ORIGINAL ARTICLE

DEVELOPMENT AND VALIDATION OF AN HPLC-UV METHOD FOR THE DETERMINATION OF GATIFLOXACIN IN BULK MATERIAL, PHARMACEUTICAL FORMULATIONS, HUMAN PLASMA AND METAL COMPLEXES

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ABSTRACT

A simple reversed phase HPLC method was developed for the quantitative determination of gatifloxacin (GTX) in the bulk material, pharmaceutical formulations and human serum using Mediterranea C18 (25 x 0.46mm, 5µm) column. The mobile phase, acetonitrile, methanol and water (40:40:20 v/v pH 2.7 adjusted by phosphoric acid), was delivered at a flow rate of 1.0 ml/min. The eluent was monitored using spectrophotometric detection at 286 nm. The method is specific to GTX and able to resolve the drug peak from formulation excipients and metal impurities. The method is accurate (99.18-101.87%), precise (intra-day variation 0.14-1.67% and inter-day variation 0.32-1.80%) and linear within the range 0.1-25µg/ml (R^2=0.999) concentration and was successfully used in monitoring left over drug in drug-metal complexes. The detection limit of GTX at a signal-to-noise ratio of 3 was 1.73 ng/ml in human plasma while quantification limit in human serum was 5.77 ng/ml. The proposed method is applicable to routine analysis of GTX in pharmaceutical formulations as well as in human plasma samples.

Keywords: Gatifloxacin; metals interaction; HPLC-UV analysis; magnesium; calcium; chromium; manganese; iron; cobalt; nickel; copper; zinc; cadmium.

INTRODUCTION

Gatifloxacin or 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-[3-methyl-1-piperazinyl]-4-oxo-3-quinolinecarboxylic acid (figure 1), is a synthetic broad-spectrum antimicrobial fluoroquinolone active against both Gram-negative and Gram-positive organisms and is used in the treatment of a wide range of infections (Blondeau et. al., 2000). The distinct difference between gatifloxacin and other fluoroquinolones is the methoxy group at position 8. It is believed that this group mediates the binding of the DNA-DNA gyrase complex to the DNA-topoisomerase complex and potentially decreases the likelihood of high-level resistance (Grasela 2000).

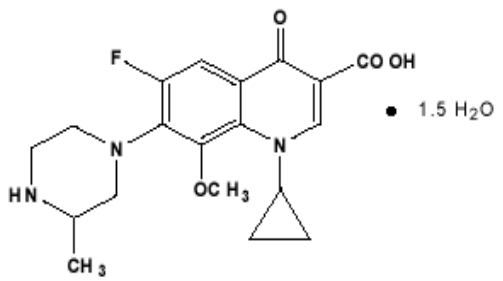

Figure 1

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Its absolute bioavailability is 96% (Nakashima *et al*., 1995; Hosaka *et al*., 1992). A number of methods have been reported for analysis of GTX in bulk drug pharmaceutical, dosage forms as well as in biological fluids such as electrospray tandem mass spectroscopy (Vishwanathan *et al*., 2001), fluorimetry (Borner *et al*., 2000), HPTLC (Shah *et al*., 2004), UV-spectrophotometry (Salgado and Olivira 2005), HPCE (Zhu *et al*., 2002), HPLC (Nguyen *et al*., 2004 ; Shirke and Nandini 2004; Saleh Al-Dgither *et al*., 2006) and microbiological assay (Salgado *et al*., 2006). In the present paper HPLC method using a C18 column was developed and validated for analysis of equivalence studies of different brands of gatifloxacin (available in Pakistan) and quantification in serum for therapeutic purposes, as well as to study gatifloxacin-metal interaction. The advantage of the proposed method is that a single method can be used for assay, clinical studies and for metal interaction studies. In addition, the method uses friendly mobile phase (MeCN:MeOH:H_2O) which is not corrosive to the column like tetrabutylammonium acetate and citric acid as used by earlier workers (Liang *et al*., 2002; Mandal *et al*., 2004; Jandera *et al*., 1980).

Metal ions play a vital role during the biological process of drug utilization in the body. A study that supported the binding of a clinically useful quinolone to the DNA gyrase

A subunit, which is mediated by Mg^{2+}, where in keto-carboxylic group of the quinolone, coordinated to Mg^{2+}, would point towards the enzyme (Reece and Maxwell 1991). Another metal ion such as Ca^{2+}, is known to interact with gyrase, at least partially during the catalytic cycle (Sissi *et al*., 2001). However chelation by multivalent cations is a concern for the fluoroquinolone class as they successfully provide various active potential donor sites, namely 4-carbonyl group and 3-caroxylic acid functionalities, which are excellent sites for chelation with divalent or trivalent metals (Domagala, 1997). In a series of structure-activity studies, the importance of a free carboxylic group for binding to active site (for antibacterial activity) has been emphasized, it could be anticipated that all of the quinolones will interact with metal ions. However, there may be differences between the quinolones regarding the extent of interaction. Therefore, many metal complexes of fluroquinolones like nalidix acid (Huber *et al*., 1980), sparfloxacin (Sivalakshmidevi *et al*., 2000), ciprofloxacin, enoxacin (Zhang *et al*., 2004; Lo´pez-Gresaa *et al*., 2002), norfloxacin (Wallis *et al*., 1994; Zhen-Feng *et al*., 2000), lomifloxacin (Riley *et al*., 1993; Turel 2002; Polk 1989) and gatifloxacin (Yong-Hua *et al* 2005) have been synthesized and characterized on the basis of spectral, magnetic and electrical measurements and in many cases their pharmacological effects were also studied. The object of present work was also to observe the possible effects of magnesium, calcium, chromium, manganese, iron, cobalt, nickel, cupper, zinc and cadmium on the *in vitro* availability of GTX and determination of their complexes if possible by RP-HPLC.

EXPERIMENTAL

Instrumentation

A Shimadzu HPLC system equipped with LC- 10 AT VP pump, DGU-14 AM on-line degasser, Rheodyne manual injector fitted with a 20 µl loop, Mediterranean C18 (25 x 0.46mm, 5µm) column, and SPD-10 A VP UV–VIS detector was utilized. Chromatographic system was integrated via Shimadzu model CBM-102 Communication Bus Module to PIV computer. Shimadzu CLASS-VP software (Version 5.03) was used for data acquisition and mathematical calculations.

Materials

GTX was a gift sample from Barrett Hodgson Pakistan Ltd and tiaprofenic acid from Aventis Pharma Pakistan. Four different formulations of GTX namely Quintec (Barrett Hodgson Pakistan (Pvt) Ltd), Gatcin (Sami Pharmaceutical (Pvt) Ltd Pakistan), Glax (Bosch Pharmaceutical (Pvt) Ltd Pakistan) and Rele (Helex Pharmaceutical (Pvt) Ltd., Pakistan) were obtained from retail pharmacies. Each product was labeled to contain 400 mg of GTX and had an expiry of not less than 365 days at the time of study. Acetronitrle, methanol gradient grade (Merk, Germany) and HPLC grade water were used to prepare the mobile phase. All other reagents used were of spectroscopic grade or analytical grade and obtained from BDH Laboratories Supplies (BDH Chemical Ltd., Poole, U.K).

Stock solutions

Stock solution of gatifloxacin was prepared by dissolving 10.00 mg gatifloxacin in 100 ml of 70:30 methanol and acetronitrile mixture and sonicated for 10 minutes. Stock solution of the internal standard, tripophanic acid, was similarly prepared. The concentration of both stock solutions were equivalent to 100µg/ml. Stock solution of essential metals salts ($MgCl_2.6H_2O$, $CaCl_2.2H_2O$, $CrCl_3.6H_2O$, $MnCl_2.H_2O$, $FeCl_3.6H_2O$, $CoCl_2.6H_2O$, $NiCl_2.6H_2O$, $CuCl_2.2H_2O$, $ZnCl_2$ and $CdCl_2.H_2O$) was prepared by dissolving 5mg of each metal salt in 100ml of di-ionized water to give concentrations of 50µg/ml.

Method validation

The method was validated for the parameters like specificity, range and linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, and precision. In addition, system suitability parameters were also calculated. To demonstrate specificity in the presence of excipients used in formulation, GTX was spiked (at approximately 25µg/ml) in drug product, chromatogram was observed and compared with that of raw material. To evaluate the linearity, the LOD and LOQ of the method in reference drug and in serum, different serial dilutions (0.0975, 0.195, 0.78, 1.56, 3.12, 6.25, 12.5 and 25µg/ml) were prepared from the standard stock solutions in 25ml volumetric flasks and volume made up with diluent which is mixture of 70:30 MeOH & MeCN. The samples were injected (20 µl) and signals from the samples were recorded at 2.01 minute which were compared with those of blank. LOD and LOQ values were calculated as signal-to-noise ratio of 3:1 and 10:1, respectively. To determine accuracy of the method, working standard of GTX was prepared in triplicate at three concentration levels (20, 25, and 30 µg/ml) and analyzed. Repeatability of the method was checked by analyzing six replicate samples of GTX (at the 100% concentration level) and calculating relative standard deviation (%RSD). To determine intermediate precision, standard solutions of GTX at eight concentration levels were analyzed three times within the same day (intra-day variation) and three other days (inter-day variation).

Assay in formulations

In case of marketed formulations, five accurately weighed tablets of each brand were crushed to a fine powder and an amount equivalent to 10 mg of GTX was added into different 100 ml volumetric flasks and volume was made up with MeCN & MeOH mixture. The samples were filtered through a 0.45-µm-membrane filter; different serial dilutions (3.12, 6.25, 12.5, 25µg/ml) were made from this solution in 25ml volumetric flask (containing 50µg/ml

tiaprophenic acid as Internal standard) and were injected for HPLC analysis

Assay in serum

One volume of plasma was de-proteinated by nine volume of acetonitrile and filtered through 0.45μm millipore filter paper that was used to make serial dilution of GTX (0.0975μg/ml to 25μg/ml while internal standard had a concentration of 50μg/ml). Three replicates of each dilution were injected to HPLC system and linearity was evaluated. Repeatability of the method was checked by analyzing six replicate samples of GTX (at the 100% concentration level) and calculating relative standard deviation (%RSD).

Metal-drug interaction studies

Equivolume solutions of metal (50μg/ml) and GTX (100μg/ml) were mixed in test tube that gave final concentration of 50 μg/ml for GTX and 25μg/ml for drug-metal in 2:1 ratio. These solutions were then placed in water-bath maintained at 37ºC for an hour and then filtered through a 0.45 μm filter paper and injected for analysis.

RESULTS AND DISCUSSION

Method validation

Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to true value. In case of the assay of a drug in a formulated product, accuracy may be determined by application of the analytical method to synthetic mixtures of the drug product components to which known amount of analyte has been added within the range of method. If it is not possible to obtain samples of all drug product components, it may be acceptable to add known quantities of the analyte to the drug product (i.e., "to spike") (USP 2004). In our studies, the later technique was adopted and GTX was spiked in drug product. The result of accuracy given in table 1 revealed that the method was found accurate for all above purposes.

Table 1: Accuracy/recovery of gatifloxacin

Parameters	Conc (μg/ml)	% Recovery	% RSD
Assay	20.0	95.02	1.65
(Spiking	25.0	101.87	4.90
method)	30.0	94.18	4.06
Assay	6.25	100.04	0.6
	12.5	100	1.6
	25	99.9	0.2
Assay	12.5	100	0.4
(in serum)	6.25	100	1.1
	3.12	100	0. 6

Precision

Precision is the degree of reproducibility or repeatability of the analytical method under normal operating conditions (USP 2004). The method passed the test for repeatability as determined by %RSD of the area of the peaks of six replicate injections at 100% test concentration. The results of intra- and inter-day variation are shown in table 2.

Table 2: Intermediate precision of the method

Concentration (μg/ml)	Assay in formulation		Serum
	Intra-day variation (%RSD)	Inter-day variation (%RSD)	Intra-day variation (%RSD)
0.098	0.14	0.91	5.12
0.195	0.37	0.32	0.07
0.78	0.37	1.80	2.78
1.56	0.88	1.10	3.27
3.12	0.28	0.22	1.17
12.5	1.67	0.75	0.42
25.0	0.26	1.10	3.39

Range and linearity

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range (USP 2004). The linearity of the method was observed with in the expected concentration range demonstrating its suitability for analysis. The regression statistics are shown in table 3. The goodness-of-fit (R^2) was found to be 0.999 and value of intercept was less than 2% of the response of 100% of the test concentration in all the cases indicating functional linear relationship between the concentration of analyte and area under the peak.

Detection and quantitation limit

The detection limit or LOD is the lowest amount of an analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. It may be expressed as a concentration that gives a signal to noise ratio of 2:1 or 3:1 (USP 2004). The lower limit of detection for GTX is 2.45 ng/ml in refrence material and formulation and 1.73 ng/ml in serum. Quantitation limit or LOQ is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. A signal to noise ratio of 10:1 can be taken as LOQ of the method (USP 2004). The LOQ values were found to be 8.18 ng/ml for raw material, formulations and 5.77 ng/ml for serum.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present in the sample matrix (USP 2004). For demonstrating the specificity of the method for drug formulation the drug was spiked and the representative chromatogram is shown in figure 2a. The excipients used in different formulation products did not interfere with the drug peak and thus, the

Table 3: Regression statistics for analysis of GTX

Parameter	Target concentration (µg/ml)	Range (µg/ml)	Goodness-of-fit (R^2)	Slope	Intercept
Assay	0.098-25	24.9	0.999	15019.01	9359.69
Serum	0.098-25	24.9	0.994	18362.47	8861.40

Table 4: Assay of GTX in marketed and formulations

S No	Product	GTX label claim (mg per tablet)	Found (mg)	Recovery (%)	%RSD
1	Quintec	400	421.71	105.42	0.77
2	Glax	400	400.38	100.09	1.5
3	Gaticin	400	539.46	134.86	1.1
4	Rele	400	407.04	101.76	0.4

method is specific for GTX. To further confirm the specificity of the method, UV scans of spiked drug were taken in the range 200–400 nm and no significant change was found by comparing the absorbance of pure drug and spiked drug at the analytical wavelength of drug.

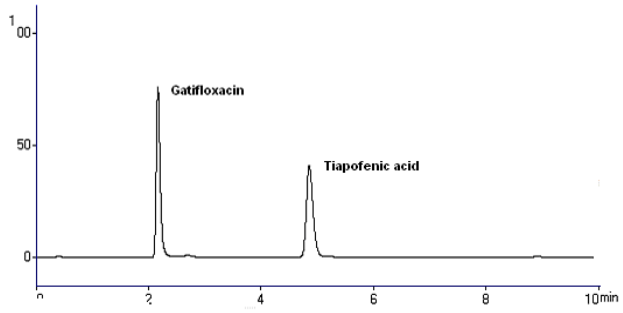

Figure 2a: A representative chromatogram of gatifloxacin: 25µg/ml with internal standard.

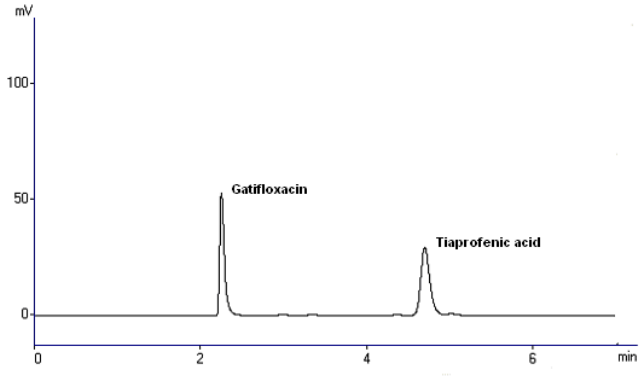

Figure 2b: Chromatogram of human plasma spiked with gatifloxacin and internal standard.

Applicability of method for GTX analysis

The developed method was applied to the determination of GTX content in marketed formulations. The assay results shown in table 4, demonstrate the suitability of method.

Analysis of GTX-metal complexes

The GTX is widely prescribed for a number of bacterial infections. Due to its complex pharmacokinetics, there is a potential for several types of drug interactions (Stein G.E 1991) Formerly, the pharmacokinetics of GTX and the influence of the antacid aluminum and magnesium hydroxide on the bioavailability of gatifloxacin have been assessed by a validated bioassay and HPLC. Co–administration of antacid reduces its bioavailability upto 45-68% (Lober *et al*., 1999). Antacids not only contain magnesium or aluminum, but can also contain calcium or bismuth ions. In the treatment of anemia, iron is orally administered while zinc, cupper and manganese are present in multivitamin mixtures. Several authors have begun to study the reasons for the reduced activity of quinolones in presence of the ions mentioned above (Polk R.E.1989; Flor S., 1999). Another object of present study was to evaluate the individual effect of above-mentioned ions on the availability of GTX, since no previous data was available on it. Moreover, binding reaction between drug and protein in the presence of metal ions (Cu^{2+} & Fe^{3+}) had been studied using fluorescence spectroscopy by two investigators (Tan *et al*., 2005), as binding phenomena is important in providing basic information on the pharmacological actions, bio-transformation and bio-distribution. They inferred that GTX forms stable complexes with $Cu^{2+}$and Fe^{3+} and these were measured at 449 nm or 473 nm and 455 nm respectively (Guo *et al*., 2004). A gatifloxacin-cadmium complex has also been synthesized and characterized by X-ray crystallographic analysis, IR and fluorescent spectroscopy, where in solid-state fluorescence was

Table 5: Quantitation of GTX-metal complexes by proposed method

Drug/Metal Complex	Retention time (min)	AUC	%Drug available	%RSD	Retention time (min)	AUC	%Drug complexed
Gatifloxacin	2.15	1170435	100	2.0	-	-	-
GTX-Fe^{3+}	2.19	616756.0	52.7	4.1	2.47	92080	13.56
GTX-Cr^{3+}	2.05	819146.0	69.98	1.5	complex not detected	-	-
GTX-Cu^{2+}	2.08	660859.5	56.46	3.0	2.37	61596	8.82
GTX-Ni^{2+}	2.07	368230.7	31.46	0.5	2.37	2918	0.79
GTXCd^{2+}	2.08	649554.7	55.49	5.7	2.37	8685	1.59
GTX-Zn^{2+}	2.09	459517.7	39.26	0.1	1.75	2386	0.50
GTX-Mg^{2+}	2.07	743452.0	63.52	0.9	complex not determined	-	-
GTX-Mn^{2+}	2.11	836175.3	71.44	0.1	complex not determined	-	-
GTX-Ca^{2+}	2.08	660158.0	56.66	0.5	1.10	3911	0.83
GTX-Co^{2+}	2.06	87747.70	74.97	1.3	complex not determined	-	-

measured at 439 nm (Yong-Hua Li *et al*., 2005). It is worth emphasizing that there may be differences between the quinolones regarding the extent of interaction. Metal: GTX ratio used in this study is 1:2 as most frequently observed interactions between metal: quinolone are in 1:1 and 1:3 ratio. During our experiment, it was observed that GTX solubility in diluents (70:30, MeOH:MeCN) was increased about twice when solution was kept on water bath maintained at 37ºC, while GTX was sparingly soluble in methanol. GTX concentration was measured after each interaction. Complex formation of GTX with metals was shown by a considerable decrease in AUC and a drift in retention time. The results are shown in table 5 and figure 3a-3c. The potential for chelation with various cations occurs in the following approximate formation order: Ni^{2+} > Zn $^{2+}$> Fe^{3+} > Cd^{2+}> Cu^{2+} > Ca^{2+}> Mg^{2+}>Cr^{2+}.> Mn^{2+}> Co^{2+}.

It is evident in figure 3c that in case of Fe-GTX complex there is another peak which is coeluted at retention time of 2.47 minutes having AUC of 13.56% of original peak. This peak can be referred to Fe-GTX complex. Similar peak was also observed for Ni^{2+}, Cd^{2+} and Cu^{2+} complexes whose characteristics are given in table 5. In other cases, a remarkable reduction was found in the concentration of GTX while no peak was observed for metal complexes. Although, this study is not sufficient to determine exact location of GTX-metal complexes and should be supplemented by measurements of UV absorptions in region of 400-600 nm. The need of multivariate HPLC method for the separation of GTX metal complexes is obvious, while present is also sufficiently accurate for the determination of GTX even in presences of metals or other product impurities.

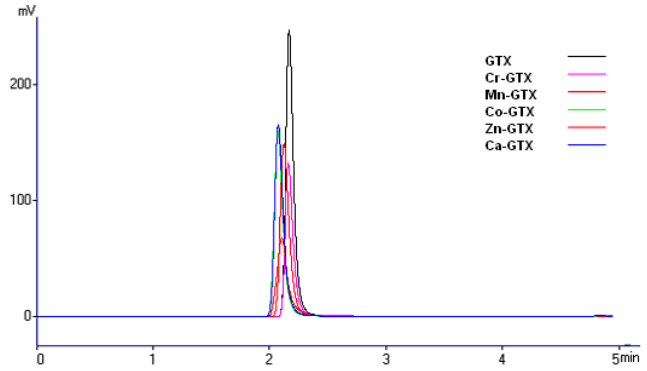

Figure 3a: Chromatogram showing differences in RT of GTX & GTX-Cr, GTX-Mn, GTX-Co, GTX-Zn, GTX-Ca complexes.

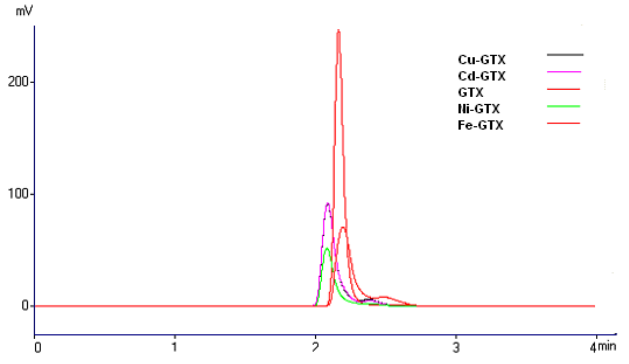

Figure 3b: Chromatogram showing differences in RT of GTX, GTX-Cu, GTX-Cd, GTX-Ni & GTX-Fe complexes.

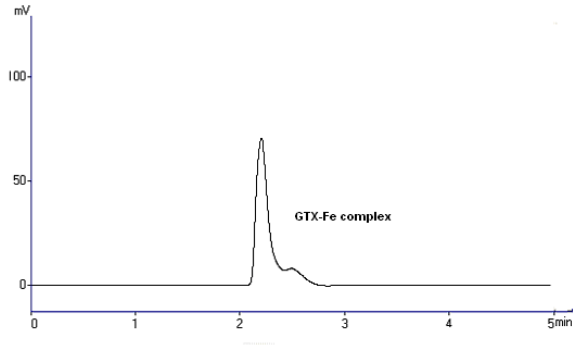

Figure 3c: Chromatogram of GTX-Fe complex and GTX

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