Journal of Chromatography B, 967 (2014) 98–101

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

HPTLC method for direct determination of gemifloxacin mesylate in human plasma

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ARTICLE INFO

Article history:
Received 9 December 2013
Accepted 2 July 2014
Available online 11 July 2014

Keywords:
HPTLC
Gemifloxacin mesylate
Fluorescence detection
Human plasma

ABSTRACT

Novel, simple and sensitive high performance thin-layer chromatography (HPTLC) with fluorescence detection has been successfully developed and validated for determination of gemifloxacin mesylate (GFX) in plasma samples without prior pretreatment. Montelukast (MK) was used as internal standard. GFX and MK in plasma samples were separated using a mobile phase consisting of a mixture of ethyl acetate:methanol:25% ammonia, (8:4.5:3, v/v/v). The emission intensity was measured using optical filter K400 after excitation at 342 nm. The R_f values for GFX and MK were 0.45 ± 0.03 and 0.79 ± 0.02, respectively. Under the optimum conditions, a linear relationship with good correlation coefficient ($r = 0.9965$, $n = 6$) was obtained in concentration range of 3–180 ng/band. The LOD and LOQ of the proposed method were 0.45 and 1.5 ng/band, respectively. The accuracy of the method was proved as the recovery % of GFX from spiked human plasma was 94.21–101.85%. The efficiency of the proposed method was confirmed by in-vivo application on human plasma in real patient samples. Moreover, the stability of GFX in plasma was carefully tested at different conditions and compared to others in aqueous solution.

1. Introduction

Gemifloxacin mesylate (GFX) is a fourth generation oral broad-spectrum fluoroquinolone antibacterial agent (Fig. 1a). It acts by dual inhibition of both DNA gyrase and topoisomerase IV, which are essential for the cellular replication and bacterial growth [1]. GFX was approved by the Food and Drug Administration (FDA) for the treatment of acute bacterial exacerbation of chronic bronchitis, mild-to-moderate pneumonia, multi-drug resistant pneumonia and community-acquired pneumonia [1,2]. Therefore, GFX has become unique among fluoroquinolone antibacterial agents and is expected to be the most clinically used one worldwide.

Different analytical methods have been developed for the quantitative determination of GFX in plasma by spectrofluorometry [3,4], LC–MS–MS [5], HPLC [6] and HPTLC [7]. Indeed, these procedures not only involved samples pretreatment, but also the cost and complexity of the instrumentation limited their applications in clinical laboratories.

HPTLC has different applications in many fields of analytical sciences [8]. A part from these applications is the determination of the active ingredients in pharmaceutical formulations, or in biological fluids with competition to HPLC methods. Most of the reported HPTLC methods in the areas of therapeutic drug monitoring, clinical pharmacokinetic and bioequivalence studies used the plasma samples after its pretreatment either by protein precipitation [7,9] or liquid–liquid extraction [10,11] methods. Indeed, the multiple extraction procedures increase the overall analysis time and negatively affect the accuracy of the method. Therefore, the need for new reliable, simple, selective, sensitive and accurate HPTLC method that can overcome the pretreatment steps of plasma samples is still urgently needed.

HPTLC is strongly polar stationary phase that can retain the highly polar contents of plasma by an appropriate selection of the mobile phase composition. Hence, this observation can be applied for determination of moderately polar or non-polar analytes in plasma directly without pretreatment using silica gel as a polar stationary phase.

Therefore, the present work describes, for the first time, a novel simple, sensitive and selective HPTLC method to avert pretreatment of plasma samples for the determination of GFX using fluorescence detection.

2. Experimental

2.1. Materials

Analytically pure GFX (99.8%) was provided from Tabuk Pharmaceutical Manufacturing Co., KSA and used as received without

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http://dx.doi.org/10.1016/j.jchromb.2014.07.004
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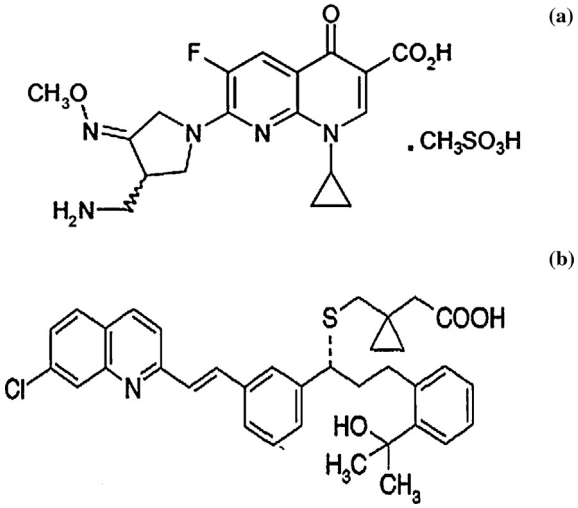

Fig. 1. Structure of (a) gemifloxacin mesylate and (b) montelukast.

further purifications. Montelukast (MK, Fig. 1b) (99.7%) was purchased from European Egyptian Pharmaceuticals, Egypt. HPLC grade methanol was purchased from Sigma–Aldrich, Seelze, Germany. Blank human plasma samples were supplied from Assiut University Hospitals (Assiut, Egypt). Other solvents and materials used throughout this study were of analytical grade.

2.2. Instrumentation

Sample application was done by using Camag Linomat V applicator and Camag 100 μl syringe (Camag, Switzerland, Germany). The chromatographic separation was performed on a HPTLC precoated silica gel Plate $60F_{254}$ (20 cm × 20 cm, 6–8 μm thicknesses) (Merck, Darmstadt, Germany) as stationary phase. The sample was sprayed in the form of narrow bands of 4 mm length at a constant rate 3 μl/s and 21 tracks can be applied on the same plate. The development was carried out in trough glass chamber (24 cm W × 14 cm H × 9.5 cm D, standard type) (Sigma–Aldrich Co., USA) and the development times does not exceed 10 min. The densitometric scanning was performed by using Camag TLC scanner III system equipped with Hg lamp and optical filters for the fluorescence detection. The system control and data acquisition were performed by winCATS software, version 1.4.4.6337. Drug-free human plasma was stored in deep-freezer (Illshin Lab. Co. Ltd, Korea) at −80 °C till use.

2.3. Preparation of standard solutions

GFX stock solution containing 1 mg/ml was prepared in water. Working standard solutions containing 6, 30, 60, 120, 240 and 360 μg/ml were prepared by suitable dilution of the stock solution with water. The internal standard was prepared by accurately weighing 25 mg of MK then transferred into a 25 ml calibrated flask, dissolved in 10 ml methanol and completed the void volume with water to produce a stock solution of 1 mg/ml. This stock solution was further diluted with water to produce final concentration of 300 μg/ml.

2.4. Preparation of plasma samples

Into 2 ml Eppendorf tubes, an aliquot of 1 ml blank human plasma was spiked with 250 μl of MK internal standard solution (300 μg/ml). Then 250 μl from each working standard solutions of GFX (6–360 μg/ml) was added to obtain final concentrations of GFX ranging from 1 to 60 μg/ml (corresponding to 3–180 ng/band). Blank plasma was spiked with only 250 μl of MK (corresponding to 150 ng/band) and completed to 1.5 ml with water. The mixtures were vortexed for 30 s and used directly for the application step without further treatment.

2.5. Stability studies of GFX

2.5.1. Quality control (QC) samples

The stability of GFX in plasma was assessed by preparing two sets of QC samples at low (1 μg/ml) and high (60 μg/ml) concentrations in the same manner as the calibration standards and stored at −80 °C until use. Another QC samples were freshly prepared and analyzed immediately at zero time (baseline) to evaluate the accuracy and precision of this method. Bench-top stability (short term stability) was assessed the two QC concentrations (low and high) by standing on the bench-top for 4 h at room temperature (25 ± 2 °C). While, long-term stability was evaluated after storing the QC samples at −80 °C for 7 weeks before being analyzed. Freeze–thaw stability was determined at the two QC concentrations after freezing (−80 °C) for 24 h and thawing completely at room temperature (25 ± 2 °C) for three cycles [12].

All the QC samples were analyzed in triplicates, and the results were compared with those obtained from the freshly prepared samples (baseline).

2.5.2. Stock solution

The stability of GFX stock solution was determined by appropriate dilution of stock solution in water to obtain low and high concentrations at 1 and 60 μg/ml, respectively and analyzed at baseline, after storage for 48 h at room temperature (25 ± 2 °C), or after storage at −80 °C for 7 weeks.

2.6. Application to real plasma samples

Five recently hospitalized patients (Chest department, Assiut University Hospitals) were enrolled in this study to confirm the applicability of the developed method to quantify gemifloxacin in future bioequivalence and/or bioavailability studies. The patients received Factive® tablets (Oscient Pharmaceuticals, USA) equivalent to 320 mg gemifloxacin/tablet as their normal drug regimen. The blood samples were drawn after 1 h (T_{max} of GFX) from the patients and placed in stoppered tubes containing K_2EDTA as anticoagulant. After centrifugation, plasma samples were separated and immediately stored in deep-freezer at −80 °C till the analysis without further handling.

2.7. Chromatographic conditions

Mobile phase consisted of ethyl acetate:methanol:25% ammonia (8:4.5:3, v/v/v) was used for the development step. Linear ascending development was carried out in trough glass chamber that was pre-saturated with the vapour of freshly prepared mobile phase for at least 20 min at room temperature (25 ± 2 °C). The HPTLC plate scanning was performed using fluorescence detection. The emission intensity was measured using optical filter K400 after excitation at 342 nm.

3. Result and discussion

3.1. Optimization of chromatographic conditions

3.1.1. Mobile phase

The separation of GFX could be adjusted by changing the composition of the mobile phase. Initially individual solvents like methanol, hexane, chloroform, and ethyl acetate were tested. The results showed that GFX was developed with methanol and ethyl

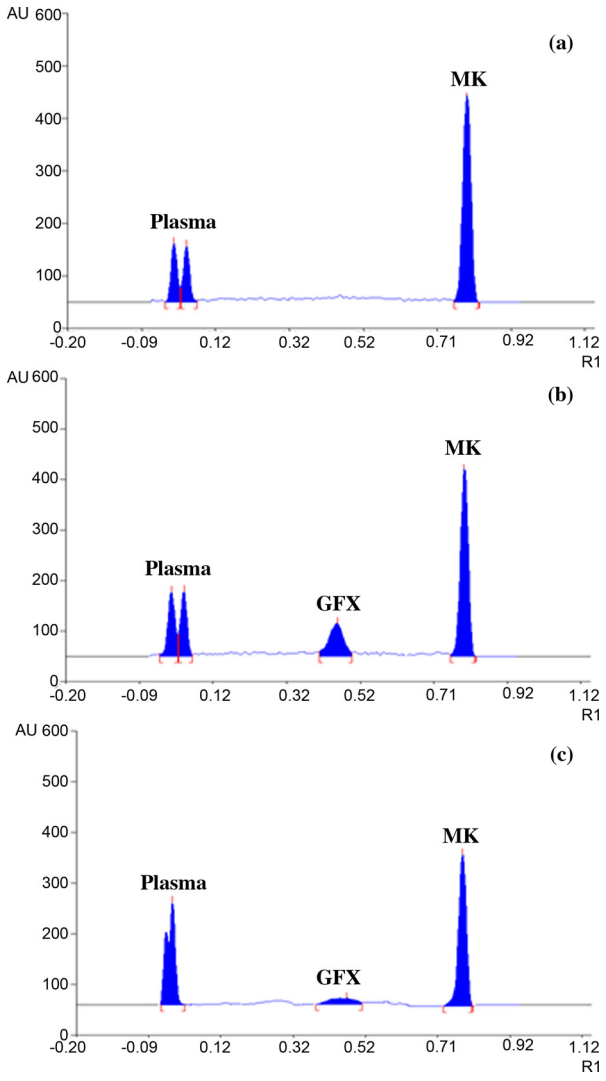

Fig. 2. HPTLC-densitogram of (a) blank human plasma spiked with MK (150 ng/band), (b) plasma sample spiked with GFX (6 ng/band) and MK (150 ng/band) and (c) human plasma obtained 1 h after oral administration of GFX in presence of MK (150 ng/band) as internal standard.

acetate but with unsuitable R_f value (lower than 0.2). Different ratios of ethyl acetate and methanol were investigated. The results indicated that the best R_f value was achieved when the ratio of ethyl acetate:methanol was 8:4.5 (v/v) but tailing was observed. Several experiments have shown that the employment of an appropriate ratio of acid or base in the mobile phase may improve the chromatographic peak resolution [13]. The acidic pH of the mobile phase was also tested using either acetic or phosphoric acid and was found that they significantly reduce the fluorescence intensity and broaden the chromatographic peak. Thus, the acid was replaced with ammonia solution where different concentrations of ammonia (10, 15, 20, 25 or 33%) were tested. The results showed that 25% ammonia was able to improve the shape of the chromatographic peak of GFX and resulting in more symmetric peak. Further experiments were performed and finally the optimum mobile phase was consisted of ethyl acetate:methanol:25% ammonia (8:4.5:3, v/v/v). Fig. 2b shows a sharp and well-defined symmetrical peak of GFX at $R_f = 0.47 \pm 0.03$ using the selected mobile phase system.

3.1.2. Fluorescence detection

GFX has a native fluorescence spectrum exhibiting two excitation maxima at 272 and 342 nm and one emission at 391 nm. The fluorescence intensity obtained at λ_{ex} 272 nm was more than that obtained at λ_{ex} 342 nm. Thus, the first trial was performed by excitation at 272 nm and optical filter K400 but it was found that the peak of GFX was inverted. This may be due to that the HPTLC plates with fluorescence indicator (F_{254}) absorb in wavelength range of 250–300 nm, resulting in fluorescence quenching [14]. So, λ_{ex} 342 nm and optical filter K400 were selected in this study for direct measurement of the fluorescence intensity of GFX.

3.1.3. Internal standard

A widely used method of quantitation involves the addition of an internal standard to compensate several of the analytical errors. Thus different fluoroquinolones (levofloxacin, gatifloxacin, ciprofloxacin, sparafloxacin and norfloxacin) were tested as internal standards in order to show its reliability. The results showed that these fluoroquinolones were unsuitable as internal standards due to significant difference in their excitation and emission wavelengths and because they had poor peak resolution with GFX. Therefore, other different fluorescent drugs such as itraconazole, ketoconazole, montelukast, naproxen, paroxetine and lisinopril were tested. Montelukast (Fig. 1b) was measured at 400 nm after excitation at 350 nm. Therefore, MK was found to be the ideal one, as its excitation and emission wavelengths are closed to those of GFX. Also, good resolution between GFX (0.47 ± 0.03) and MK (0.79 ± 0.02) was achieved under the applied chromatographic conditions (Fig. 2). So, MK was selected for all the subsequent experiments as an internal standard.

3.2. Analytical method validation

The procedure was fully validated in accordance with ICH guidelines [15]. All results were expressed in percentages, where n represents the number of values. For the statistical analysis Excel 2007 (Microsoft Office) was used. A 5% significant level was selected. The developed HPTLC method was validated for the following parameters:

3.2.1. Calibration curve and linearity

Calibration curve was constructed by plotting the peak area ratio versus different concentrations of GFX in plasma. The calibration curve shows a good correlation coefficient (0.9965) over the concentration range 3–180 ng/band with respect to raw volume. The parameters for the analytical performance of the proposed method are summarized in Table 1. Allen et al. [16] and Gee et al. [17] studied the pharmacokinetic parameters of GFX in healthy volunteers after a single oral dose administration. These methods indicated that the maximum concentration (C_{max}) of the drug was achieved approximately 1 h after dosing and the mean C_{max} value was found as 1.48 ± 0.39 μg/ml and 2.33 ± 0.50 μg/ml; respectively following a single oral dose of 320 mg GFX. Therefore, the drug level in plasma is within the working linearity range of the proposed method. By application in real samples, it was found that GFX can be detected clearly and well separated from the endogenous plasma constituents that indicate the efficiency of the proposed method.

Table 1
Quantitative parameters and statistical data for determination of GFX by the proposed HPTLC method.

Parameter	HPTLC-FL
Range (ng/band)	3–180
Intercept (a) ± SD[a]	$0.029 \pm 3.2 \times 10^{-3}$
Slope (b) ± SD[a]	$0.021 \pm 3.8 \times 10^{-5}$
Correlation coefficient (r)	0.9965
LOD (ng/band)	0.45
LOQ (ng/band)	1.51

[a] Mean of six determinations.

Table 2
The accuracy and precision of the proposed method at three concentration levels of GFX.

GFX conc. (ng/band)	Recovery (%) ± SD[a]	Intra-day precision		Inter-day precision	
		Mean ± SD[a]	%RSD	Mean ± SD[a]	%RSD
3	94.21 ± 3.51	98.96 ± 2.49	2.25	98.41 ± 3.47	3.52
30	101.85 ± 1.93	101.13 ± 1.71	1.69	99.99 ± 1.95	1.95
180	98.59 ± 1.52	98.30 ± 1.49	1.51	98.41 ± 1.46	1.49

[a] Mean of six replicates.

Table 3
Stability of GFX under various storage conditions.

Concentration (μg/ml)	[*]Stability (%) ± SD				
	Plasma samples			Stock solution[a]	
	Short-term (4 h)	Long-term (7 weeks)	Freeze–thaw cycle	48 h at room temp.	7 weeks at −80 °C
1	98.12 ± 1.77	99.64 ± 1.91	96.34 ± 1.56	99.17 ± 0.85	100.86 ± 1.84
60	99.29 ± 1.63	96.63 ± 1.83	97.66 ± 1.49	99.90 ± 0.99	102.97 ± 1.70

[a] GFX stock solution, 100 μg/ml in water.
[*] Stability (%) represents the recovery (%) and equals mean measured concentration ($n = 3$) at the indicated time divided by mean measured concentration ($n = 3$) at baseline × 100.

3.2.2. Limits of detection and quantitation

The values obtained for the limits of detection (LOD) and quantitation (LOQ) were indicative of the high sensitivity of the method. The LOD and LOQ values were 0.45 and 1.51 ng/band, respectively (Table 1). Thus, this method is more sensitive than the reported HPTLC [7] method. This may be due to the benefit of the fluorescence detection compared to UV detection used in the reported one.

3.2.3. Accuracy and precision

The accuracy of the method was determined by investigating the recovery of GFX at three concentrations levels covering the specified range (six replicates of each concentration). The results shown in Table 2 depict good accuracy and recovery percentage ranged from 94.21–101.85% that indicates the high efficiency of the proposed method for extraction of GFX without pretreatment. Therefore, this method is superior to the other reported methods introduced for determination of GFX either in spiked [7] or real [18] plasma samples which yielded low % recovery ranged from 80–86 or 68–71% respectively. In addition to the small values % RSD point to high precision of the proposed method.

3.2.4. Selectivity

Selectivity of the method was evaluated by analyzing blank, spiked and real human plasma samples containing GFX in presence of MK as internal standard. The densitograms were visually examined for any interfering peaks from the endogenous compounds. It was found that there were no interfering peaks from the biological matrix. In addition, the R_f values of both, GFX and MK remained unchanged in presence of plasma. This indicates that GFX can be determined in plasma without any pre-treatment.

3.2.5. Stability

Plasma QC samples of GFX at two concentrations (1 and 60 μg/ml) were used for the stability experiments. The results shown in Table 3 indicate that no significant degradation occurred at these conditions. Thus, it is suggested that the plasma samples containing GFX can be handled under normal laboratory conditions without any significant loss of compound.

Stock solution of GFX, stored at room temperature (25 ± 2 °C) for 48 h and at −80 °C for 7 weeks, were stable under the studied conditions and there was no significant changes in the peak area of analytes (Table 3).

4. Conclusion

The high sensitivity attained by the proposed HPTLC method allowed the determination of GFX in plasma. The method introduces an innovative idea which is able to determine GFX without prior pretreatment from plasma. The statistical analysis confirms the absence of any interference from the plasma components with high throughput of GFX. Thus, it would have a great value when it is applied in the pharmacokinetic and bioavailability studies of GFX. Furthermore, this result proves the suggested concept that the polar stationary phase of HPTLC plates will be a powerful tool for determination of moderately polar and non-polar drugs in plasma.

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