-(2,8-diazabicyclo[4.3.0]nonane) - 6 - fluoro-8-methoxy-1,4-dihydro-4-oxo-3-quinoline carboxylic acid hydrochloride (I, Fig. 1) is a new enantiomerically pure 8-methoxyquinolone with potent antimicrobial activities against both Gram-negative and Gram-positive bacteria and anaerobes. The compound was first synthesised by Petersen et al. [1]. In a series of

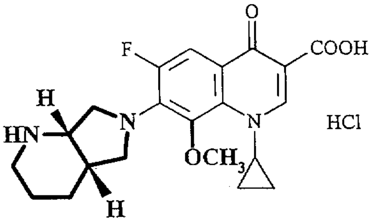

Fig. 1. Structure of BAY 12-8039 (I).

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preclinical in vivo and in vitro experiments an excellent activity against clinically relevant microorganisms has been found. Efficacy was shown in several animal models after oral as well as after intravenous administration of BAY 12-8039 [2–4]. Based on these results the clinical evaluation of this drug is currently being carried out.

In order to assess human pharmacokinetics of I a sensitive and specific analytical assay was required to quantify the unchanged drug following separation from metabolites in human body fluids [6–11]. Plasma concentrations ranging from 0.01 to >5 mg/l were regarded as clinically relevant and should be obtained with single oral doses of 50–400 mg.

Since I exhibits a major native fluorescence, liquid chromatographic separation on a RP-18 stationary phase with fluorescence detection was the obvious choice, first utilised in preclinical studies [5]. Unlike other quinolones that required on-line photothermal post-column derivatisation to yield fluorescent decomposition products feasible for detection [6,7], I shows direct native fluorescence. With the corresponding method concentrations down to a limit of quantification (LOQ) of 2.5 μg/l [5]. As the assay was optimised for plasma samples from various mammalian species and had to be adapted for the workup of human biofluids under clinical study conditions. The chromatographic parameters did not allow to separate the drug from the inactive metabolites and endogenous compounds characteristic for humans, which interfered with the analytes. Therefore, a new assay was developed and validated to quantify I in human body fluids (plasma, saliva, urine) by high-performance liquid chromatography (HPLC) with fluorescence detection using on-column focusing [12–14]. Relatively large sample volumes containing significant amounts (>60%) of acetonitrile due to sample preparation (deproteination) had to be injected to quantify the low concentrations typically prevalent during the terminal phase of the plasma concentration vs. time profiles in clinical studies. On the other hand, chromatography was very sensitive to the amounts of organic solvent injected onto the column and was prone to errors in the quantitative results due to band broadening, shifting retention times and interfering peaks originating from the matrix. The technique of on-column focusing that proved very useful to reliably quantify trace amounts of analytes in environmental samples (e.g., waste waters, [12–14]) was successfully used to minimise these influences. This enabled the high sample throughput required in clinical studies at a high quality standard and was the key prerequisite to respond in an adequate time frame to the demands of a fast track clinical development programme (e.g., decision on safety issues related to plasma concentrations during ongoing studies).

Microbiological assays were described in the literature for fluoroquinolones [15,16]. They bear several disadvantages such as the long analysis time and lack of discrimination between the drug and other antimicrobially active sample constituents due to the nature of this methodology. For ciprofloxacin a discrepancy was noted between HPLC analysis and bioassay of plasma and urine samples due to the presence of microbiologically active ciprofloxacin metabolites [16]. However, it was demonstrated that BAY 12-8039 exhibits identical antibacterial activity irrespective of whether the racemic mixture or the enantiomers as well as the metabolites were tested [1,2]. Therefore, a comparison of bioassay and HPLC was also performed, since the first method is a technique frequently used in clinical investigations of antimicrobial agents.

The new method was tested and applied to determine plasma and urine concentrations of I after administration of 200 mg of the drug to healthy male volunteers.

2. Experimental

2.1. Chemicals

The reagents used were of analytical grade. BAY 12-8039 (I), was obtained as a certified reference compound (Bayer, Wuppertal, Germany) for quantitative analysis. Since I is a hydrochloride (M_w 438.5) but found as betaine in biological fluids (M_w 400), all concentrations were calculated based on the betaine. Further human metabolites of I [M1 sulfocompound (II), M2 acyl-glucuronide (III)] were provided as solutions by Dr. A. Kern (Department of Preclinical Metabolism and Isotope Chemistry, Bayer). Their structure was confirmed by NMR (^{1}H, ^{13}C) and mass spectroscopy (MS). Ofloxacin was purchased from Sigma (Munich, Germany) as a certified standard. HPLC solvents were purchased from J.T. Baker (Gross-Gerau, Germany). Sodium hydroxide (analytical-reagent grade), phosphoric acid 85% and sodium dihydrogenphosphate were obtained from Merck (Darmstadt, Germany). Hydrochloric acid (36.5%) was purchased from Riedel-de Haen (Seelze, Germany) and tetrabutyl ammonium hydrogen sulphate from Fluka (Neu-Ulm, Germany). Water was purified by the Milli-Q system (Millipore Waters, Eschborn, Germany). Blank plasma was either obtained from fasted healthy volunteers or purchased from the German Red Cross (DRK, Hagen, Germany). Nitrogen 5.0 was obtained from Messer Griessheim (Griessheim, Germany).

For microbiological experiments Iso Sensitest Agar and *B. subtilis* pore suspension No. 2 Agar (Difco-Laboratories, Augsburg, Germany) were used.

2.2. Instrumentation and operating conditions

2.2.1. Chromatography

A HP 1090 instrument (Hewlett-Packard, Waldbronn, Germany) was used equipped with a fluorescence detector HP 1046A (excitation at 296 nm, emission at 504 nm for all analytes). The autosampler temperature was kept at 8°C using a Haake D8 water bath (Haake, Karlsruhe, Germany). A Nucleosil 100 C_{18} (5 μm particle size, 250×4.6 mm I.D.) capillary column preceded by a guard column (17×4.6 mm I.D.) of the same material (M.u.W. Chromatografietechnik, Berlin, Germany) was used for separation. The column oven temperature was set to 50°C.

The mobile phase consisted of an aqueous solution of 0.01 mol/l tetrabutyl ammonium sulphate and 0.05 mol/l sodium dihydrogenephosphate (a, pH=3.0) and acetonitrile (b). The flow-rate was set at 1.1 ml/min for the separation and at 1.3 ml/min for the wash gradient (11–14 min, see Table 1). The gradient employed for elution of I from plasma is given in Table 1. For analysis of urine and saliva samples chromatography was slightly modified to achieve separation from endogenous compounds [elution of I using 20% b (urine) and 23% b (saliva) for 9 min].

Table 1
Gradient elution conditions for determination of I in human plasma

Time (min)	Flow-rate (ml/min)	Eluent composition (% acetonitrile)
0	1.0	5.0
1.0	1.0	25.0
5.0	1.0	25.0
5.1	1.0	70.0
6.5	1.0	70.0
6.6	1.3	70.0
10.3	1.3	5.0
10.4	1.0	5.0

In each time interval the gradients were linear.

2.2.2. Bioassay

The conventional cup-plate agar diffusion method was used to quantify I. For this purpose 150 ml *B. subtilis* spore suspension were added to 75 ml Iso Sensitest Agar and poured into diffusion plates. After solidification of the agar holes of 8 mm in diameter were punched into the agar; the distance between the holes was 35 mm. Every diffusion plate contained 18 holes. Each hole was filled with 100 ml of the sample and standard, respectively. The range of standard concentrations was comprised within 2 mg/l to 0.06 mg/l (1:1 dilution) so that six reference concentrations per plate and twelve samples with concentrations to be determined were analysed per plate. Plates were incubated at 37°C for 18 h.

2.3. Sample preparation

For microbiological experiments the plasma and urine samples were either used without any workup procedure or following dilution, with blank plasma, respectively, urine. For chromatography the subsequent sample preparation procedures were used.

2.3.1. Plasma

A 250-μl aliquot of plasma was placed in a glass tube and 750 μl dilution solvent consisting of acetonitrile–0.1 mol/l aqueous phosphoric acid (9:1, v/v) added. The resulting suspension was vortexed for 10 s on a laboratory vortex followed by centrifugation (10 min, 1500 *g*) while cooling (8°C). The supernatant was directly injected onto the column.

2.3.2. Urine

Urine samples were diluted in brown glass vials using 0.1 mol/l dihydrogenephosphate solution pH 4. A dilution factor of 1:20 was usually applied to yield solutions with concentrations feasible for quantitative analysis.

2.3.3. Saliva

Saliva samples were collected with cotton wool swabs, which had to be chewed for 1 min by the volunteers. Saliva was recovered from the swabs by centrifugation (5 min, 1000 *g*) while cooling (8°C). After this procedure the swabs were treated twice with 500 μl of acetonitrile–0.1 mol/l aqueous phosphate buffer solution, pH 4–0.1 mol/l phosphoric acid (45:50:5, v/v/v). Extraction was achieved by centrifugation of the treated wool swabs (5 min, 1000 *g*, 8°C). The extracts were combined and 50 μl of internal standard (I.S.) solution containing 5 mg/l ofloxacin dissolved in 0.1 mol/l dihydrogenphosphate pH 4 solution was added. After homogenisation the solution was injected onto the column.

2.4. Calibration and quality control

2.4.1. Chromatography

For quantification of unknown plasma and urine concentrations the external standard method was applied. Calibration (CAL) samples were obtained by spiking aliquots of working solutions (phosphate buffer, pH 4) into blank urine or plasma, respectively to produce at least seven concentrations in the range of 2.5–1000 μg/l (urine) and in the range of 5–1500 μg/l (plasma).

For quantification of unknown saliva concentrations the I.S. method was applied. the I.S. was ofloxacin which was completely separated under the chromatographic conditions chosen. CAL samples were obtained by spiking aliquots of methanol working solutions into aqueous phosphate buffer pH 4 to produce at least seven concentrations in the range of 10–1000 μg/l.

Quality control (QC) samples were prepared at three concentration levels covering the whole range expected for the unknown samples. QC samples were stored together with samples of the respective study at −20°C. Two replicates of each QC sample were analysed together with calibration and unknown samples in the same analytical sequence. CAL samples were freshly prepared prior to each sequence. Storage duration was limited to a maximum of 12 months. CAL, QC and unknown samples were processed as described in Section 2.2.

Calibration curves were obtained by plotting the peak heights relative to the I.S. peak height (saliva) or the absolute peak heights (plasma, urine) of I against the concentrations added. Linear regression was performed using $1/y^2$ as a weighting factor (Concalc software, Department of Clinical Pharmacokinetics, Bayer, Wuppertal, Germany). For unknown or QC samples the concentrations of I were calculated using the regression function.

2.4.2. Bioassay

For quantification of unknown plasma and urine concentrations the external standard method was applied. CAL samples were obtained by spiking aliquots of aqueous working solutions of I into blank urine or plasma, respectively to produce six concentrations per agar plate in the range of 0.06–2.0 mg/l (urine and plasma). Higher concentrations were covered by appropriate dilution of the sample using blank matrix. For every plate a linear calibration curve was plotted from the zones of inhibition which were read optically. The sample concentrations were calculated using the established calibration function of that plate.

2.5. Assay validation

Validation was performed according to the guidelines for development of bioanalytical assays in human biomatrices [17–22]. Short term stability was investigated in whole blood, plasma, urine and in the solutions for injection. Within the corresponding experimental programme, investigations on light and freeze–thaw stability (plasma only) were performed. For the investigation of light stability, the corresponding solutions were exposed to sunlight and yellow light for a period several days under different conditions (see Table 2). Freeze–thaw stability was assessed by analysing plasma samples repetitively thawed and frozen over a range of five cycles. Samples which were only thawed once prior to sample preparation were used as reference.

Long term stability in plasma and urine (storage at

Table 2
Short term stability of I in whole blood, plasma and organic solvent under different conditions

Storage conditions	Recovery of I (%)		
	Whole blood	Plasma	Stock solution
Duration	4 h	75 h	22 days
Room temperature/white glass/daylight	99.0	90.0	39.8
Room temperature/brown glass[a], yellow light[b]	100.02[b]	98.0[b]	97.1[a]
Reference I (4°C, refrigerator)	98.0	102.0	102.4
Reference II (37°C, dark)	n.d.[c]	n.d.	99.3

[c] n.d. = Not determined.

−20°C) was assessed by repeated analysis of blank samples spiked with concentrations of 100 and 200 μg/l over a range of 12 months.

For plasma six spiked samples at concentrations of 2.5, 5, 10, 100 and 1000 μg/l were analysed within one day to assess intra-day variability. Inter-day variability was evaluated by assaying three sets of six spiked plasma samples (see above for concentration ranges) on three consecutive days.

For urine the validation procedure described above was also applied using solutions of 50, 100, 100, 200, 2000 and 10 000 μg/l. To validate saliva assay six spiked samples at concentrations of 10, 25, 50 and 100 μg/l were analysed to determine the intra-day variability.

Specificity of the method was assessed by determination of the relative retention times of peaks from endogenous compounds and relevant known metabolites (M1, M2) of I. For this purpose urine samples from preclinical experiments with known concentrations of the corresponding metabolites were injected onto the HPLC system.

During routine analysis of study samples QC samples served as means to evaluate inter-day precision and accuracy of the method.

HPLC and bioassay results derived from a clinical study (see Section 2.6) were compared.

2.6. Application

Compound I was determined in plasma and urine in six volunteers who received 200 mg of I as a single dose in a clinical phase I study approved by an ethics committee [23]. The trials were conducted according to the German Medicines Act, the Helsinki Declaration and Good Clinical Practice rules. All subjects gave written informed consent prior to participation. Blood samples were collected at 0, 0.25, 0.5, 0.75, 1, 1.5, 2,3, 4, 6, 8, 12, 16 and 24 h following administration and urine was collected up to 24 h. Within 20 min after blood draw, plasma samples were prepared as described in Section 2.2.

3. Results

3.1. Chromatography

Compound I was readily separated from matrix constituents with reversed-phase (RP) chromatography (C_{18} stationary phase) and gradient elution with an aqueous phosphate buffer containing tetrabutyl ammonium sulphate and acetonitrile. Methanol was also tested as organic solvent instead of acetonitrile, but resulted in a decreased sensitivity due to additional band broadening and worse resolutions. A gradient profile as outlined in Table 1 was employed. On-column focusing step [12–14] permitted to concentrate the sample on top of the column and to strip off the relatively high amount of organic solvent needed for sample preparation. Elution of the analyte then was performed under isocratic conditions in two steps. Under the conditions chosen, interference from matrix components or from possible relevant metabolites of I known from preclinical experiments could be excluded. Simple sample preparation by dilution including a protein precipitation step was sufficient.

3.2. Validation

3.2.1. Sample preparation

The light stability of I was investigated in plasma and organic solvent (Table 2). Stability was adequate in whole blood and plasma to allow easy sample preparation. I did not show any significant decomposition when stored for 22 days under different conditions as long as impact of direct sunlight was avoided (brown glass).

Long term storage stability was assessed for I in plasma under the standard storage conditions of −20°C. The results are summarised in Table 3. No significant loss of substance due to instability was noticed in these experiments. The experiments on freeze–thaw stability covered a stability of I up to five freeze–thaw cycles.

3.2.2. Additional validation parameters

Fig. 2 depicts representative chromatograms of a volunteer receiving a 200 mg oral dose of I. The chromatograms of the blank matrix samples did not show a significantly altered pattern of interfering compounds. Although the analytes were diluted, the sensitivity of the method was appropriate to fully describe the concentration vs. time profiles; hence, a tedious and time consuming extraction method could be avoided for plasma and urine samples. Sample preparation by diluting the biofluid with a solvent that includes extraction of the analytes and precipitation of proteins offers a simple straight forward work up procedure for routine analysis. For the saliva sample a 3-fold extraction was needed to achieve adequate recovery of I from the wool swabs. Recovery was 93±5% throughout the whole concentration range investigated.

In order to prove the ruggedness of the chromatography during routine plasma samples were assayed with and without the use of the on-column focusing gradient. In the latter case integration of the peaks was biased by coeluting peaks originating from the matrix especially when injection volumes of more than 50 μl were injected. Additionally, peak broadening and shift of retention times were observed after multiple injections.

Table 3
Long term stability of I plasma at −20°C over 12 months using samples spiked with a nominal concentration of 100 μg/l

Storage duration (months)	Recovery of I (%)	Container material
12	104	Glass
8	96	Polystyrene
9	104	Polypropylene

Calibration was done by plotting concentration vs. absolute respectively relative (saliva) peak-height and linear curve fitting with a $1/y^2$ weighting function. Fig. 3 presents an example of typical calibration curves. For plasma and urine it was found that due to the simple sample preparation use of an I.S. was not necessary to generate valid results. Relative residuals were comparable when calibration was performed using an I.S. For saliva, however, extraction from the wool swabs introduced a high variability caused by variable recovery. Adding ofloxacin as I.S. to the solution for injection significantly decreased relative residuals, because the volumes of extraction solvent recovered could be determined more accurately.

The results of a formal validation are presented in Table 4 (plasma, urine). The data of the validation for saliva are summarised in Table 5. Precision was always below 10% for all matrices investigated. Accuracy was between 104–109% for plasma and urine, for saliva it ranged between 92 and 97%. Based on these findings a LOQ of 2.5 μg/l can be regarded as meaningful, for lower concentrations accuracy will become unacceptable due to interference caused by signals derived from matrix constituents (acceptance criteria for LOQ: precision ±20%, accuracy 85–115% of theory [22]). The limit of detection (LOD) was estimated as 1 μg/l based on a signal-to-noise ratio of 3:1.

3.2.3. Method ruggedness

HPLC columns were used for more than 1000 injections over a period of half a year without significant decrease of separation efficiency or peak shape. Data on the long term performance were generated routinely during analysis of unknown samples during a period of six months by analysing QC samples (at least two replicates at three concentration levels per analytical sequence). Fig. 4 shows an overview of the results which confirmed that the specified limits for precision and accuracy can easily by kept (see previous Section 3.2.2, [17–22]).

3.2.4. Correlation of data from chromatography and bioassay

In addition to the validation presented above the results of the HPLC were compared to data gener-

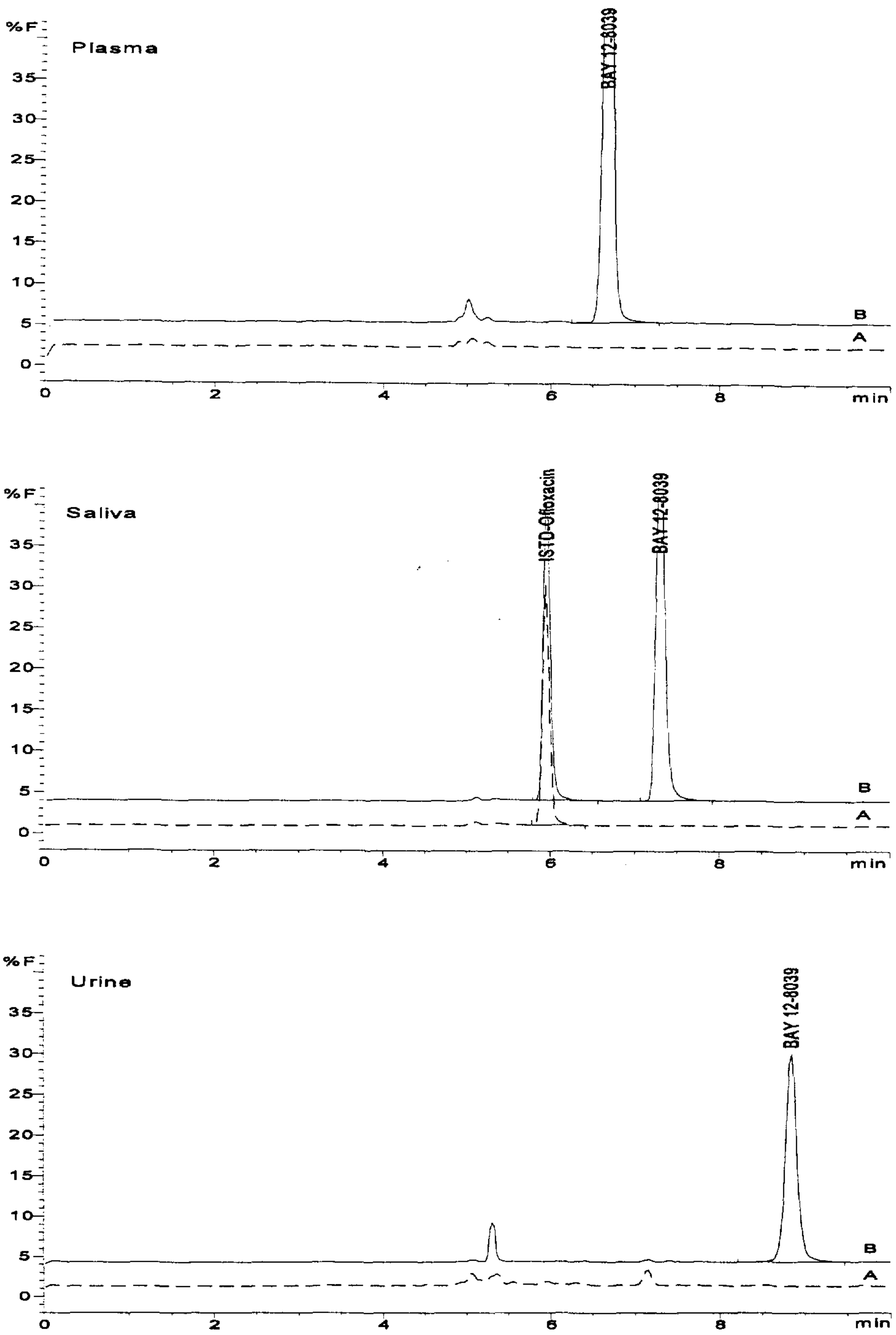

Fig. 2. Chromatograms of plasma, saliva [before (A) and 1.5 h after administration (B)] and urine [before (A) and fraction collected 4 h after administration (B)] of a volunteer receiving a 200 mg oral dose. The signals for I correspond to concentrations of 1.5 mg/l (plasma), 1.0 mg/l (saliva) 0.3 mg/l (urine).

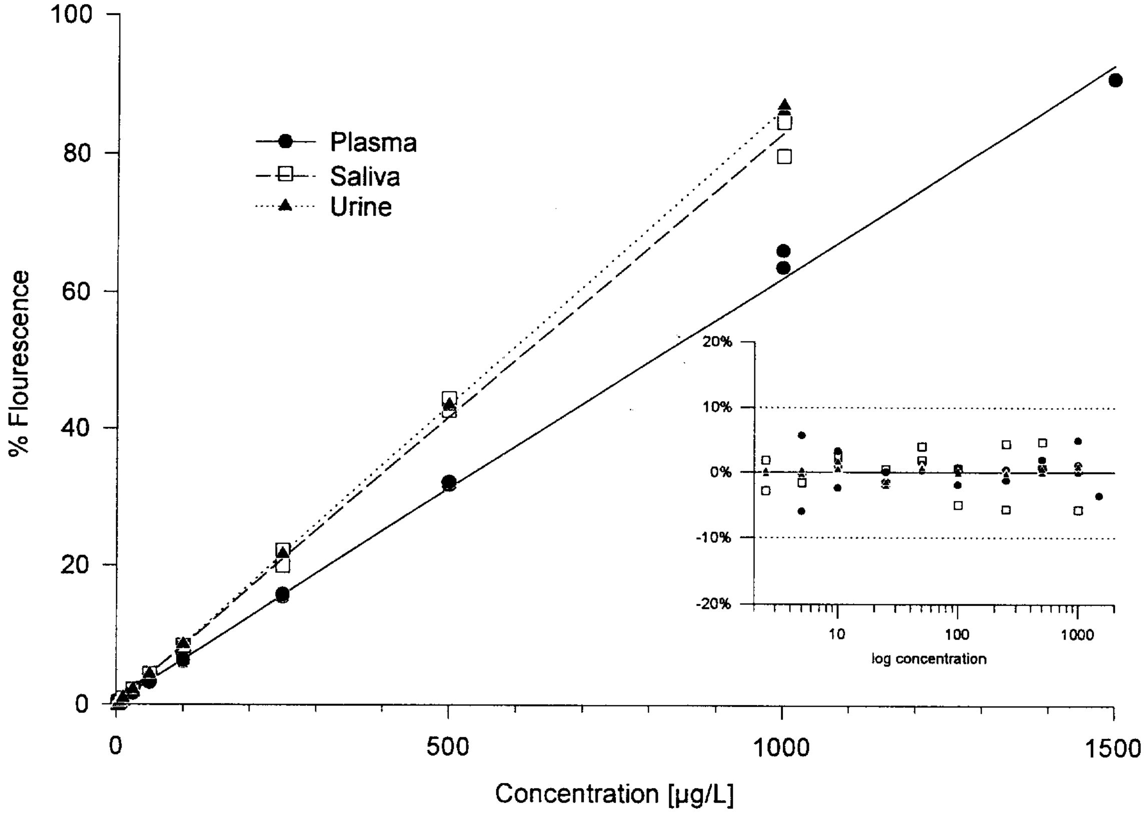

Fig. 3. Typical calibration curves for I and residuals of back-calculated concentrations.

ated with the bioassay. Fig. 5 presents the results of the evaluation. An excellent correlation with a slope of 1.05 and a low variability was found ($r^2=0.993$) indicating that quantification of I by HPLC provides adequate data on antimicrobial activity of the drug. Since the bioassay is not able to discriminate between I and metabolites and matrix constituents as it measures antimicrobial activity in total, the conclusion can be drawn that there are no pharmacologically active metabolites in human body fluids. Thus the HPLC assay provides the advantage of higher selectivity and better sensitivity (LOQ: 2.5 µg/l vs. 60 µg/l, see Section 2.4) and is therefore applicable more universally in clinical development.

4. Application

The assays described above were used in pharmacokinetic studies to analyse plasma, saliva and urine samples obtained after oral administration of I to more than 100 healthy volunteers [23–30]. Interindividual variability of the pharmacokinetics was low in these studies. Typical results for one subject are illustrated in Fig. 6 [23].

I was rapidly absorbed and distributed into extravascular compartments following oral administration. While maximal concentrations (C_{max}) of approximately 1.5 mg/l were reached in plasma 2.5 h following drug ingestion, higher concentrations (2.2 mg/l) were detected in saliva 1.5 h post application. Concentrations of I declined in a biphasic manner with a terminal half-life of approx. 15 h, resulting in quantifiable data over at least 72 h following drug administration. Urinary recovery in this time period amounted to 21% of the dose.

Kinetics of saliva were investigated to assess the drugs ability of penetrating into extravascular body compartments, where usually the target of infection is located. Though easier to collect than plasma

Table 4
Inter- and intra-day precision and accuracy of the determination of I in human plasma and urine

	Nominal concentration of I (μg/l)									
	Plasma calibration range 2.5–1000 μg/l					Urine calibration range 50–10 000 μg/l				
	2.5	5	10	100	1000	50	100	200	2000	10 000
Concentration found (μg/l, arithm. mean value)										
Day 1 ($n=6$)	2.91	5.56	10.94	104.8	1051	53.8	109.8	211.4	2098	10 399
Day 2 ($n=6$)	2.85	5.28	10.66	105.7	1054	55.3	107.7	209.7	2059	10 197
Day 3 ($n=6$)	2.83	5.39	10.79	102.4	1040	55.0	108.9	209.2	2064	10 230
Inter-day ($n=18$)	2.86	5.41	10.80	104.3	1048	54.7	108.8	210.1	2074	10 275
Accuracy (%, arithm. mean value)										
Day 1 ($n=6$)	116.3	111.2	109.4	104.8	105.0	107.5	109.8	105.7	104.9	104.0
Day 2 ($n=6$)	114.2	105.6	106.6	105.7	105.4	110.5	107.7	104.8	103.0	102.0
Day 3 ($n=6$)	113.2	107.7	107.9	102.4	104.0	110.1	108.9	104.6	103.2	102.3
Inter-day ($n=18$)	114.6	108.2	108.0	104.3	104.8	109.4	108.8	105.0	103.7	102.8
Precision (%, arithm. mean value)										
Day 1 ($n=6$)	4.7	3.0	4.6	1.7	2.1	4.5	3.6	2.0	0.8	0.8
Day 2 ($n=6$)	6.0	2.5	3.4	1.2	1.8	4.6	2.5	1.4	1.4	1.8
Day 3 ($n=6$)	2.6	3.0	5.1	2.4	2.6	1.9	1.4	1.5	1.1	1.3
Inter-day ($n=18$)	4.5	3.4	4.3	2.2	2.1	3.9	2.6	1.6	1.4	1.5

(noninvasive method) the use of saliva is prone to misinterpretation. Besides artefacts (e.g., residues of the drug in the mouth during oral intake) interpretation of data has to be done carefully taking into regard physiological mechanisms (e.g., active secretion from the glands, degree of protein binding...). Nevertheless, analytical results on saliva allow the conclusion that the drug is distributed almost instantaneously to spaces other than the plasma, a very important prerequisite to take advantage of its bactericidal activity.

Table 5
Intra-day precision and accuracy of the determination of I in human saliva

	Nominal concentration of I (μg/l)			
	Saliva calibration range 10–100 μg/l			
	10	25	50	100
Concentration found (μg/l, arithmetic mean value)				
($n=6$)	9.65	23.1	45.8	92.3
Accuracy (%, arithmetic mean value)				
($n=6$)	96.5	92.4	91.6	92.3
Precision (%, arithmetic mean value)				
($n=6$)	9.2	2.0	2.7	3.1

5. Discussion and conclusions

Based on the preclinical experience with bioanalysis of I in several mammalian species [5] the development of a HPLC assay with fluorescence detection applicable in a clinical setting was straightforward. Although sample preparation includes dilution of the analyte solution, RP chromatography with fluorescence detection was sensitive enough to provide adequate concentrations in the ranges relevant for bioanalysis. The technique of on-column focusing contributed to the ruggedness of the chromatography allowing sample preparation steps with large portions of organic solvents. During the injection of variable analyte volumes containing acetonitrile in concentrations much higher than in the mobile phase no deterioration of chromatographic performance was observed even after analysis of large sample batches. Peak broadening, interferences and shift of retention times were inevitable when the focusing step was skipped in validation experiments. Thus,

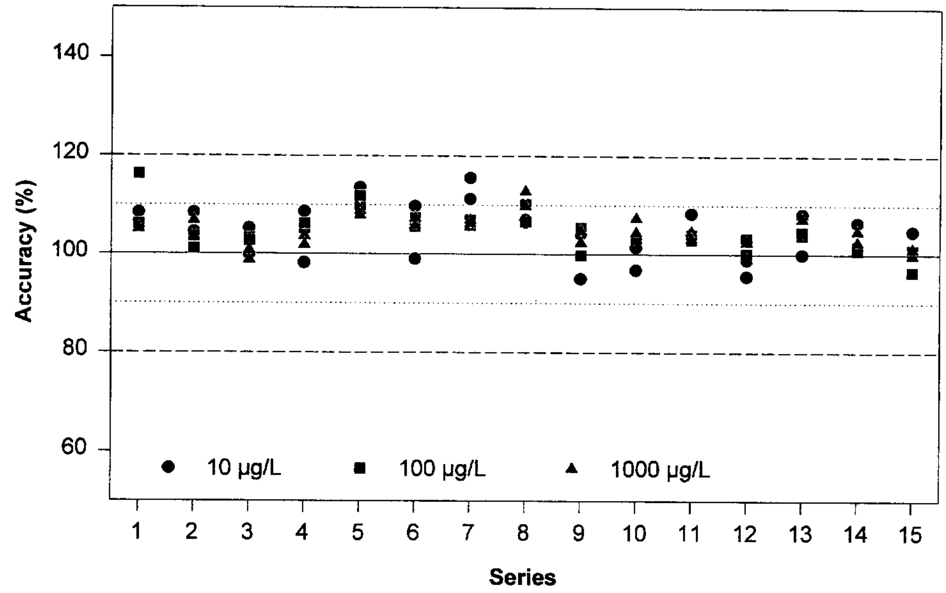

Fig. 4. Quality control chart of I in plasma confirming the performance specifications of the assay during 6 months [accuracy calculated relative to the nominal (spiked) concentrations].

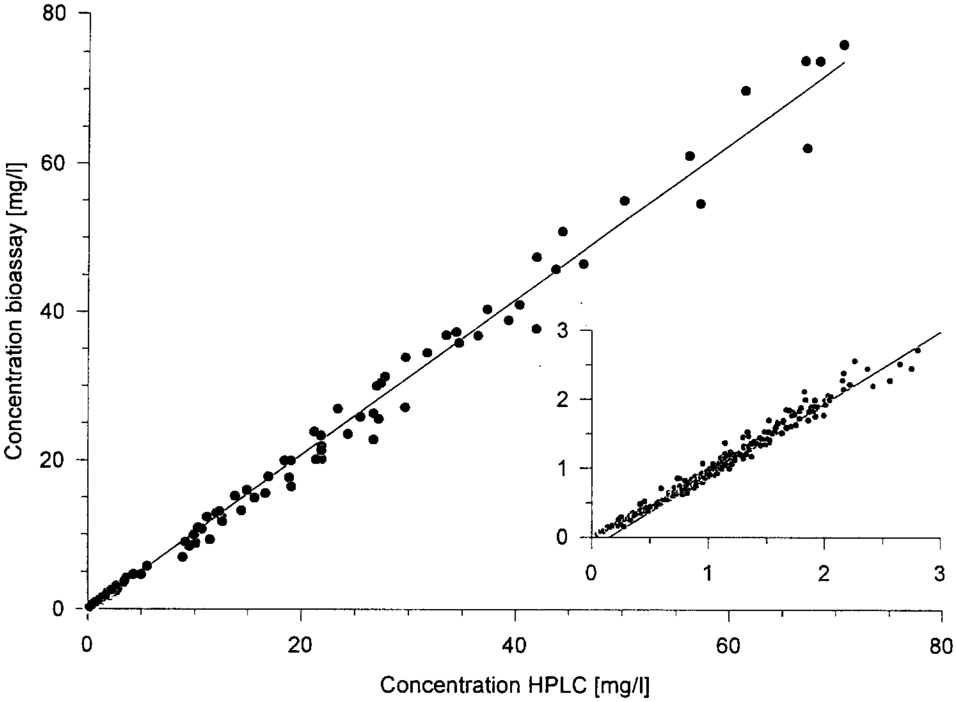

Fig. 5. Correlation of concentration of I in plasma and urine obtained with HPLC and bioassay ($r^2 = 0.993$, slope 1.05, intercept = −0.16).

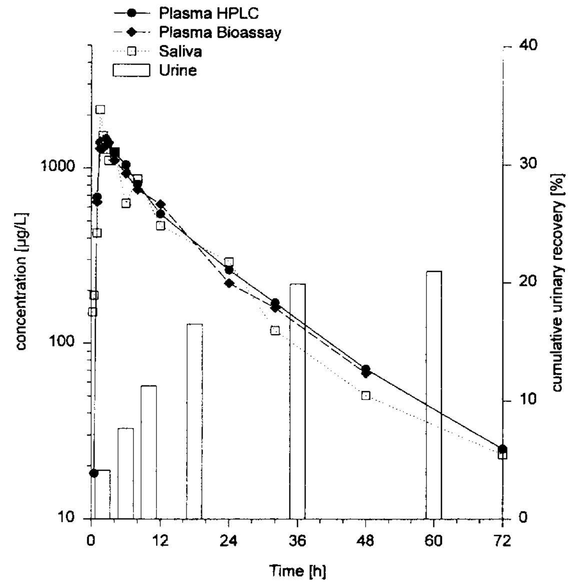

Fig. 6. Plasma and saliva concentration–time profiles and urinary recovery of I from a healthy male volunteer receiving a 200 mg oral dose of BAY 12-8039 determined by HPLC and bioassay.

time consuming optimisation of chromatographic conditions during analysis of samples with variable concentrations were avoided. Chromatographic conditions allowed short analysis times with a long column shelf life, which were the key factors for the high sample throughput realised with this method. The method can easily be used in a clinical setting.

Formal validation according to the recommendations of the EU (CPMP guidelines) and FDA [17–22] proved that the assay is appropriate for use in clinical studies. Precision and accuracy of the bioanalytical results were below 10% of theory in the whole working range of the method. The LOQ was adequate to enable a full description of the human pharmacokinetics.

Sample stability seems to acceptable over the range of long term storage (−20°C) investigated. No significant degradation of I or other interference due to a possible contribution of matrix constituents was detected in the stability samples. Working solutions of I can be stored for 21 days when protected against direct sunlight (brown glass).

HPLC separates the analyte from other matrix constituents, detection is based on its physico-chemical properties. Contrarily, the bioassay uses total antimicrobial activity of the sample without discriminating between analyte and other constituents. Therefore, by comparing results from both methods the question could be answered whether HPLC quantification of I alone is sufficient to provide meaningful information for clinical development with respect to antimicrobial efficacy. The excellent correlation proves that the method is even superior to the bioassay with a better selectivity and sensitivity (lower LOQ) with a broader range of possible applications.

All the factors discussed above lead to the conclusion that the method presented allows fast analysis of BAY 12-8039 in human biofluids. It is providing data with adequate quality for secondary (e.g., pharmacokinetic) analysis and is currently being used in clinical studies [22–30].

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