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High performance capillary electrophoresis (HPCE) determination of FR 900098, an anti-malarial compound, in aqueous solutions and human serum

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ABSTRACT

A high performance capillary electrophoresis (HPCE) method with UV detection at 214 nm was developed for the assay of FR 900098 (a promising better antimalarial drug in the class of fosmidomycin). Optimization of the analytical parameters resulted in an organic modifier (methanol) concentration of 15%, 0.2 mM hexadecyl trimethyl ammonium bromide (HTAB) and phosphate buffer (14 mM KH_2PO_4/56 mM K_2HPO_4) with adjusted PH to 10.9 by 1M sodium hydroxide. Under these conditions the migration time of FR 900098 was 7.6 minutes in human serum. Validation of the analytical method showed good recoveries, precision and accuracy. The limit of detection (LOD) and limit of quantification (LOQ) in human serum was 0.5 µg/ml and 1.0 µg/ml respectively. FR 900098 solutions were stable for short time (a day), freeze-thaw and long term (six months). This technique will be useful as a fast and accurate analytical method for FR 900098 pharmaceutical dosage assays and pharmacokinetic studies.

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1. INTRODUCTION

Worldwide many people (especially those living in developing countries) are affected by malaria with an estimated 200-450 million cases and 0.5 to 3 million deaths per annum. Hence new, safe and effective antimalarial drug discovery is a priority area of research due to resistance development to the currently available drugs and mosquitoes resistance to the available insecticides [1].

FR 900098 is an antimalarial compound in development showing promising activity *in vitro* and *in vivo*. It belongs to the class of fosmidomycin antimalarial compounds. Chemically it is a methyl derivative of fosmidomycin as shown in Figure 1. It has antimalarial activity twice more than the parent compound fosmidomycin with an IC_{50} value ranging from 70-180 nM in different strains of Plasmodium falciparum [2].

It was first isolated as an antibiotic from *Streptomyces rubellinomurinus* active against gram negative bacteria (P*seudomonas, Proteus*, *Escheria coli*) [3]. It has also antimicrobial activity against gram positive bacteria and is found to be safe in acute toxicity studies [4].

Regarding antimalarial mechanism of action, these compounds act on 1-deoxyl-D-xylulose 5-phosphate(DOXP) reductoisomerase enzyme, which is a non mevalonate pathway to isoprenoids

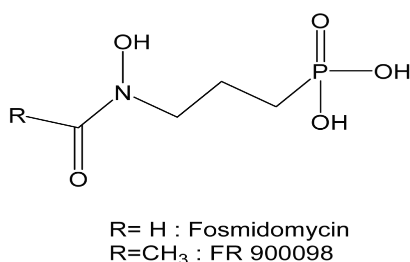

Figure 1. Chemical structures of Fosmidomycin and FR 900098.

present in many bacteria, algae, plants and the malarial parasite Plasmodium falciparum [5]. As this pathway is absent in humans and animals which depend on mevalonate pathway of isoprenoid biosynthesis, these drugs are assumed to be safe. This pathway is localized inside a plastid like organelle called apicoplast which is a drugs target for many parasitic diseases like infection by malaria (Plasmodium); toxoplasma (leading opportunistic infection in AIDS); congenital neurological birth defects; and Eimeria (significant disease of poultry and cattle) [6].

The model drug in this class of chemicals, fosmidomycin, underwent pharmacokinetic evaluation by biological assay using the microorganism *Enterobacter cloacae* [7-9]. Although the same microorganism was used in the previous pharmacokinetic studies, the bioassay method has been detailed recently [10]. But biological assay methods generally suffer complexity, less precision and inability to attribute the required effect to a particular compound [11]. Hence relying on these methods in drug monitoring and pharmacokinetics studies of antimalarial drugs where the case management requires combination drug therapy is vague [12]. For such purpose, validated HPCE analytical method has been developed by our group for fosmidomycin [13].

For further biological and/or chemical evaluation of FR 900098, availability of very sensitive, validated analytical method is of paramount significance. Thus here, we report an analytical method using high performance capillary electrophoresis (HPCE) for determining FR 900098 in aqueous and spiked serum samples.

2. EXPERIMENTAL

2.1. Materials and reagents

FR 900098 [3-(N acetyl-N-hydroxyamino) propyl phosphonic acid monosodium salt] was synthesized by a combination of the methods mentioned elsewhere [14, 15]. Dichloromethane and acetonitrile, HPLC grade, were from Merck (Darmstadt, Germany); hexadecyl trimethyl ammonium bromide (HTAB) was obtained from Fluka (Buchs, Switzerland) and methanol was from Prolabo (VWR international, SAS, Fontenay-Sous-Bois, France). Phosphate salts and sodium hydroxide are from Merck (Darmstadt, Germany).

2.2. High performance capillary electrophoresis system

The electrophoresis experiments were carried out using a Beckman PACE MDQ system (Beckman-coulter, Fullerton, CA, USA) with direct UV detection at 214 nm wavelength. The thermostat of the capillary cartridge system was set at 25°C. Fused silica capillary having 61 cm total length (50 cm to detection window) with 75 µm i.d. was used. The capillary when new was equilibrated with the buffer system for 2 hours. Otherwise for day to day experiments, it is equilibrated for 30 minutes. Before sample injection, it was rinsed with 0.1 M NaOH and then ultra pure water for 10 minutes each at 20 psi. Sample injection was by hydrodynamic means at pressure of 0.5 psi for 15 s. Before the injection of the first sample and between two runs the capillary was rinsed successively with 0.1 M NaOH and the working buffer for half minute each. Finally samples underwent electrophoresis at 15 kV voltage in a reverse mode from the cathode towards the anode.

2.3. Electrophoresis buffer

The optimized buffer composition is 14 mM KH_2PO_4/56 mM K_2HPO_4, 0.2 mM HTAB and 15% methanol. PH was adjusted to 10.9 using 1 M NaOH. The observed current is 150 µA in aqueous and 130 µA in serum samples.

2.4. Sample preparation

Serum

A method developed by Rudrick and Bawdon was employed with slight modification [16]. Briefly,

serum samples (500 µl) were deproteinized by mixing with acetonitrile (500 µl). The mixture was vortexed briefly. Then, the tubes were mixed by a rotating shaker at 20 rpm for 10 minutes. This was followed by centrifugation (2000x g) for ten minutes. The supernatant was taken and mixed for 10 minutes at 20 rpm with 3.5 ml dichloromethane to remove the excess of acetonitrile followed by centrifugation (2000x g) for 10 minutes. The resulting top layer was used for analysis after passing it in ultra free-mc filter units (molecular mass cut off 5000 Da, Milliford Corporation, Bedford, MA, USA) for 15 minutes at 23000x g. Samples were stored either in refrigerator or at 10°C in the storage compartment of the PACE MDQ CE system before analysis. Sample analyses were carried out by placing 150 µl of the sample in the sample holder vials and then pressure injection at 0.5 psi for 15 seconds.

2.5. Validation procedure

2.5.1. Ohms law

This law where V=IR, was applied to determine the optimum voltage used.

2.5.2. Serum

2.5.2.1. Linearity and accuracy

Seven linearity curves were determined with seven concentrations (2.5, 5, 7.5, 25, 50, 75, 100 µg/ml) and three concentrations were used as quality control samples (1, 10, 100 µg/ml)

The accuracy was determined as the deviation of the mean results obtained by the method from the spiked concentrations determined with the mean calibration curves.

2.5.2.2. Limit of detection and quantification

The limit of quantification (LOQ) was the minimum FR 900098 concentration resulting in an accuracy of higher than 80% as determined with the standard mean curve.

The limit of detection (LOD) in the spiked human serum was defined as the minimum detectable concentration with a signal to noise ratio of three.

2.5.2.3. Precision

Repeatability

In order to assess the repeatability of injection, each of three samples containing 1, 10, 100 µg/ml FR 900098 were measured ten times. Between the two runs the capillary was rinsed as described in the sample preparation protocol.

Day to day and intraday reproducibility

The day to day reproducibility was determined on serum samples spiked with three different concentrations (100, 10, 1 µg/ml). For each concentration, eleven samples were extracted and analyzed one by one at different days.

The intraday reproducibility was studied by analyzing eleven aliquots of the same concentration simultaneously extracted of from 500 µl of each three spiked serum concentrations at 1, 10, 100 µg/ml.

2.5.2.4. Extraction recovery

The recovery of the extraction procedure was defined as the ratio of the corrected area determined after extraction to the corresponding corrected area determined in water at the same concentration ($n = 7$). The recovery was assessed for three concentrations (1, 10, 100 µg/ml).

2.5.2.5. Stability

Short term stability

Short term stability (24 h) of three concentrations of FR 900098 (1, 10, 100 µg/ml) in human serum was assessed at room temperature and at 4°C. At each time, samples were extracted in triplicate and the stock solution was stored at room temperature or at 4°C.

Long term stability

Long term stability in serum was determined at three concentrations (1, 10, 100 µg/ml). At day 0, samples were extracted in triplicate and for each concentration the resulting solutions were divided before freezing at -80°C. For each time point, three aliquots of each concentration were thawed and extracted until 6 months of storage.

Freeze thaw stability

Freeze thaw stability was assessed with three concentrations (1, 10, 100 µg/ml). At day 0 samples were extracted in triplicate immediately after the preparation of the solution and before freezing at -80°C. The solutions were thawed at room temperature, and aliquots extracted in triplicate at day 1, 2, 3 and 4. On each day the solutions were refrozen.

2.5.2.6. Exogenous interferences

Various molecules with a potential to be co-administered with FR 900098 (Clindamycin, Clarithromycin, hydroxy clarithtromycin and rifampicin) and used in blood sampling tubes as anticoagulant, EDTA(K3E, BD, Becton Dickson) were assessed for their potential interference in FR 900098 HPCE assays.

2.6. Analyzing and integrating data

Peak integration was performed using the 32 karat ™ software (Beckman-coulter). To compensate for potential mobility changes between the single runs, results were expressed as corrected areas (Ac), which correspond to the ratio of the peak area to the migration time (Arbitrary units).

3. RESULTS

3.1. Ohms law

The optimal voltages that obey Ohms law called ohmic conductor or a linear conductor is between 10 and 15 kV as shown in Figure 2.

3.2. Migration time and specificity

Typical electropherogrames of human serum spiked with 1 µg/ml, 10 µg/ml, and blank serum are shown in Figures 3-5. The migration time for FR 900098 is 7.6 minutes. There were no interfering endogenous signals with FR 900098 peak as seen in the diagrams.

3.3. Stability of stock solution

Both long term (6 months) and short term stability of three concentrations (1, 10, 100 µg/ml) were assessed. At day 0, samples were extracted in triplicate, and for each concentration the resulting solutions were divided before freezing at -80°C. After 6 months of storage, the differences of the mean corrected area to the corresponding value of day 0 were +1, -4.7, -6.2 % for concentrations 1, 10, 100 µg/ml respectively as shown in the Table 2.

3.4. Serum

3.4.1. Linearity and accuracy

The assay was linear from concentrations ranging from 2.5 µg/ml to 100 µg/ml. The correlation coefficient of the mean calibration (n = 7) curve was R^2 = 0.99997 and the equation was y = 0.0565 + 0.0070 x, where x correspond to the corrected area measured by HPCE for each assay. The accuracy as determined by the coefficient of variation (CV) of the quality control samples from the mean standard calibration curve is

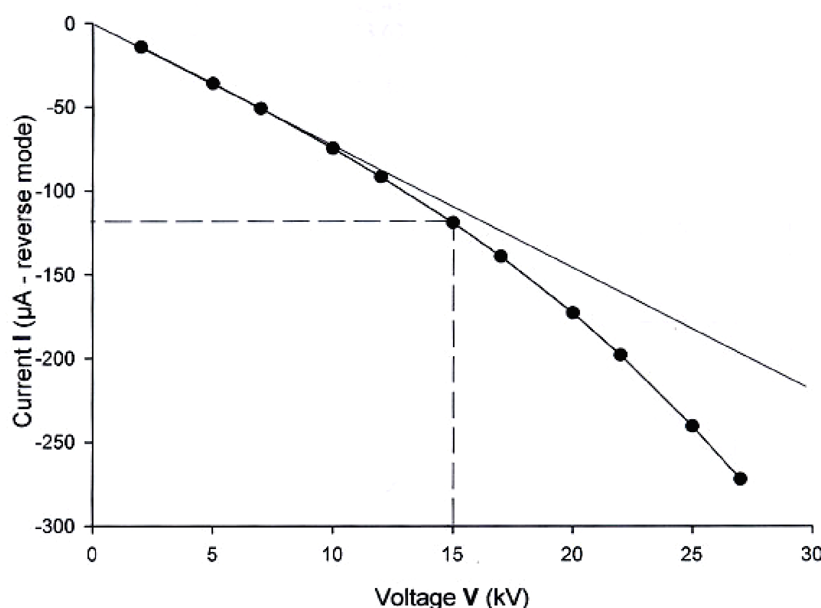

Figure 2. Ohms Law plot of Current versus Voltage.

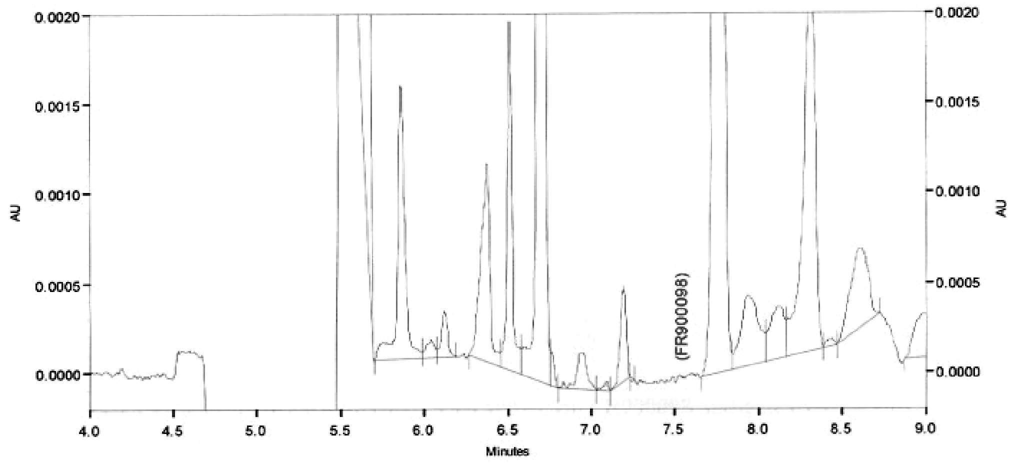

Figure 3. Blank serum control run.

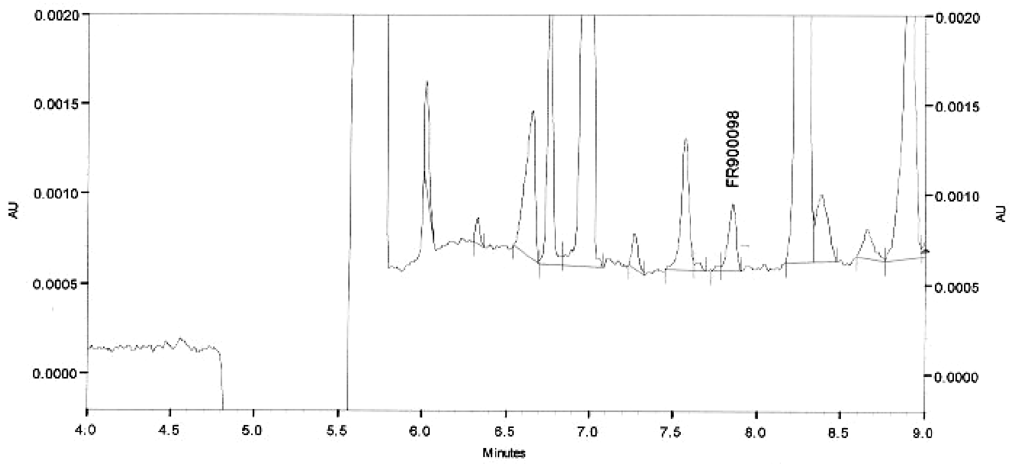

Figure 4. Serum sample spiked by 1 µg/ml FR 900098.

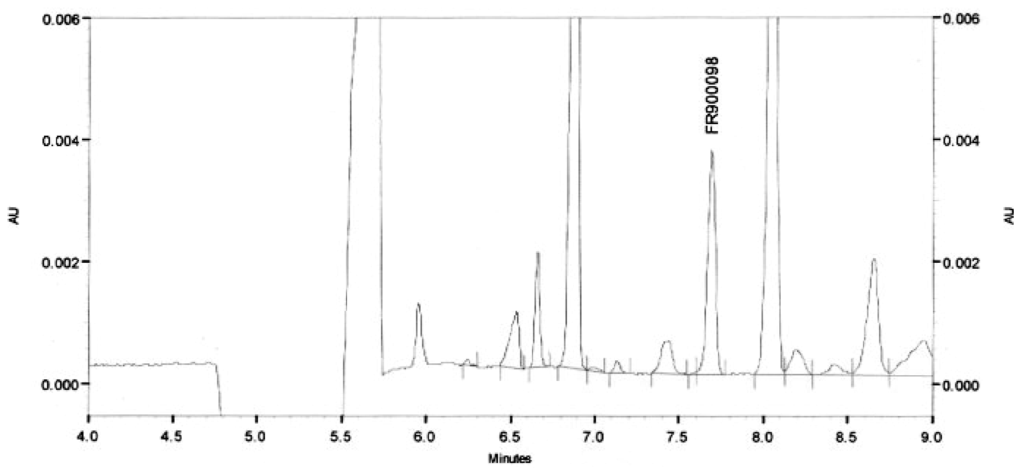

Figure 5. Serum sample spiked by 10 µg/ml FR 900098.

Table 1. Short term stability of FR 900098 in serum at room temperature and 4°C.

Time (hours)	Difference to hour 0 (%)					
	1 µg/ml		10 µg/ml		100 µg/ml	
	Rt	4°C	Rt	4°C	Rt	4°C
1	+2.5	+1.3	-4.6	-2.1	-3.1	-3.5
2	+3.2	-6.3	-2.7	-2.2	-2.0	-2.2
4/5*	+10.6	+0.3	-2.1	-3.7	-1.2	-0.3
7	-0.1	-0.5	-1.4	-0.1	-1.2	+5.9
24	-1.7	-3.7	-3.7	-2.2	-0.9	+1.5

Rt: room temperature, *: done only for 1 µg/ml Rt

Table 2. Long term and freeze thaw stability of FR 900098 in human serum.

Time	Difference to day 0 (%)					
	1 µg/ml		10 µg/ml		100 µg/ml	
	Long term	Freeze thaw	Long term	Freeze thaw	Long term	Freeze thaw
Day 1	+2.1	+2.4	-5.0	+1.1	-8.8	+1.1
Day 2	-0.7	-1.8	-3.6	-0.4	-7.0	-2.9
Day 3		-0.6		-4.6		-5.3
Day 4	+2.6	-2.5	+0.2	+1.0	-7.9	-5.1
Day 8	+8.0		-0.2		-2.2	
Day 15	+5.0		+1.2		+0.1	
Month 1	+3.8		+0.4		-1.1	
Month 2	+2.7		-5.9		-8.7	
Month 5	+2.4		-5.4		-6.3	
Month 6	+1.0		-4.7		-6.2	

0.91%, 0.13%, 3.06% for 100, 10, and 1 µg/ml respectively. For other concentrations the percent deviation has been indicated in Table 3.

3.4.2. Limit of detection and quantification

The limit of detection of the method in HPCE system is 0.5 µg/ml. The LOQ value was 1.0 µg/ml with an accuracy of 97.00% as shown in Table 4.

3.4.3. Precision

Repeatability

The coefficient of variation between multiple injections (n = 10) were 0.7, 0.9, 4.2% for concentrations 100, 10 and 1 µg/ml respectively in aqueous solutions. For serum spiked samples of FR 900098 the CV is 1.8, 1.7 and 4.4% at the concentrations 100, 10, and 1 µg/ml in the same order.

Day to day reproducibility

The day to day reproducibility resulted in coefficient of variation of 2.1, 2.0, and 3.2% for 100, 10 and 1 µg/ml respectively (n = 11).

Intraday reproducibility

The intraday reproducibility (CV %) was 0.6, 1.8, and 4.9% for the concentrations 100, 10 and 1 µg/ml respectively (n = 11).

Recovery

The mean recovery of the extraction procedure from serum compared to aqueous solutions was 108.4, 112.7 and 101% for 100, 10 and 1 µg/ml respectively (n = 8).

Stability

FR 900098 is stable in the long term (6 months) in human serum at -80°C. It was also stable at room

Table 3. Deviation of the concentration values with the mean calibration curve in human serum spiked values.

Spiked concentration (µg/ml)	Mean concentration determined with the mean standard curve	Deviation (%)
100	99.98	0.02
75	74.29	0.95
50	49.76	0.48
25	24.83	0.68
7.5	7.71	2.80
5	5.07	1.40
2.5	2.53	1.20

Table 4. Deviation of the concentration values with the mean calibration curve in quality control (QC) samples.

Quality control samples (µg/ml)	Mean concentration determined with the mean standard curve	Deviation (%)
100	98.63	1.37
10	9.99	0.11
1	0.97	3.00

temperature and at 4°C as shown in the Table 1. Besides, FR 900098 was resistant to loss during many cycles of freezing and thawing. After four freeze thaw cycles (4 days) 97.5, 101.0, 96.4% of FR 900098 were recovered from 100, 10, 1 µg/ml samples respectively as described in the Table 2.

3.5. Exogenous interferences

Potential interferants which can be possibly co-administered and used in blood sampling tubes as anticoagulant with FR 900098 were assessed. At the examined 10 µg/ml concentration of FR 900098 with fosmidomicin, then clindamycin, no interference was observed. The same is true for clarithromycin, hydroxyclarithromycin and rifampicin at concentrations of 50 µg/ml. EDTA (K3E, BD, Becton-Dickinson) highly interferes with FR 900098 assay thus it should not be used in blood sampling procedure for FR 900098 determination.

4. DISCUSSION

HPCE is an accepted routine analytical technique used in the analysis of drugs, metabolites as well as endogenous substances in body fluids. Although in most cases a complementary technique to chromatography, HPLC, the former is advantageous in being a micro analytical technique requiring small volumes, having excellent resolving capabilities, short analysis time, requiring less extensive sample preparation steps in some cases to the degree of using biological fluids directly and minimization of hazardous organic solvent usage [17-19]. A typical example of the better resolving power of CE over HPLC in the analysis of cephalosporins has been described by Mrestani *et al.* [20].

FR 900098 is a simple very polar compound making it an unsuitable candidate for routine reverse phase HPLC. However, it possesses ionizable functional groups in its structure (Figure 1) making it suitable for HPCE. Thus we prioritize in developing an HPCE analytical method for this molecule than HPLC.

The HPCE assay of FR 900098 offers good advantages like short analysis time, high separation and direct UV detection. Although direct detection at 214 nm is possible, other endogenous samples

being UV active at this wavelength, so many peaks are observed in the electropherogram. But FR 900098 peak is clearly separated from other peaks.

The extraction procedure allows a good recovery. The ultra filtration step is important to extend the life time of the capillary otherwise more or less similar peaks are observed by avoiding this step. Moreover it makes the analysis protocol further less time consuming and economical. The mean life time of the capillary was 200 runs.

HTAB was chosen for this electrophoresis experiment due to its negligible absorbance property by the UV detector. Higher P^H buffer is used as FR 900098 absorbance behavior in UV increases with P^H. Various buffer compositions (K_2HPO_4 and KH_2PO_4) and organic modifiers (methanol, acetonitrile) were tested. The optimized concentration is mentioned in the experimental section. FR 900098 being negatively charged at P^H 10.9, as its pKa's are at 2.0, 7.2 and 9.4 [3], its migration is from cathode towards anode because of its electrophoretic mobility slowed down by the opposite electro osmotic flow. HTAB in its optimized concentration helps to get a good peak shape and reasonable migration time.

Temperature was also set to be constant at 25°C as it affects analytical results like migration time, peak shape, separation efficiency, reproducibility and robustness. Variation in temperature can lead to change in P^H and viscosity of buffer [21].

Methanol added as organic modifier show strong influence on the migration due to modification in viscosity. The current also varies with the percentage of methanol.

The UV response of FR 900098 in serum was linear over a large range of concentration 1.0-150 µg/ml with an excellent correlation coefficient. The methods LOD and LOQ were in accordance with other HPCE methods reported [22].

CONCLUSION

A convenient HPCE technique for quantification and detection of FR 900098 in serum has been developed. The technique offers reasonable speed, sensitivity and accuracy. Therefore it can be applied in pharmacokinetic studies, therapeutic drug monitoring and analyses of its pharmaceutical dosage forms.

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