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HPLC analysis of ciprofloxacin and ciprofloxacin metabolites in body fluids ☆

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Abstract

An improved high-performance liquid chromatography (HPLC) procedure for the analysis of ciprofloxacin and three of its metabolites in plasma, serum and urine samples was developed. The previously published HPLC procedure described the isocratic separation of ciprofloxacin and three ciprofloxacin metabolites in urine samples on a polystyrene–divinylbenzene reverse-phase column followed by quantitation using a UV detector. The present procedure involved the same chromatographic separation, but is also applicable to the analysis of plasma and serum as well as urine samples, and quantitation was based on fluorometric detection after postcolumn induction of fluorescence instead of UV detection. The post-column induction of fluorescence was necessary because the M2 and M3 metabolites of ciprofloxacin have relatively weak native fluorescence, and induction enhanced the fluorometric signals of metabolites M2 and M3 forty-four-fold and eleven-fold, respectively. The observed enhancement of fluorescence may be attributed to the partial conversion by UV light of metabolites M2 and M3 to metabolite M1 which has intense native fluorescence. The lower quantitation limits of ciprofloxacin and metabolites M1, M2 and M3 were 0.05 μg ml^{-1}, 0.01 μg ml^{-1}, 0.05 μg ml^{-1}, and 0.5 μg ml^{-1}, respectively. All four analytes were quantitated using one isocratic elution of either plasma or serum supernatant after the precipitation of proteins or the isocratic chromatography of diluted urine samples.

Keywords: Ciprofloxacin; Metabolites; HPLC; Body fluids assay; Fluorescence induction

1. Introduction

Ciprofloxacin (see Fig. 1) is a quinolone carboxylic acid derivative that has Gram-negative and Gram-positive bactericidal activity [1–3], exhibits a rapid onset of action, and lacks cross-reactivity with penicillins, cephalosporins, and aminoglycosides [4]. Although there are several published procedures for the analysis of ciprofloxacin and three ciprofloxacin metabolites (depicted in Fig. 1) in body fluids [5–12], there is still a need for a simple procedure that can be used for the simultaneous analysis of ciprofloxacin and ciprofloxacin metabolites in

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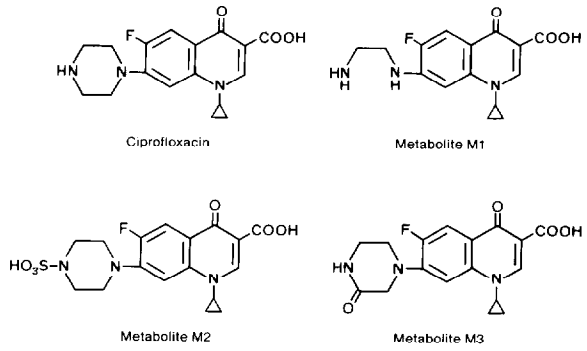

Fig. 1. The chemical structures of ciprofloxacin and ciprofloxacin metabolites.

serum and plasma as well as in urine. Such a procedure can save a considerable amount of time when concentrations of ciprofloxacin and ciprofloxacin metabolites have to be assayed in thousands of serum and urine samples collected during clinical and pharmacokinetic studies.

The previously published procedure for the analysis of ciprofloxacin and ciprofloxacin metabolites in urine samples required only the dilution of urine samples and the isocratic elution of all analytes on the polystyrene divinylbenzene (PLRP-S) reverse phase column [7]. However, several sample preparation steps and either two isocratic elutions [9] or a gradient elution [5,12] were necessary for the analysis of ciprofloxacin and ciprofloxacin metabolites in serum and plasma when octadecylsilane (C18) reverse-phase columns were used.

The present procedure required two modifications of the previously published procedure [7] that was used primarily for the analysis of ciprofloxacin and ciprofloxacin metabolites in urine samples. The modifications involved the precipitation of serum proteins with trichloroacetic acid and acetonitrile prior to chromatography, followed by the direct injection of the supernatant on the polymeric reverse-phase column and fluorometric detection after postcolumn UV induction of fluorescence. The precipitation step prolonged the PLRP-S column stability while postcolumn UV induction facilitated the quantitation of two metabolites (M2 and M3) that have relatively weak native fluorescence. A forty-four-fold increase in the fluorescence signal of metabolite M2 and an eleven-fold increase of metabolite M3 signal were observed after UV induction. A similar postcolumn induction of ciprofloxacin metabolite fluorescence was described by Scholl et al. in a previous publication [9]. However, the present procedure does not require the filtration of serum and/or plasma prior to chromatography and involves only one isocratic separation of ciprofloxacin and ciprofloxacin metabolites on a polystyrene–divinylbenzene reverse-phase column rather than two isocratic or gradient elutions on octadecylsilane reverse-phase columns.

2. Experimental

2.1. Materials and reagents

Analytical grade potassium dihydrogen phosphate and 85% *o*-phosphoric acid were purchased from Fisher Scientific (Springfield, NJ). HPLC grade acetonitrile, methanol, reagent grade ammonium hydroxide, sodium hydroxide and trichloroacetic acid were purchased from J.T. Baker (Phillipsburg, NJ). A Milli-Q reagent water system (Millipore, Bedford, MA) was used for the purification of deionized water. Polystyrene–divinylbenzene (PLRP-S) columns were purchased from Polymer Laboratories (Amherst, MA). Human plasma and serum were purchased from Biological Specialty Corp. (Colmar, PA). Ciprofloxacin, ciprofloxacin metabolites, and the *N*-isopropyl analog of ciprofloxacin that was used for internal standard purposes were obtained from Bayer AG, Institute of Clinical Pharmacology (Wuppertal, Germany).

The primary reference standard stock solution of ciprofloxacin was prepared in the purified deionized water at about a 0.3 mg ml^{-1} concentration. The corresponding stock solution of Metabolite M1 was prepared in 0.05 M potassium phosphate monobasic (pH 3.0) also at about 0.3 mg ml^{-1} concentration. Subsequently, M1 stock solution was diluted with the phosphate buffer to yield 0.03 mg ml^{-1} working standard solution. Metabolites M2 and M3 were first dissolved in 1 N ammonium hydroxide at about 0.5–0.8 mg ml^{-1} concentration, and then diluted

with 0.05 N potassium phosphate monobasic (pH 3.0) to yield 0.2–0.4 mg ml^{-1} concentrations. All solutions were stored at 4 °C. The final stock solution of the *N*-isopropyl analog of ciprofloxacin internal standard was dissolved in 0.1 M phosphoric acid to yield a 0.02 mg ml^{-1} concentration.

2.2. Sample preparation

Each 0.5 ml aliquot of the serum or plasma sample was diluted with 0.1 ml of a 0.02 mg ml^{-1} solution of internal standard in 0.1 M phosphoric acid and 0.3 ml of 5.0 M trichloroacetic acid–acetontrile (1:1, v/v) solution. The mixture was vortexed and diluted again with 0.1 ml of acetonitrile and 0.3 ml of water. The final solution was vortexed and centrifuged for 15 min at 2800 rev min^{-1} (1500*g*). The supernatant was transferred with a Pasteur pipette into a glass autosampler vial for HPLC analysis.

Urine samples were diluted at least 1:20 (v/v) with a 0.05 M potassium phosphate monobasic buffer solution (pH 3.0) and a 0.5 ml aliquot of the diluted solution was transferred into a glass HPLC autosampler vial. The diluted urine samples were diluted again with 0.1 ml of the 0.02 mg ml^{-1} internal standard solution and 0.7 ml of 0.1 M trichloroacetic acid solution. The vials were capped and vortexed prior to injection on to the HPLC column.

2.3. Chromatography

Aliquots (10 μl) of reference standards, quality control samples, and clinical study samples were injected onto the 15 cm × 4.6 mm i.d. reverse-phase PLRP-S column which was packed with 5 μm beads of polystyrene–divinylbenzene support. A 5.0 mm × 3.0 mm guard column packed with the same support was connected between the injector and the 15 cm analytical column. The guard and the analytical column were eluted with 0.02 M trichloroacetic acid (pH 3.0)–acetonitrile–methanol (74:22:4, v/v/v) mobile phase at a flow rate of 0.7 ml min^{-1} and a temperature of 30 °C.

2.4. Detection and quantitation

The column eluent was circulated through a 10 m, 0.25 mm i.d. knitted Teflon coil that was wrapped around a 254 nm UV low pressure lamp. An Aura Industries (Staten Island, NY) PHRED photochemical reactor was used for the post-column induction of fluorescence. The fluorescence was monitored with a Waters 470 detector (Waltham, MA) at 277 nm excitation and 418 nm emission wavelengths. The detector gain was 1 and the time constant was 1.5 s. The detector output was recorded with a Spectra Physics model SP4270 (San Jose, CA) chromatographic data module. Quantitations of ciprofloxacin and ciprofloxacin metabolite concentrations were based on the relative peak height response ratios of each compound and the internal standard.

3. Results and discussion

3.1. Chromatographic selectivity and efficiency

Figs. 2 and 3 illustrate representative chromatograms of reference standard and subject serum samples. The chromatograms of urine samples are depicted in Figs. 4 and 5. According to these chromatograms, the ciprofloxacin and ciprofloxacin metabolites and the structurally related internal standard were completely resolved from each other and their relative sequence of elution reproduced the previously published results [7]. Negligible interferences from endogenous compounds were observed at the retention times of ciprofloxacin, ciprofloxacin metabolites M1 and M3, and the internal standard in the reference standards and serum samples collected from clinical study subject. Partial interference was observed at the retention time of the M2 metabolite in the serum and plasma samples. However, since metabolite M2 has a relatively high fluorometric response, the observed interference was equivalent to less than 10% of the signal obtained with the reference standards, even at the lowest quantitation limit. Partial interference was also observed at the retention time of metabolite M1 in some of

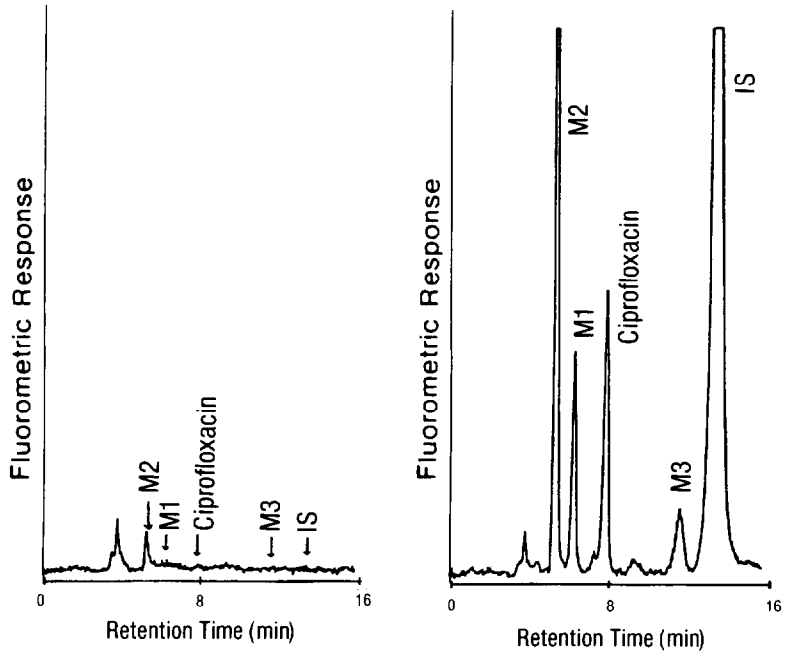

Fig. 2. HPLC chromatograms of a blank plasma standard (left) and a plasma calibation standard containing 0.50 μg ml^{-1}, 0.05 μg ml^{-1}, 0.50 μg ml^{-1}, 1.5 μg ml^{-1}, and 4.0 μg ml^{-1} of metabolite M2, metabolite M1, ciprofloxacin, metabolite M3, and internal standard, respectively (right).

the urine samples of subjects with severe renal impairment.

The chromatographic efficiency of the PLRP-S column was relatively constant and ranged from about 4000–6000 theoretical plates after over 500 injections of plasma, serum, and/or urine samples. However, a significant decrease in the response signal of metabolite M2 was observed after several hundred sample injections. This observed decrease might be attributed to the gradual entrapment of the metal ions by the polystyrene–divinylbenzene matrix and subsequent complexation of the negatively charged sulfo group of metabolite M2 by the trapped metal ions. This hypothesis is supported by the observation that the chromatographic response of metabolite M2 is significantly enhanced when the column is reconditioned with ethylenedinitrilotetraacetic acid (EDTA) at pH 7 (see Fig. 6).

3.2. Relative fluorometric responses and quantitation limits of ciprofloxacin and its metabolites

Table 1 lists the relative fluorometric peak height responses of ciprofloxacin and ciprofloxacin metabolites corrected for variable concentrations. Fig. 7 illustrates the chromatograms obtained with the postcolumn UV reactor lamp on and off. It is apparent that UV induction of fluorescence was most significant for metabolites M2 and M3 which have relatively weak native fluorescence. About a forty-four-fold enhancement of metabolite M2 fluorescence and an eleven-fold enhancement of metabolite M3 were observed. In contrast, only about a 30% enhancement of the ciprofloxacin fluorescence and about a 40% decrease in the fluorescence of metabolite M1 were observed with the UV reactor lamp on. We have also observed

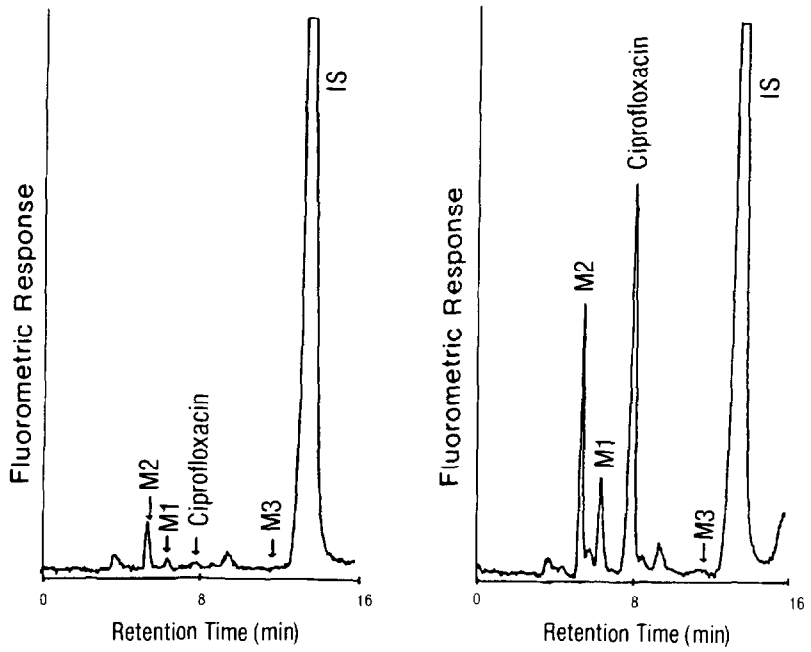

Fig. 3. HPLC chromatograms of a predose subject serum sample (left) and a 10 h postdose subject serum sample (right).

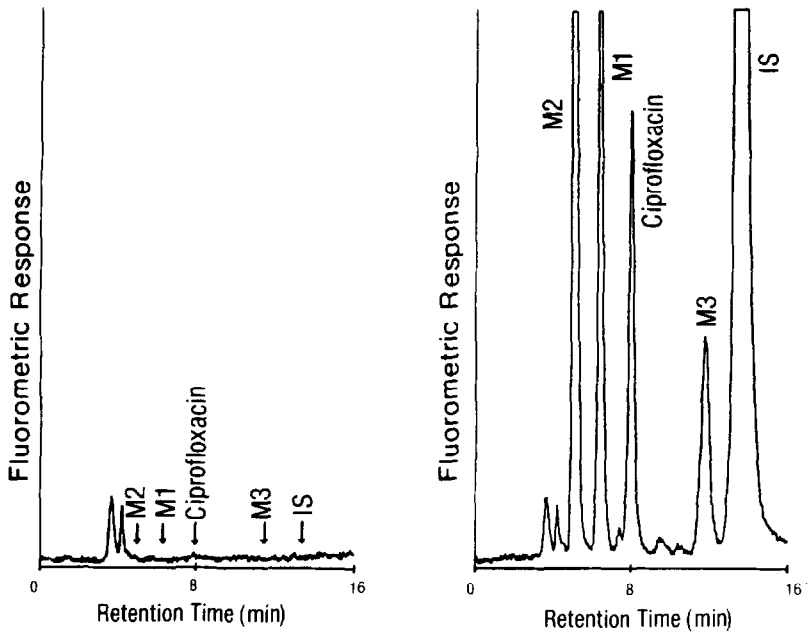

Fig. 4. HPLC chromatograms of a blank urine standard (left) and a urine calibration standard containing 0.50 μg ml^{-1}, 0.20 μg ml^{-1}, 0.50 μg ml^{-1}, 2.00 μg ml^{-1}, and 4.00 μg ml^{-1} of metabolite M2, metabolite M1, ciprofloxacin, metabolite M3, and the internal standard (right).

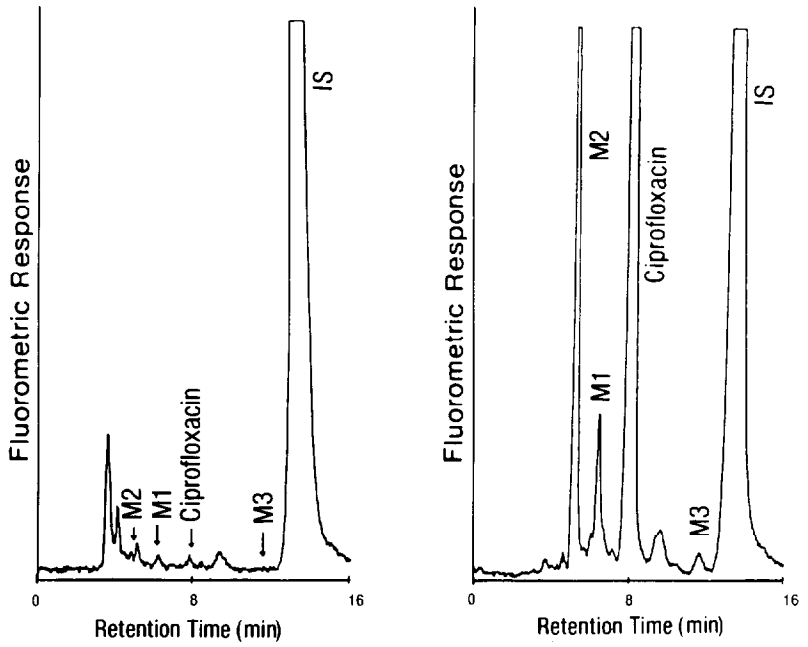

Fig. 5. HPLC chromatograms of a predose subject urine sample (left) and a 16–24 h postdose subject urine sample (right).

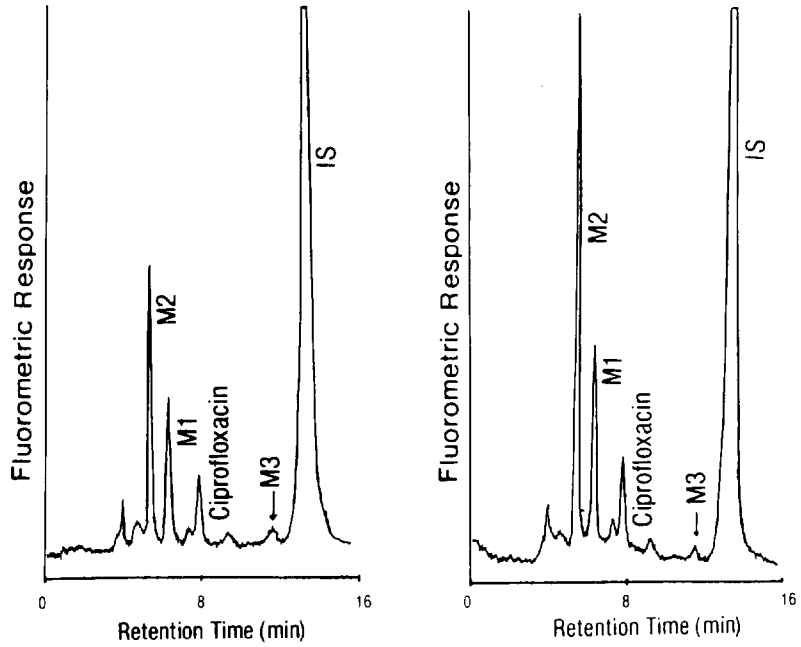

Fig. 6. HPLC chromatogram of a serum sample chromatographed on the polystyrene–divinylbenzene column after about 500 injections (left) and a chromatogram of the same sample after the same column was reconditioned with EDTA at pH 7.0 (right).

Table 1
Relative peak heights of ciprofloxacin and ciprofloxacin metabolites with UV reactor on and off

Analyte	Peak height conc.$^{-1}$ UV reactor off	Peak height conc.$^{-1}$ UV reactor on	On/off ratio
Ciprofloxacin	1400	1830	1.31
Metabolite M1	23470	13790	0.58
Metabolite M2	147	6450	43.88
Metabolite M3	11	120	10.91

that a significant percentage (about 70%) of metabolite M2 was converted to metabolite M1 after UV irradiation, while UV irradiation of ciprofloxacin and metabolite M3 yielded a considerably lower percentage (5% and 1%, respectively), of metabolite M1. The above observations were based on the chromatographic analysis of ciprofloxacin and metabolite fractions that were collected after postcolumn UV irradiation. Since metabolite M1 has a relatively intense native fluorescence, the observed formation of metabolite M1 in the irradiated ciprofloxacin and metabolite M2 and M3 chromatographic fractions accounts for some or most of the observed UV induction of ciprofloxacin, metabolite M2 and M3 fluorescence. However, at present, we do not know why the native fluorescence of metabolite M1 is considerably more intense than the native fluorescence of ciprofloxacin and metabolites M2 and M3, and we could not detect and identify any UV irradiation products of metabolite M1. The fact that the native fluorescence of metabolite M1 decreases about 40% after UV irradiation also indicates that the UV irradiation products of metabolite M1 are either non-fluorescent or have relatively weak fluorescence at the specified wavelengths.

The lower quantitation limits of the procedure were 0.01 μg ml^{-1} of metabolite M1, 0.05 μg ml^{-1} for ciprofloxacin and metabolite M2, and 0.5 μg ml^{-1} for metabolite M3. The sensitivity of the procedure can be enhanced further by the injection of larger sample volumes (50 μl instead of 10 μl).

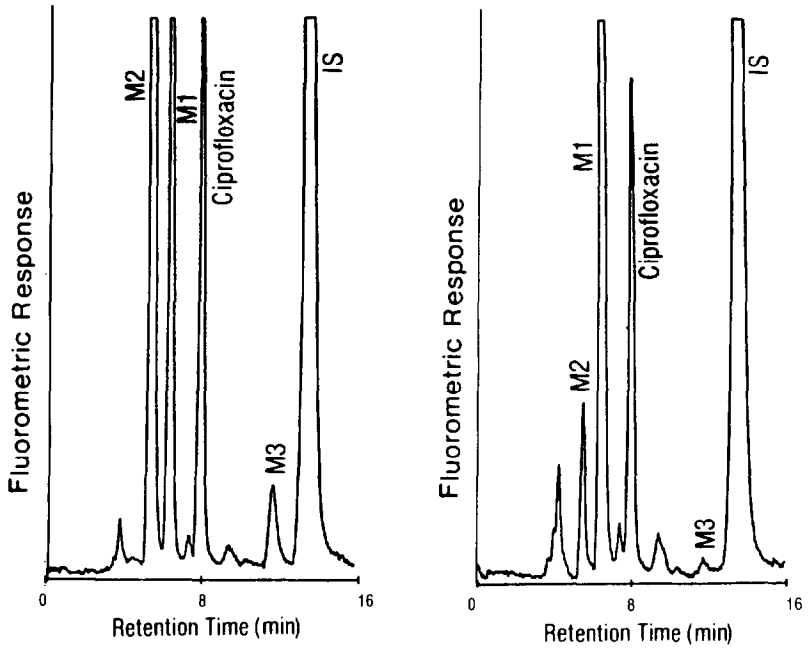

Fig. 7. HPLC chromatogram of a plasma calibration standard containing 1.0 μg ml^{-1}, 0.2 μg ml^{-1}, 1.0 μg ml^{-1}, 2.0 μg ml^{-1}, and 4.0 μg ml^{-1} of metabolite M2, metabolite M1, ciprofloxacin, metabolite M3, and the internal standard, respectively, obtained with the postcolumn UV reactor on (left) and with the reactor off (right).

Table 2
Intraday precision and accuracy observed with plasma calibration standards containing ciprofloxacin and ciprofloxacin metabolites

Analyte	Theoretical[a] conc. ($\mu g\ ml^{-1}$)	Mean observed conc. ($\mu g\ ml^{-1}$)	RSD (%)	Accuracy (%)
Ciprofloxacin	0.05	0.052	6.7	104.0
	7.5	7.36	0.6	98.1
Metabolite M1	0.01	0.01	4.5	100.0
	5.0	4.90	0.7	98.0
Metabolite M2	0.05	0.054	8.3	108.0
	5.00	4.80	1.1	96.0
Metabolite M3	0.50	0.50	5.8	100.0
	7.50	7.22	1.5	96.3

[a] Six replicates at seven concentration levels were analyzed; only the lowest and highest levels are listed.

3.3. Accuracy and precision of plasma and urine assay procedures

The intra-day and inter-day accuracy and precision of the plasma assay procedure are summarized in Tables 2 and 3. The intra-day accuracy and precision data were based on the analysis of six replicate calibration standards at seven concentration levels. The inter-day data were obtained with qualtiy control samples which were analyzed concurrently with the study samples. Tables 4 and 5 present the corresponding intra-day and inter-day accuracy and precision data that were obtained with urine calibration standards and quality control samples. All plasma and urine quality control samples were within 15% of nominal values.

3.4. Recovery

Table 6 summarizes the observed recoveries of ciprofloxacin and metabolites after the precipita-

Table 3
Interday precison and accuracy observed with plasma quality control samples analyzed concurrently with the study samples

Analyte	Theoretical conc. ($\mu g\ ml^{-1}$)	Replicates N	Mean observed conc. ($\mu g\ ml^{-1}$)	RSD (%)	Accuracy (%)
Ciprofloxacin	0.07	99	0.07	2.5	100.0
	1.00	100	1.01	2.8	101.0
	5.00	100	4.97	1.6	99.4
Metaboite M1	0.015	99	0.015	3.3	100.0
	0.20	22	0.21	2.1	105.0
	3.00	78	2.95	1.6	98.3
Metabolite M2	0.07	99	0.071	3.9	101.4
	1.00	78	1.07	3.2	107.0
	3.00	78	3.15	3.0	105.0
Metabolite M3	0.70	99	0.72	3.6	102.9
	3.00	78	2.95	4.4	98.3
	5.00	78	4.87	5.0	97.4

Table 4
Intraday precision and accuracy observed with urine calibration standards containing ciprofloxacin and ciprofloxacin metabolites

Analyte	Theoretical[a] conc. ($\mu g\ ml^{-1}$)	Mean observed conc. ($\mu g\ ml^{-1}$)	RSD (%)	Accuracy (%)
Ciprofloxacin	0.025	0.027	8.1	108.0
	7.50	7.23	0.3	96.4
Metabolite M1	0.005	0.005	3.2	100.0
	5.00	4.99	4.3	99.8
Metabolite M2	0.025	0.025	3.2	100.0
	5.00	4.91	4.3	98.2
Metabolite M3	0.25	0.25	7.4	100.0
	7.50	7.81	1.3	95.9

[a] Six replicates at eight concentration levels were analyzed; only the lowest and highest levels are listed.

Table 5
Interday precision and accuracy observed with urine quality control samples analyzed concurrently with study samples

Analyte	Theoretical conc. ($\mu g\ ml^{-1}$)	Replicates N	Mean observed conc. ($\mu g\ ml^{-1}$)	RSD (%)	Accuracy (%)
Ciprofloxacin	0.7	28	0.073	3.0	104.3
	1.00	28	1.00	1.3	100.0
	5.00	28	4.95	1.2	99.0
Metabolite M1	0.015	28	0.016	5.4	106.7
	1.00	28	1.00	2.0	100.0
	3.00	28	2.97	2.0	99.0
Metabolite M2	0.07	28	0.071	3.5	101.4
	1.00	28	1.08	2.6	108.0
	3.00	28	3.31	1.3	110.0
Metabolite M3	0.70	28	0.69	6.0	98.6
	3.00	28	2.98	2.2	99.3
	5.00	28	5.17	2.4	103.4

tion of plasma proteins. Although about 20% of ciprofloxacin and the metabolite M3 was lost during precipitation, the observed loss was relatively constant and was corrected by the concurrent analysis of appropriate calibration standards. Recoveries of analytes in urine samples were not determined since only the dilution step was necessary for the analysis of urine samples.

3.5. Sample stability

The stability of ciprofloxacin and its metabolites in plasma and serum samples was analyzed after 14–18 weeks of storage at −20 °C. The observed mean percent deviations from the initial concentration values were −5.6% for ciprofloxacin, −3.8% for metabolite M2, and −5.0% for metabolite M3. The corresponding mean percent deviations after three freeze–thaw cycles were +0.2%, −0.4%, +0.5%, and +0.8% for ciprofloxacin and metabolites M1, M2, and M3, respectively. The observed percent deviations were within the experimental error of the assays.

Table 6
Percent recoveries of ciprofloxacin and ciprofloxacin metabolites from plasma

Analyte	Analyte conc. ($\mu g\ ml^{-1}$)	Mean recovery[a] (%)	Standard deviation
Ciprofloxacin	0.05	81	5.6
	7.50	78	0.2
Metabolite M1	0.01	91	5.4
	5.00	88	0.2
Metabolite M2	0.05	104	9.7
	7.50	98	0.8
Metabolite M3	0.50	80	5.4
	7.50	78	1.1

[a] $N = 6$.

4. Conclusions

The described procedure is specific, accurate, precise and sufficiently sensitive for the analysis of ciprofloxacin and ciprofloxacin metabolites in plasma, serum and urine. A relatively simple sample preparation step and only one isocratic chromatographic elution that separates and quantitates all four analytes enhance the efficiency of the procedure. The procedure was used for the analysis of about 5000 samples collected during pharmacokinetic and clinical studies.

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