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Determination of moxifloxacin in growth media by high-performance liquid chromatography

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Abstract

A direct injection high-performance liquid chromatographic method with column switching has been developed to determine moxifloxacin in Mueller–Hinton broth. A LiChrocart 4-4 pre-column filled with a LiChrospher 100 RP 18, 5 μm and a 150×4.6 mm I.D. column packed with a Supelcozil ABZ+ Plus were used and led to a retention time of 5.70 min. Fluorescence detection allowed one to reach a quantification limit of 0.05 μg/ml with a 100-μl sample size. The standard curves were linear from 0.05 to 3.2 μg/ml. Intra- and inter-day imprecisions within the linearity range were ≤4.76 and ≤5.75%, respectively. The mean relative errors for the same day and the day-to-day inaccuracies ranged from −2.93 to +4.50% and from −1.10 to +6.00%, respectively. The method was demonstrated to be useful for pharmacokinetic–pharmacodynamic studies of moxifloxacin in an in vitro model.

Keywords: Mueller–Hinton broth; Moxifloxacin

1. Introduction

Moxifloxacin (BAY 12-8039) is a new fluoroquinolone with a broad spectrum of activity encompassing gram-negative and gram-positive bacteria [1]. The mechanism of acquired resistance to the quinolones is consistent among currently available drugs in this class and is expected to be similar for new and developmental agents as well. In the laboratory, selection of bacterial resistance depends on the particular quinolone, its concentration, and the species of bacterium involved [2]. Several in vitro pharmacokinetic–pharmacodynamic (PK–PD) models were developed to evaluate the efficacy of antimicrobial agents and possible emergence of resistance. These models allows for comparisons of antimicrobial efficacy of newly developed antibiotics with the efficacy of older drugs. Mutation rates in the presence of clinically relevant concentration time curves may be compared for various drugs and regimens [3]. Such experiments are time-consuming because of the need to examine a large number of

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samples. Therefore, rapid analytical methods are required for labor-saving.

High-performance liquid chromatography (HPLC) methods involving deproteinization of human body fluids samples followed by centrifugation [4,5] or solid-phase extraction [6] and a capillary electrophoresis technique [7] were proposed for moxifloxacin determination. A new assay using column switching was developed to allow on-line HPLC by direct injection of Mueller–Hinton broth (M.–H.B.) sample, shortening greatly the sample processing. The on-line method consists of a first step of trapping the analyte in the pre-column (PC) and elution of biological matrix to the waste. In a second step, the analyte is transferred to the analytical column (AC) and separation occurs.

2. Experimental

2.1. Chemicals

Moxifloxacin hydrochloride was a gift from Bayer (Puteaux, France). Compounds obtained from pharmaceutical companies and used for analytical interference studies were: amikacine, cefepim (Bristol-Myers Squibb, Paris, France), tobramycine (Lilly, Saint Cloud, France), gentamicine (Dakota, Paris, France), cefotaxime, cefpirome (Roussel, Paris, France), ceftazidime (Glaxo Wellcome, Marly-le-Roi, France) and ceftriaxone (Roche, Neuilly-sur-Seine, France). Analytical-grade (Normapur) dipotassium hydrogenphosphate, potassium dihydrogenphosphate, 85% orthophosphoric acid were provided by Prolabo (Fontenay-sous-Bois, France). Chromanorm-grade acetonitrile and methanol were also purchased from Prolabo. Tetrabutylammonium bromide (TBAmBr) was provided by Sigma (Saint Quentin Fallavier, France). HPLC-grade water was obtained with a Milli-Q water purification unit, Millipore (Saint Quentin Yvelynes, France).

2.2. Chromatographic system and conditions

A schematic diagram of the column switching system is given in Fig. 1. A HP 1050 liquid chromatograph (Hewlett-Packard, Evry, France) composed of an isocratic pump (pump 1) and an autosampler and a HPLC pump 420 Kontron (pump 2) (Kontron Instruments, Milan, Italy) delivered mobile phases 1 and 2, respectively. An electric-actuated switching system with a six-port Rheodyne valve was controlled by the external time events of the HP 1050 pump. Sample injection starts its programmable time-relay.

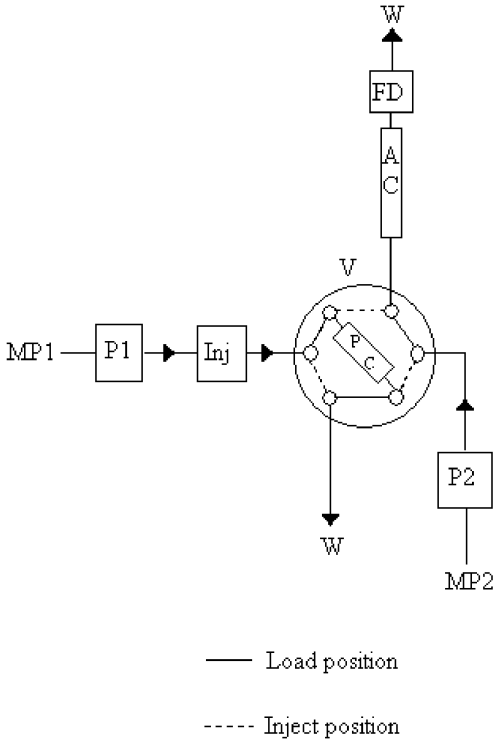

Fig. 1. Schematic diagram of the column switching system for the assay of moxifloxacin. MP1 and MP2, mobile phase 1 and 2; P1 and P2, pumps 1 and 2; Inj, injector; PC, pre-column; AC, analytical column; V, six-port switching valve; FD, fluorescence detector; W, waste.

The PC was a LiChrocart 4-4 filled with LiChrospher 100 RP-18, 5 μm (Merck–Clevenot, Nogent-sur-Marne, France). The AC was a 150×4.6 mm I.D. stainless steel column filled with Supelcosil ABZ+ Plus, 5 μm (Supelco, Saint Quentin Fallavier, France). An Upchurch 5 μm pre-column filter (Cluzeau Info Labo, Sainte-Foy-La-Grande, France) was inserted between the PC and the AC.

Detection was performed with a Spectroflow 980 fluorimetric detector (ABI Analytical/Kratos Division, Fontenay-sous-Bois, France) set at 296 nm

excitation and equipped with a 550 nm emission cut-off filter. A recorder output range of 0.010 μA full scale was used. Data acquisition was performed with a HP 3396 A integrator (Hewlett-Packard).

Mobile phase 1 consisted of 10 m*M* K_2HPO_4 buffer adjusted to pH 5.4 with orthophosphoric acid-methanol (97:3, v/v). Mobile phase 2 was a mixture of 10 m*M* KH_2PO_4 buffer, pH 2.5 containing 2 m*M* TBAmBr-acetonitrile (85:15, v/v). The pH of the buffer was adjusted to 2.5 with orthophosphoric acid. The flow-rates were 1.00 ml/min and 1.25 ml/min for mobile phases 1 and 2, respectively.

Injection volume was 100 μl and broth samples from PK–PD in vitro models, containing bacteria in suspension, underwent a 5-min centrifugation at 2000 *g* and the supernatant was injected.

The total sequence of automated sample analysis required 12 min and included the following three steps:

Step I (0–2 min, valve in load position): injection of the sample and transfer of the analyte to the PC where it was retained. The PC was purged with the mobile phase 1 and unwanted components of the broth were directly vented to waste.

Step II (2–11 min, valve in injection position): mobile phase 2 with high elution strength allowed analyte transfer in backflush mode from PC to the AC where it was separated for quantification.

Step III (11–12 min, valve in load position): the PC was equilibrated with mobile phase 1 for the next injection.

2.3. Stock solutions and spiked broth samples

Moxifloxacin was made up as 320 μg and 200 μg free base per ml stock solutions in water. The 320 μg/ml solution was diluted with blank M.–H.B. to obtain calibration samples at 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 μg/ml. Quality control samples containing 0.05, 0.25, 0.50, 1 and 2 μg/ml were prepared by successive dilution of the 200 μg/ml stock solution with the same blank matrix. Portions of 200 μl were transferred to Eppendorf tubes and stored at −20°C.

For analytical interference studies, stock solutions of reference compounds were prepared by dissolving 10.0 mg in 10 ml water and stored at −20°C. Working solutions of 62.5 μg/ml were obtained by successive dilution of the stock solution just before injection.

2.4. Calibration and calculations

The concentration of unknown samples was calculated from a linear calibration curve. This curve was obtained by computing a nonweighted least-squares regression of the peak area *y* versus moxifloxacin concentration *x* from seven standard samples: 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 μg/ml. A mean regression curve was determined by assaying the seven standards in six separate assay runs within 2 weeks. The slopes of log–log plots of the concentration versus the peak area were calculated to evaluate linearity.

2.5. Recovery

The recovery was established for M.–H.B. by six analyses of five moxifloxacin concentrations: 0.05, 0.25, 0.50, 1, and 2 μg/ml. The response of the worked-up sample was compared with that obtained by injection of moxifloxacin aqueous solution directly in the AC.

3. Results and discussion

3.1. HPLC system

In order to assay many experimental samples of moxifloxacin, we adopted direct injection with a column switching technique which was practical without the disadvantages of classical labor-intensive and time consuming preparation process [8,9]. The pre-column and the 5 μm filter were replaced after injection of about 100 samples. All the development and validation tests, that is to say at least 500 samples were analyzed with the same analytical column without appreciable decrease of its efficiency, and, therefore, confirmed its stability under the chromatographic conditions retained as an elution buffer pH of 2.5.

Table 1
Extraction recovery of moxifloxacin from Mueller–Hinton broth

Concentration (μg/ml)	Recovery (%) (mean±SD) (n=6)
0.05	115.01±4.60
0.25	95.65±0.79
0.50	94.24±1.20
1.00	95.41±0.65
2.00	91.32±1.13

3.2. Recovery

The recovery (Table 1) was 91.32±1.13–115.01±4.6% (n=6) in the concentration range 0.05–2.0 μg/ml. A comparable value (93±5%) was obtained by Stass and Dalhoff [4] for a saliva sample.

3.3. Selectivity

Simple column switching methods often suffer from decreased selectivity compared to extraction methods [10]. This drawback was compensated in the proposed method by taking advantage of fluorescence detection technique selectivity. Typical chromatograms of blank M.–H.B., M.–H.B. spiked with moxifloxacin (1.00 μg/ml) and supernatant of *Staphyloccocus aureus* ATCC 25923 suspension in M.–H.B. after 48-h incubation at 37°C are shown in Fig. 2A–C, respectively. Moxifloxacin retention time was approximately 5.70 min. No interference of broth components or culture by-products was noted.

In attempts to minimize the emergence of quinolone resistance, several approaches might be taken, including monitoring for development of bacterial resistance, careful targeting of drug use, use of optimal dosing regimens, use of combination drug therapy, development of more potent derivatives, and cycled use of quinolones with structurally unrelated drugs [11]. With respect to combination drug therapy, the results of many studies are relatively consistent about the effects of quinolone activity. The effects of quinolones combined with β-lactams or aminoglycosides tend to be additive, may be synergistic, and are uncommonly antagonistic for most bacterial species [12]. Therefore, analytical interference study was done on different aminosides (amikacine, tobramycine, gentamicine) and cephalosporins (cefotaxime, ceftazidime, ceftriaxone, cefepime, cefpirome). No peak was detected for

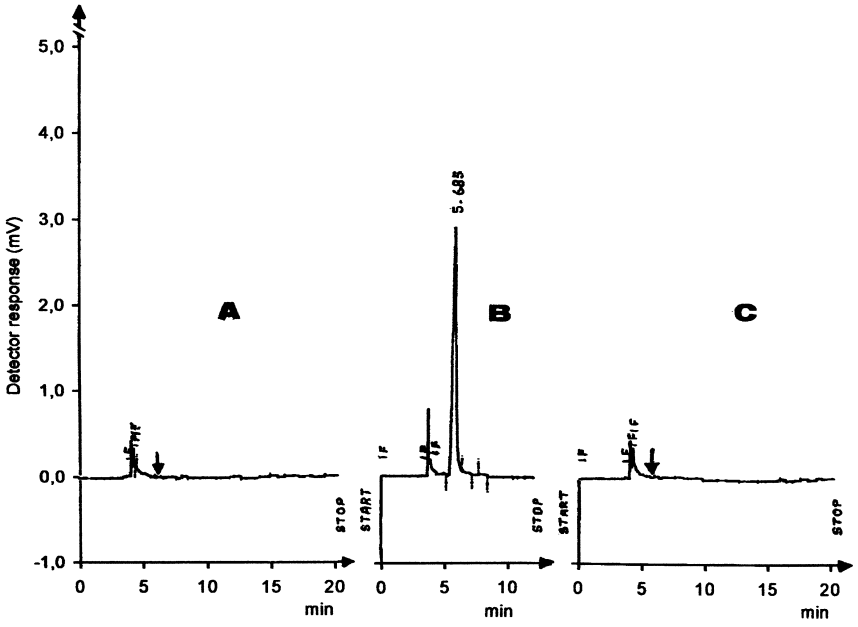

Fig. 2. Typical chromatograms from (A) blank Mueller–Hinton broth, (B) Mueller–Hinton broth spiked with moxifloxacin (1.00 μg/ml) and (C) supernatant of *Staphylococcus aureus* ATCC 25923 suspension in blank Mueller–Hinton broth after 48-h incubation at 37°C.

Table 2
Intra- and inter-assay imprecision and inaccuracy of moxifloxacin determination in Mueller–Hinton broth

Added concentration (μg/ml)	*n*	Concentration found (mean±SD) (μg/ml)	RSD (%)	Inaccuracy[a] (%)
Intra-assay				
0.05	6	0.052±0.002	4.76	+4.50
0.25	6	0.25±0.002	1.13	+0.15
0.50	6	0.485±0.006	1.24	−2.93
1.00	6	0.977±0.035	3.65	−2.31
2.00	6	1.945±0.033	1.70	−2.76
Inter-assay				
0.05	12	0.053±0.003	5.75	+6.00
0.25	12	0.247±0.004	1.63	−1.10
0.50	12	0.495±0.010	2.15	−1.10
1.00	12	1.003±0.016	1.67	+0.31
2.00	12	2.010±0.035	1.76	+0.49

[a] Mean relative error.

these potential comedications under the selected chromatographic conditions.

3.4. Linearity

The correlation between moxifloxacin concentration (x) and peak area (y) in the range 0.05–3.2 μg/ml led to a mean slope (±SD) of 445.56 (±19.04) and a mean y-intercept (±SD) of 1.36 (±3.65) from six separate assay runs. The mean correlation coefficient was 0.9998. The use of internal standard was not necessary to generate valid results because of a good reproducibility of moxifloxacin transfer into the PC and its elution from.

The mean slope (±SD) of log–log plots for the concentration versus the peak area was close to 1 (1.0006±0.0099) and then, allowed us to confirm the linearity of standard curves.

3.5. Imprecision and inaccuracy

The intra- and inter-assay imprecision (given by the relative standard deviation of replicate analyses) and the inaccuracy (given as mean relative error, i.e., the percentage deviation between found and added concentration) of the method were evaluated using control broth samples for concentrations ranging from 0.05 μg/ml to 2.0 μg/ml. The intra-assay repeatability was determined by analyzing six specimens of spiked broth samples on the same day. The inter-assay repeatability was obtained by analyzing two specimens of broth samples on six days over a period of 2 weeks. The data in Table 2 demonstrate

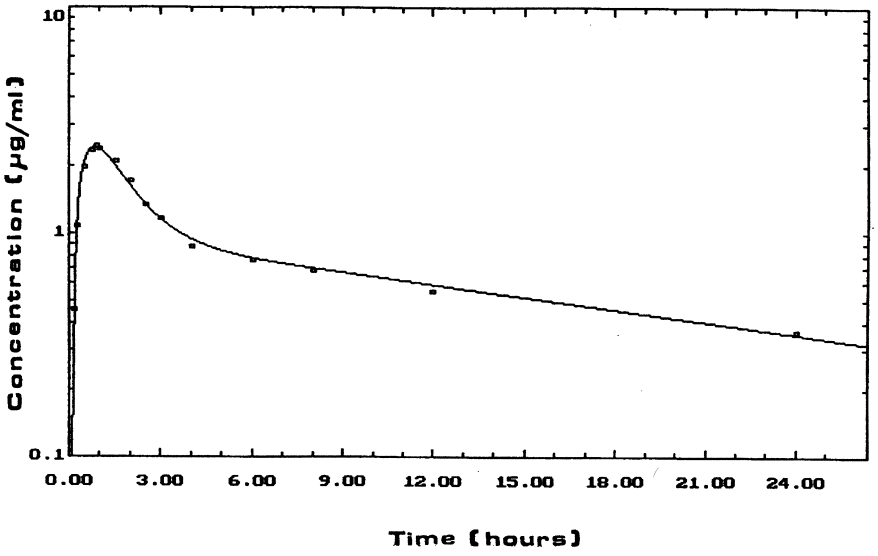

Fig. 3. Pharmacokinetic profile from simulation of oral administration of a 400 mg dose of moxifloxacin in the peripheral compartment of an in vitro pharmacokinetic–pharmacodynamic model using Mueller–Hinton broth.

the good precision and accuracy for moxifloxacin in M.–H.B. over the concentration range investigated.

4. Application

The method described was successfully applied to the analysis of M.–H.B. samples from an in vitro PK–PD model derived from the two-compartment kinetic model with artificial capillary units proposed by Blaser et al. [13]. The model was designed to expose bacteria to changing antibiotic concentrations, without dilution of the bacterial inoculum together with the antibiotic. The central compartment (CCp) consisted of a thermostatizable flask with magnetic stirrer containing culture broth, tubing and the lumina of the capillaries within a disposable dialyser unit. The peripheral compartment (PCp) consisted of extracapillary space of the dialyser unit plus tubing. A peristaltic pump provided a fast equilibrium of antibiotic concentration between the CCp and the PCp in order to simulate blood levels also in the PCp. Broth flows into and out of CCp were controlled to simulate human concentration–time profile of the antibiotic. Fig. 3 shows the pharmacokinetic profile from simulation of oral administration of 400 mg dose of moxifloxacin in the PCp of the in vitro PK–PD model. Pharmacokinetic parameters obtained from the model (Table 3) were close to that obtained in healthy subjects [14–16]. Extensive treatment of these experimental data will be the subject of a further publication.

Table 3
Moxifloxacin pharmacokinetic parameters in the in vitro pharmacokinetic–pharmacodynamic model and in healthy subjects

Parameter	In vitro model	Healthy subjects, Refs. [14–16] (n=12)
C_{max} (μg/ml)	2.42	2.50±1.29 (1.62–3.80)
$C_{12\ h}$ (μg/ml)	0.55	0.90
$C_{24\ h}$ (μg/ml)	0.36	0.40
t_{max} (h)	0.90	2.0 (0.50–6.0)
$T_{1/2}\beta$ (h)	16.28	15.6±1.15 (12.1–19.1)
k_a (h^{-1})	2.63	(2–3)[a]

[a] Dose=200 mg, n=6.

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