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Fully automated high-performance liquid chromatography of ciprofloxacin with direct injection of plasma and Mueller–Hinton broth for pharmacokinetic/pharmacodynamic studies

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Abstract

An isocratic high-performance liquid chromatographic method with column switching and direct injection has been developed to determine ciprofloxacin in plasma and Mueller–Hinton broth. An on-line dilution of the sample was performed with a loading mobile phase consisting of 173 m*M* phosphoric acid. The analyte was retained on a LiChrocart 4-4 precolumn filled with a LiChrospher 100 RP18, 5 μm. An electric-actuated system with two six-port valves allowed a clean-up step with a mixture 20 m*M* phosphate buffer (pH 3.5)–methanol (97: 3, v/v) and the transfer of the analyte by a back-flush mode to a 150×4.6 mm I.D. column packed with a Kromasil C_8 5 μm, using a mobile phase of 20 m*M* phosphate buffer (pH 3.5)–acetonitrile (85:15, v/v). Fluorescence detection allowed a quantification limit of 0.078 μg/ml with a 40-μl sample size. The method was evaluated to determine its usefulness in studying the pharmacokinetic/pharmacodynamic behaviour of ciprofloxacin in an in vitro model.

Keywords: Mueller–Hinton broth; Column switching; Ciprofloxacin

1. Introduction

Ciprofloxacin is a broad spectrum fluoroquinolone antibacterial agent. It is effective in the treatment of a wide variety of infections, particularly those caused by gram-negative pathogens. Gram-positive bacteria are generally susceptible or moderately susceptible [1].

In developing an analytical method for pharmacokinetic studies, not only the sensitivity and reproducibility suitability for the purpose but also labour-saving factors must be considered because of the need to examine a large number of samples [2].

Several high-performance liquid chromatography (HPLC) methods involving liquid–liquid extraction have already been described for the determination of ciprofloxacin in serum or plasma. Pou-Clave et al. [3] proposed an extraction with methylene chloride at serum pH, evaporation of the organic layer and re-extraction of the residue with methylene chloride in the presence of phosphoric acid (pH 2). Davis et al. [4] used a similar technique but the residue was reconstituted in the mobile phase prior to injection. Weber et al. [5] proposed deproteinization with acetonitrile and direct injection of the supernatant.

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This technique presented the drawback to dilute the analyte. Lowdal et al. [6] suggested evaporation of the supernatant and reconstitution of the residue with a small volume of mobile phase. Mack [7] withdrew acetonitrile by re-extraction of the supernatant with a mixture of methylene chloride–2-propanol. Trichloroacetic acid was also used as deproteinization reagent [8,9]. All these methods required relatively time-consuming extraction and/or concentration steps.

Sequential separation techniques using switching devices are well suited to such a time problem. The column-switching technique allows on-line high-performance liquid chromatography by direct injection of the plasma sample [10]. The manual sample preparation steps are eliminated and the time required for processing the sample is greatly shortened. As far as we are aware, no HPLC method for determination of ciprofloxacin by direct injection of biological samples has been published.

We described a four-step on-line method consisting of firstly, trapping the sample that contains the analyte in a precolumn (PC). In a second step, the compounds of the biological matrix were eluted to waste. Then, the analyte was transferred to the analytical column (AC). The PC was reconditioned in a last step. This method takes advantage of the intrinsic fluorescence of ciprofloxacin, enhancing its specificity and sensitivity.

Clinical trials and in vitro pharmacokinetic/pharmacodynamic (PK–PD) studies of ciprofloxacin are needed to evaluate the efficacy against pathogens and possible emergence of resistance. This improved direct-injection method was developed both for plasma and Mueller–Hinton broth (M.–H.B) and therefore was suitable for PK–PD studies in, in vitro models and correlations with human plasma data.

2. Experimental

2.1. Chemicals

Ciprofloxacin hydrochloride monohydrate was a gift from Bayer Pharma (Puteaux, France). Compounds used for analytical interference studies, amikacine, tobramycine, gentamicine, cefotaxime, ceftazidime, ceftriaxone, cefizoxime, were obtained from Bristol–Myers–Squibb (Paris, France), Lilly France s.a.. (Saint Cloud, France), Schering–Ploug (Levallois–Perret, France), Roussel (Paris, France), Glaxo (Paris, France), Roche (Neuilly-sur-Seine, France) and Bellon (Neuilly-sur-Seine) respectively. Acetonitrile was purchased from Touzart and Matignon (Courtaboeuf, France). Methanol, 85% orthophosphoric acid and potassium dihydrogenphosphate were provided by Prolabo (Fontenay-sous-Bois, France). Mueller–Hinton broth was provided by A.D.L. (Tresses, France). All reagents were analytical grade. HPLC grade water was obtained with a Milli-Q water purification unit, Millipore (Saint Quentin Yvelines, France). Plasma standards were prepared using fresh frozen plasma from citrated human blood which were obtained from a blood bank (E.T.S.A., Bordeaux, France).

2.2. Chromatographic system and conditions

A schematic representation of the column-switching system is given in Fig. 1. An HP 1050 liquid chromatograph (Hewlett–Packard, Evry, France) equiped with an isocratic pump (pump 1) and an autosampler delivered mobile phase 1.

The pump 2 was a Constametric III Milton Roy pump (LDC, Paris, France) delivering mobile phase 2.

An HPLC pump 420 Kontron (Kontron Instruments, Milano, Italy) delivered mobile phase 3.

An electric-actuated switching system with two six-port Rheodyne valves (Touzart and Matignon) was controlled by the external time events of the autosampler and the pump of the HP 1050 liquid chromatograph. Sample injection starts the programmable time-relay of the autosampler and the pump by the mean of a connection between the two modules.

The PC was a Lichrocart 4-4 filled with a 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 packed with Kromasil C_8, 5 μm (Touzart and Matignon).

The eluted compound was detected with a spectroflow 980 fluorometric detector (ABI Analytical/Kratos Division, Fontenay sous Bois, France) set at 278 nm excitation and equiped with a 418-nm

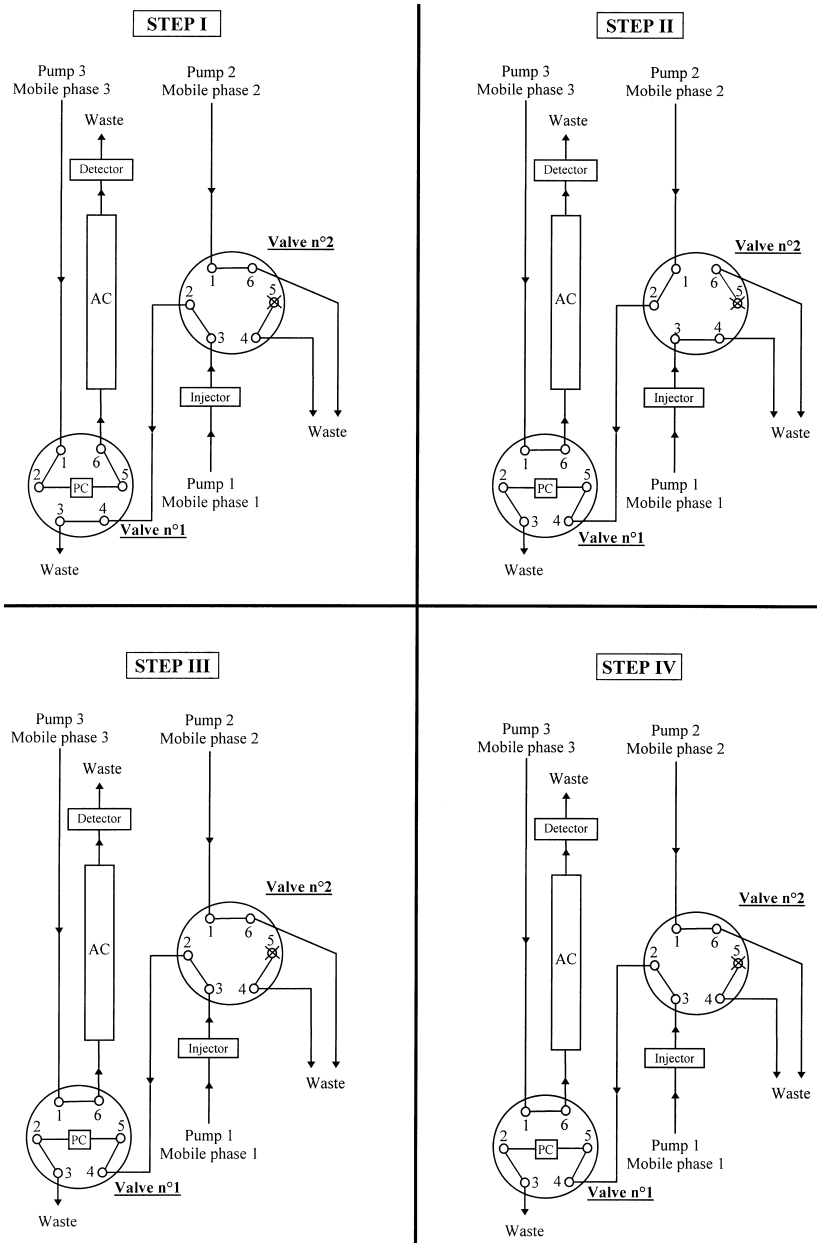

Fig. 1. Schematic representation of the column-switching system.

emission cut-off filter. A recorder output range of 0.020 μA full scale was used.

Data handling was performed by means of a HP 3396 A integrator (Hewlett–Packard).

The mobile phase 1 was a 173 m*M* orthophosphoric acid solution and was obtained by the mixture of 20 ml of 85% orthophosphoric acid and purified water.

The mobile phase 2 consisted of 20 m*M* KH_2PO_4 buffer adjusted to pH 3.5 with orthophosphoric acid and methanol (97:3, v/v).

The aqueous component of mobile phase 3 was the same as mobile phase 2 but the organic modifier was acetonitrile in a proportion of 15%.

All the mobile phases were pumped at 1.0 ml/min.

The total sequence of automated sample analysis required 12 min for plasma and 15 min for M.–H.B. (a component of the broth was eluted at 14.0 min) and included the following four steps.

Step I (0–1 min, valve 1 and valve 2 in load position): injection of the sample and dilution of its proteins in the mobile phase 1. The analyte was transferred to the PC where it was retained.

Step II (1–3 min, valve 1 in load position and valve 2 in injection position). The PC was purged with mobile phase 2: the unwanted components directly vented to waste. The low elution strength of mobile phase 2 avoided analyte loss during this clean-up step.

Step III (3–5 min, valve 1 in inject position and valve 2 in load position). Mobile phase 3 with high elution strength, enabled analyte transfer in back flush mode from PC to the AC where it was separated for quantification.

Step IV (5–12 or 15 min, valve 1 and valve 2 in load position). The PC was equilibrated with mobile phase 1.

The injection volume was 40 μl.

2.3. Stock solutions and spiked plasma and broth samples

Ciprofloxacin was made up as 1.0 mg free base per ml stock solution in water. Ciprofloxacin was diluted with blank human plasma or M.- H.B. to make spiked samples of 10 μg/ml. Then, plasma and broth samples containing 5.0, 1.25, 0.625, 0.312, 0.156 and 0.078 μg/ml were prepared by successive dilution with the corresponding 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. Working solutions of 31.25 μg/ml were obtained by successive dilution of the stock solutions and aliquots were stored at −20°C.

2.4. Sample preparation procedure

Plasma or broth samples with concentrations expected to be below 1.25 μg/ml were injected directly. Samples with concentrations expected to be above 1.25 μg/ml underwent an 1/10 dilution with the corresponding blank matrix.

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.

2.5. Calibration and calculations

The concentration of unknown samples was calculated from a linear calibration curve. This calibration curve was obtained by computing a nonweighted least-squares regression of the peak area *y* versus ciprofloxacin concentration *x* from five standard samples. For diluted sample, dilution factor was taken into account for the calculation.

Linearity was determined by assaying five standards in six separate assay runs within a week.

2.6. Recovery

The recovery was established for plasma and M.–H.B. by six analyses of three ciprofloxacin concentrations, 0.078, 0.312, and 1.25 μg/ml. The response of the worked-up sample was compared with that obtained by injection of ciprofloxacin aqueous solution directly in the AC.

3. Results and discussion

3.1. Sample clean-up procedure

When direct injection of untreated sample is implemented, a diluting fluid such as water or buffer is usually added prior injection. The diluting fluid can have several functions, it can reduce the signal due to endogenous material in the matrix, reduce the viscosity or ionic strength of the sample, ensure the compatibility of the sample with mobile phase or

disrupt weak bonding between the analyte and plasma proteins [11]. In the work of Scholl et al. [12], liquid matrices were chromatographied directly after dilution with 33 m*M* phosphoric acid. Thus, we adapted the idea of sample dilution as a simple and effective on-line sample preparation by using 173 m*M* phosphoric acid solution as loading mobile phase.

The number of injections on the precolumn varied, depending on the injection volume. The total number of injections of 40-μl untreated samples before the PC had to be changed, because of increasing back-pressure, was about 150 for plasma and M.–H.B..

Memory effects from the precolumn ('substance bleeding') may be identified by injecting blank solutions after a run with definite amounts of drug. No memory effect was observed.

The life-time of the analytical column was the same as if conventional liquid–liquid extraction was used.

3.2. Recovery

The recovery (Table 1) was 86.77±2.78%–98.07±9.90% (n=6) for plasma and 76.52±2.72%–86.54±3.23% (n=6) for M.–H.B. in the concentration range 0.078–1.25 μg/ml. These results were comparable to those obtained by Lovdall et al. [6] for chinchilla plasma (89.4±1.2%) and middle ear effusion (91.4±1.6%). Davis et al. [4] obtained a recovery of 79.5±6.9%–88.9±9.4% for a concentration range 0.5–5 μg/ml.

3.3. Selectivity

Typical chromatograms of blank plasma, plasma spiked with ciprofloxacin at a concentration of 0.312 μg/ml, blank M.–H.B., M.-H.B. spiked with ciprofloxacin (0.312 μg/ml), and the supernatant of *Klebsiella pneumoniae* suspension in M.–H.B. after 24-h incubation at 37°C are shown in Fig. 2A–E, respectively. Ciprofloxacin retention time was approximately 9.60 min. No interference of plasma endogenous compounds, broth components or culture by-products was noted.

Table 1
Extraction recovery of ciprofloxacin from plasma and Mueller–Hinton broth

Concentration (μg/ml)	Recovery (%) (mean±S.D.) (n=6)	
	Plasma	Mueller–Hinton broth
0.078	98.07±9.90	86.54±3.23
0.312	87.67±7.47	81.26±4.73
1.25	86.77±2.78	76.52±2.72

Interference with other antibiotics used as synergistic co- medications has been determined. Bacterial isolates refractory to ciprofloxacin monotherapy with subsequent development of resistance have been reported. Crook et al. [13] have seen a case where long-term oral ciprofloxacin for *Pseudomonas aeruginosa* infection seemed to have led to the development of clinically significant resistance and they suggested caution in the use of this drug alone and the need for double drug chemotherapy. Chapman et al. [14] observed the emergence of resistance in five patients with *Pseudomonas* or other serious gram-negative infections given shorter courses. Antimicrobial combinations may prevent the emergence of isolates resistant to single agents and provide broad coverage when treating unidentified or polymicrobic infections [15]. In animal studies, ciprofloxacin combined with beta-lactam agents has demonstrated the best two-agent therapeutic results [16]. Synergistic interactions were observed with ciprofloxacin in combination with tobramycin against *P. aeruginosa* [17]. Therefore, analytical interference study was done on different cephalosporins (cefotaxime, ceftazidime, ceftriaxone, cefizoxime) and aminosides (amikacine, tobramycine, gentamicine) by injection of each compound aqueous solution in the chromatographic system. No peak was observed for these potential comedications.

3.4. Linearity

The correlation between ciprofloxacin concentration (x) and peak area (y) was linear in the range 0.078–1.25 μg/ml. For plasma, the mean slope (±S.D.) was 0.2715 (±0.008) and the y-intercept (±S.D.) was 0.0026 (±0017) with an average correlation coefficient of 1.0000. For M.-H.B., the mean slope (±S.D.) and the y-intercept (±S.D.) were 0.1640 (±0.0078) and 0.0027 (±0.0009) respectively, with an average coefficient of 0.9999.

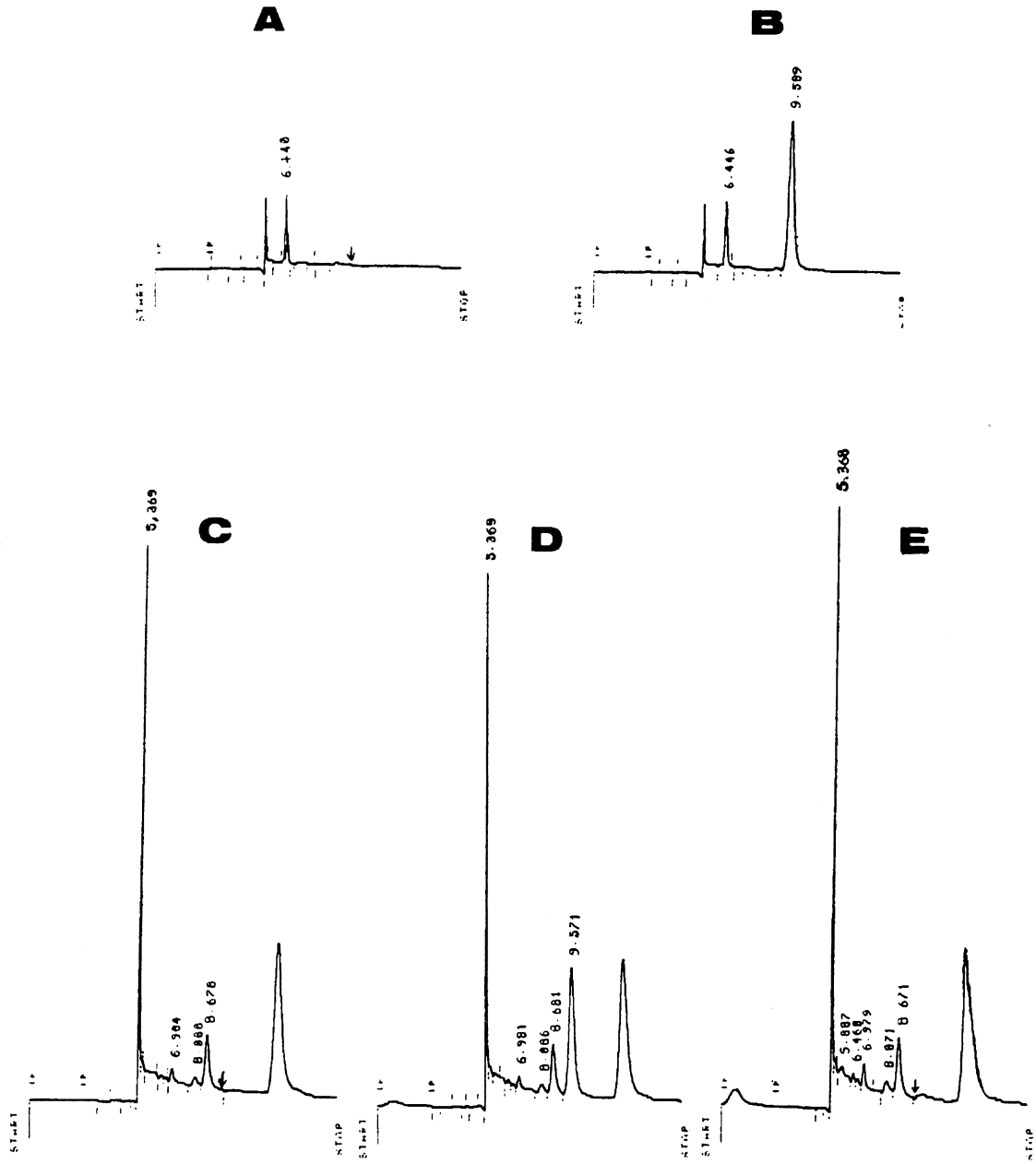

Fig. 2. Typical chromatograms from (A) human blank plasma, (B) human plasma spiked with ciprofloxacin (0.312 μg/ml), (C) blank Mueller–Hinton broth, (D) Mueller–Hinton broth spiked with ciprofloxacin (0.312 μg/ml) and (E) supernatant of *Klebsiella pneumoniae* suspension in blank Mueller–Hinton broth after 24-h incubation at 37°C.

3.5. Precision and accuracy

The intra-assay and inter-assay precision (given by the relative standard deviation) and the accuracy (given as inaccuracy, i.e. the difference between found and added concentration) were checked using control plasma and broth samples for concentrations ranging from 0.078 μg/ml to 10.0 μg/ml (1/10

dilution was performed for concentrations above 1.25 μg/ml). The intra-assay repeatability was determined by analysing at least seven specimens of spiked plasma and broth samples on the same day. The inter-assay repeatability was obtained by analysing one specimen of spiked plasma and broth samples on eight days over a period of three weeks. The results (Table 2) were acceptable within the concentration range checked.

3.6. Limit of quantification

The limit of quantification defined by a signal-to-noise level of c.a. 10:1 was 0.0078 μg/ml. The intra-assay coefficients of variation (C.V.) of spiked plasma and broth samples at this concentration were 3.2% ($n=9$) and 1.O4% ($n=7$) respectively, with deviation from the nominal value of +4.36% ($n=9$) and −1.92% ($n=7$), respectively. The inter-assay precision and inaccuracy were 5.00% and −2.00%, respectively for plasma ($n=8$) and 7.07% and −0.38% respectively, for M.–H.B. ($n=8$).

Both oral and intravenous ciprofloxacin are normally administered in a twice-daily regimen. Intravenous ciprofloxacin is usually administered in dosages of 200 to 400 mg every 12 h [1]. In a bioavailability study [18], at 12-h post infusion of 200 mg, serum concentrations were 0.10±0.03 μg/ml ($n=12$) and at 12 h after oral administration of the same dose, serum concentrations averaged 0.09±0.03 μg/ml. The quantification limit of 0.078 μg/ml fitted quite favourably with these data.

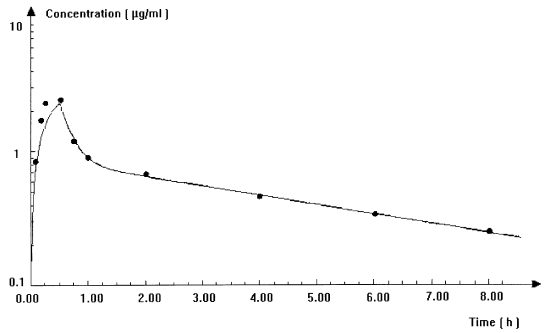

Fig. 3. Pharmacokinetic profile from simulation of a 30-min intravenous infusion of 200 mg of ciprofloxacin in an in vitro pharmacokinetic/pharmacodynamic model using Mueller–Hinton broth.

4. Application

The method described was successfully applied to the analysis of M.–H.B. samples from an in vitro PK–PD model as described by Blaser et al. [19] and Mouton et al. [20].

Fig. 3 showed the pharmacokinetic profile from simulation of a 30-min intravenous infusion of 200 mg of ciprofloxacin in an in vitro PK–PD model.

Table 2
Intra-assay and inter-assay precision and accuracy of ciprofloxacin determination in plasma and Mueller–Hinton broth

Theoretical concentration (μg/ml)	Plasma				Mueller–Hinton broth			
	n	Concentration found (mean±S.D.) (μg/ml)	R.S.D. (%)	Accuracy (%)	*n*	Concentration found (mean±S.D. (μg/ml)	R.S.D. (%)	Accuracy (%)
Intra-assay								
0.078	9	0.081±0.026	3.19	+4.36	7	0.076±0.001	1.04	−1.92
0.312	9	0.304±0.011	3.58	−2.53	7	0.308±0.002	0.81	−1.12
1.25	8	1.23±0.01	0.73	−1.55	7	1.24±0.01	0.50	−0.16
5.00	8	4.94±0.19	3.99	−1.13	7	5.20±0.09	1.82	+4.04
10.0	8	10.10±0.15	1.54	+0.99	7	9.73±0.03	0.34	−2.64
Inter-assay								
0.078	8	0.076±0.003	5.00	−2.00	8	0.077±0.005	7.07	−0.38
0.312	8	0.302±0.076	2.54	−3.23	8	0.315±0.010	3.19	+1.25
1.25	8	1.21±0.02	2.10	−3.44	8	1.24±0.04	3.37	−0.06
5.00	8	5.06±0.26	5.15	+1.22	8	4.98±0.14	2.88	−0.44
10.0	8	9.78±0.20	2.05	−2.15	8	9.64±0.20	2.09	−3.54

Extensive treatment of these experimental data will be the subject of a further publication.

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