Journal of Chromatography
Separation Techniques

Rote and Kumbhoje, J Chromatograph Separat Techniq 2011, 2:4
DOI: 10.4172/2157-7064.1000115

Research Article Open Access

Development and Validation of HPTLC Method for Simultaneous Estimation of Gatifloxacin and Ornidazole in Human Plasma

Ambadas R Rote* and Prasanna A Kumbhoje

Department of Pharmaceutical Chemistry, M. G. V.'s Pharmacy College, Panchavati, Nashik, Mumbai - Agra Road, Nashik- 422003, Maharashtra, India

Abstract

A simple, rapid, sensitive and economic chromatographic method have been developed for simultaneous estimation of gatifloxacin and ornidazole in human plasma by using diclofenac sodium as internal standard The method employes a 20cm x10cm TLC plate precoated with silica gel 60F254 with 250µm thickness on aluminium sheets. The plasma sample was extracted using chloroform:formic acid (4.0:0.4, v/v). The mobile phase used consists of methanol: chloroform: ammonia solution [25%] (8.5: 1: 0.4 v/v/v), having chamber saturation for 20 min at room temperature. The R_f values were found to be (R_f=0.28±0.00178),(R_f=0.77 ±0.00427) and(R_f=0.50±0.00337) for GTFX, ORNZ and DICLO respectively. Densitometric analysis was carried out at wavelength 297.86 nm. The linear detector response was observed between 100 ng/band to 600 ng/band for both GTFX and ORNZ. The stability of gatifloxacin and ornidazol in plasma was confirmed during three freeze-thaw cycles (-20˚C), on bench during 12 hr and post preparative after 48 hr. The method was validated according to the FDA Bioanalytical guidelines with respect to selectivity, linearity,sensitivity precision and accuracy, recovery and stability.

Keywords: High Performance thin layer chromatography; Gatifloxacin; Ornidazole; LLE

Introduction

Gatifloxacin (GTFX) (Figure 1) [1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-(3-methyl-1-piperazinyl)-4-oxo-3-quinoline carboxylic acid sesquihydrate] is a synthetic broad-spectrum antimicrobial fluoroquinolone that is active against both gram-negative and gram-positive bacteria. [1,2] It is official in IP [3] .Ornidazole (ORNZ), chemically 1-chloro-3-(2-methyl-5-nitro-imidazol-1-yl) propan-2-ol, is an antimicrobial agent [1,2], used in treatment of susceptible protozoal infections and anaerobic bacterial infection. It is official in IP [3]. Several methods are reported for the individual estimation of gatifloxacin and ornidazole. For GTFX various other methods are reported on instruments like HPLC-UV [4,5], plasma [6], LC-Electrospray Tandem MS [7] individually and in combination with other drugs. Literature survey also reveals different methods like spectrophotometrically [8], HPTLC [9],HPLC [10] for estimation of ORNZ individually and in combination with other drugs.No HPTLC method is reported for the simultaneous estimation of gatifloxacin and ornidazole in human plasma. The proposed research work describes the development and validation of a method to simultaneously quantify GTFX and ORNZ by using HPTLC in human plasma.

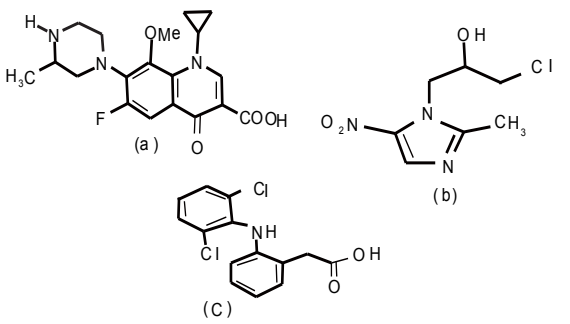

Figure 1: Structures of gatifloxacin sesquihydrate (a), ornidazole (b) and diclofenac sodium (c).

Experimental

Instrumentation

HPTLC Camag with precoated silica gel Plate 60F254 (20cm×10 cm)250µm thicknesses (E. Merck, Darmstadt, Germany) was used as stationary phase. Sample application was done by Camag 100 µl syringe using Camag Linomat 5 applicator. The sample was sprayed in the form of narrow bands of 6 mm length at a constant rate 0.2µl/s. Linear ascending development was carried out in 20cm×10cm twin trough glass chamber (Camag, Muttenz, Switzerland). The densitometric scanning was performed by using Camag TLC scanner 3 supported by win CATS software (V 1.4.2.8121 Camag). Evaluation of chromatogram was done by using peak areas.

Reference substances, sample, reagents and chemicals

Active pharmaceutical ingredient (API) working standards of gatifloxacin (GTFX) was obtained from Piramal Healthcare Limited, Pithampur ,Madhya Pradesh, India.Likewise ornidazole(ORNZ) and diclofenac sodium(DICLO) were obtained from Kirti Pharmachem,Sinnar,Nashik.Chloroform HPLC grade, Toulene AR and methanol HPLC grade were obtained from Fisher Scientific, India. Ammonia solution [25 %] and Formic acid were obtained from S. D. Fine Chem. Ltd., India.TLC plates precoated with silica gel 60 F_{254} with 250µm thickness on aluminium sheets were purchased from Merck India Pvt. Ltd. Human plasma used for research work was supplied by Arpan Blood Bank, Nashik, Maharashtra, India.

***Corresponding author:** Ambadas.R.Rote, Department of Pharmaceutical Chemistry, M. G. V.'s Pharmacy College, Panchavati, Nashik, Mumbai - Agra Road, Nashik- 422003, Maharashtra, India, Tel: +91 9579574199; Fax: +91 2532511931; E-mail: roteambadas@gmail.com

Received November 26, 2011; **Accepted** December 28, 2011; **Published** December 31, 2011

Citation: Rote AR, Kumbhoje PA (2011) Development and Validation of HPTLC Method for Simultaneous Estimation of Gatifloxacin and Ornidazole in Human Plasma. J Chromatograph Separat Techniq 2:115. doi:10.4172/2157-7064.1000115

Solution preparation

Standard working stock solution: Standard stock solutions of GTFX, ORNZ and DICLO were prepared by dissolving 100 mg of drug in 100 ml of methanol to get a concentration of 1000 µg/ml for each drug seperately.

Plasma sample preparation: In a 15ml centrifuge tube 50,100,150,200,250 and 300µl of GTFX standard working stock solution was added to 1.0 ml drug-free plasma to obtain calibration standards 100,200,300,400,500 and 600 ng/band for GTFX similarly 50,100,150,200,250 and 300µl of ORNZ standard working stock solution was added to drug-free plasma to obtain calibration standards 100,200,300,400,500 and 600 ng/band for ORNZ and 400ng/ band of DICLO (internal standard) was kept constant. The quality control (QC) samples were prepared in plasma in concentration range 200, 400, 600 ng for both GTFX and ORNZ respectively. Protein precipitation and extraction was carried out by using chloroform:formic acid (4:0.4 v/v) and with help of vigorous vortex using Remi mixer for 2 min and centrifuged at 5,000rpm for 10min. The organic phase was recovered and evaporated to dryness on hot plate. The residual mass was reconstituted with 1 ml methanol. Lastly 2 µl from each reconstituted solution was applied on HPTLC plate to analyze the sample.

Chromatographic conditions

Merck HPTLC plates coated with silicagel 60 F_{254} (0.2 mm thickness) on aluminium sheets were used as stationery phase. The mobile phase consisting of a mixture of methanol: chloroform: ammonia solution[25%] (8.5: 1: 0.4 v/v/v)was used throughout the analysis. Plates were pre-washed using methanol as solvent and activated at 120° C for 30 min in hot air oven. Chamber saturation time was optimized at 20 min and Plate development time was fixed at 15 min with migration distance of 8 cm. Slit dimension was constant at 5.0 x 0.45mm with scan speed of 20 mm/s. Isobestic point of 297.86 nm was selected as the scanning wavelength.

Validation procedure

The method was validated for sensitivity, selectivity, precision, accuracy, linearity, recovery and stability. The validation of the method was based on FDA guidelines and on standard bioanalytical method validation recommendation. The selectivity of method was investigated by analyzing six blank plasma samples. Each blank sample was tested for interference using proposed extraction procedure.Five replicate of three QC sample low, mid and high were used for the determination of precision and accuracy. Intra-day and inter-day precision were carried out at three (low,mid high) level in triplicate. Precision and accuracies showed less than 15% relative standard deviation (RSD) and %15 relative error from nominal values, at LLOQ there were less than 20% for both analytes. Linearity solutions were prepared at six concentration levels from 100-600 ng/band for both GTFX and ORNZ. Recovery of the drugs were obtained by comparing results of extracted sample with unextracted standard solution.Three types of stability studies, namely freeze-thaw (three thaw cycles at -20°C), bench top(for12 hr) and post-preparative(after 48 hrs) were determined at two QC concentrations (low, high) and the procedure were followed as stated in FDA Guidelines. LOD and LOQ were calculated from the slope and constant obtained from the graph of linearity of individual drugs.

Results and Discussions

Extraction procedure optimization

Different solvents were tried for the extraction of GTFX and ORNZ from human plasma. First 4 ml each of hexane tried for the precipitation of plasma but the recovery was very less.After that toulene was tried but it did not give any result. Ethyl acetate also tried up to 5.0 ml. It gave 50-60% of recovery because of less precipitation of protein from plasma. At the last chloroform was tried and 70–80% of recovery was obtained. It was found by literature survey that addition acid increases precipition of protein.So formic acid (strong acid) 0.4 ml increases the precipitation of protein and also the recovery which is reproducible and high as compare to other solvents. So chloroform and formic acid (4.0ml: 0.4ml) was kept as final solvent for extraction of GTFX and ORNZ.

Optimization of chromatographic conditions

Initially plane solvents like Methanol, Ethyl acetate, Chloroform n-Butanol were tried. The spots were moved with ethyl acetate and methanol, but R_f of ornidazole observed was maximum.Then n-Butanol and Methanol but no proper resolution observed between GTFX, ORNZ and DICLO also shows the tailing. Then n-Butanol and Toulene was tried.Sepration observed was good ,but it was not only taking too much time for development (to run) plate but also giving continous interference peak in plasma. So mobile phase had to change. Chloroform and Methanol in the ratio of (9.5:1, v/v) was tried but again there is no proper sepration obtained between DICLO and ORNZ.Then proportion of Chloroform was decreased showing good resolution but GTFX was showing tailing effect. Lastly tailing effect was overcome by addition of Ammonia solution.Then good resolution with symmetrical peaks of GTFX, ORNZ and DICLO were obtained. Finally mobile phase used consisted of Chloroform: Methanol :Ammonia sloution[25%] (8.5: 1: 0.4, v/v/v) which gave good resolution of peaks for GTFX, ORNZ and DICLO. The Rf values for GTFX, ORNZ and DICLO were found to be(R_f=0.28±0.00178),(R_f=0.77 ±0.00427) and (R_f =0.50±0.00337) respectively (Figure 2). Well defined spots were obtained by prewashing the plate using Methanol followed by activating at 120°C for 20min. Chamber was saturated with mobile phase for 20 min at room temperature, which gave reproducible R_f values for GTFX,ORNZ and DICLO . The densitogram was recorded at 297.86 nm.

Method validation

The newly developed method was validated according to the FDA

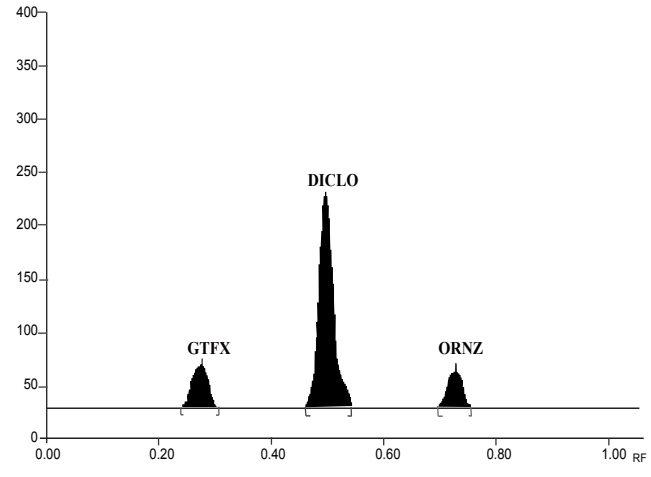

Figure 2: Densitogram of gatifloxacin sesquihydrate (GTFX) (R_f =0.28±0.00178) and ornidazole (ORNZ) (R_f=0.77 ±0.00427) and IS diclofenac (DICLO) (R_f =0.50±0.00337) in human plasma.

guidelines with respect to Selectivity, linearity, sensitivity, recovery studies, precision and stability [11].

Selectivity: The selectivity of this method was investigated by analyzing pooled blank human plasma. The blank sample was checked for its interference using the proposed LLE procedure and HPTLC conditions and compared with spiked sample concentration of GTFX and ORNZ at lower limit of quantification (LLOQ) in human plasma. There was no any interference found in biological matrix in the quantitation of GTFX and ORNZ.

Linearity: GTFX and ORNZ showed linearity in the range f 100-600 ng/band. Linear regression equations and correlation coefficient (r^2) are: Y_{GTFX} = 0.0045x-0.0941 (r^2 = 0.9963) and Y_{ORNZ} = 0.0019x + 0.0729 (r^2 = 0.9957). The LOD 6.1848 and 5.9248 for GTFX and ORNZ and LOQ 18.7418 and 17.9541 for GTFX and ORNZ was found respectively (Table 1)

Sensitivity: Sensitivity was determined by calculating accuracy and precision at LLOQ (100 ng) by analyzing 5 replicates. The lower limit of quantitation which could be detected were found to be 100 ng/band with %RE = 9.83,11.54 and %RSD= 18.90,13.68 for GTFX and ORNZ respectively, is within acceptable limit.

Precision and Accuracy: Precision of the method was determined by repeatability (intraday) and intermediate precision (inter-day) and accuracy for set of quality control (QC) sample (low, mid, high) in replicate (n = 5).Intra-day precision was found in the range of 0.72-2.28% and the inter-day precision was 1.65-6.39% and the accuracy was within 0.5-14.68%.The low percent standard deviation (%RSD) and percent relative error (%RE) were within the acceptable limit. Statistical Evaluation of Precision study was found to be in the range. The Percent relative error (%R.E.) and percent standard deviation (%RSD) are reported in (Table 2).

Extraction efficiency (Recovery): Absolute recoveries were calculated by comparing peak areas obtained from freshly prepared samples extracted with unextracted standard solutions of same concentration. Recovery data was determined in triplicate at three concentrations (low, mid, high) as recommended by FDA guidelines. The recovery at three concentrations 200, 400, 600 ng/band was found to be 73.43% , 71.73% and 67.03% for GTFX and recovery at three concentrations 200,400, 600 ng/band was found to be 71.79%, 72.92% and 85.27% for ORNZ (Table 3).

Parameter	GTFX	ORNZ
Beer's law range (ng/band)	100-600	100-600
Regression Equation (y = mx + c)		
Slope (m)	0.004508	0.001980
Intercept (c)	-0.0941	0.0729
Correlation coefficient ($r^{2)}$	0.9963	0.9957
LOD (ng/band)	6.1848	5.9248
LOQ (ng/band)	18.7418	17.9541

y=absorbance and x=concentration

Table 1: Calibration Parameters of Gatifloxacin and Ornidazole. For HPTLC in human plasma.

Parameter	Concentration (ng/band)		%RSD		%RE	
	GTFX	ORNZ	GTFX	ORNZ	GTFX	ORNZ
INTRA	200	200	1.99	1.1350	12.0	0.5
	400	400	0.94	0.2434	1.5	6.5
	600	600	0.72	2.2852	5.3	13.26
INTER	200	200	6.39	3.32	13.9	1.9
	400	400	2.88	3.5	3.354	7.4275
	600	600	1.65	1.1560	6.4	14.68

Table 2: Results of precision of GTFX and ORNZ in human plasma

Sr. No	Concentration (ng/band)		Absolute Recovery (%)±S.D.		%RSD	
	GTFX	ORNZ	GTFX	ORNZ	GTFX	ORNZ
1	200	200	73.43 ± 2.46	71.79 ± 3.5	3.35	5.01
2	400	400	71.73 ±1.13	72.92 ± 3.9	1.57	5.44
3	600	600	67.03 ± 0.50	85.27 ± 4.7	0.75	5.49

Table 3: Result of recovery of GTFX and ORNZ in human plasma.

Stability parameters	Amount of analytes spiked (ng/band)	Amount of analytes found after stability condition exposure (ng/band)	RSD (%)
Freeze-thaw	Low 200	73.86	3.5072
	High 600	119.32	6.2624
Bench top 12 hr	Low 200	100.94	2.9421
	High 600	152.76	7.2652
Post-preparative	Low 200	77.86	8.9198
	High 600	112.80	8.6165

Table 4: Stability study of GTFX in human plasma.

Stability parameters	Amountof analytes spiked (ng/band)	Amount of analytes found after stability condition exposure(ng/band)	RSD (%)
Freeze-thaw	Low 200	104.82	7.0896
	High 600	117.92	11.1399
Benchtop 12 hr	Low 200	109.30	10.016
	High 600	124.68	6.2042
Post-preparative	Low 200	94.14	7.7310
	High 600	105.90	6.6010

Table 5: Stability study of ORNZ in human plasma.

Stability

Freeze thaw stability: Analyte stability was determined after three freeze and thaw cycles for three replicate of low and high QC sample. The samples were stored at -20°C temperature for 24hr.Then thawed at room temperature. No significant difference between freeze–thaw sample and freshly prepared sample was observed.The results obtained were within limits and reported in Table 4 and Table 5.

Short-Term Temperature Stability (Bench top stability): Three aliquots of each of the low and high concentrations was thawed at room temperature and kept at this temperature from 4 to 24 hours and analyzed. Comparison of the results for QC sample (low and high) with freshly prepared stock solution showed that there was no significant difference between response of freshly prepared solution and sample of GTFX and ORNZ after 12 hr. The results obtained were within limits and reported in Table 4 and Table 5.

Post preparative stability: The extracted low and high QC samples

(ready to inject) were kept for the 48 hrs, and analyzed. No significant difference observed between post preparative sample and freshly prepared sample.The results obtained were within limits and reported in Table 4 and Table 5.

Analysis speed

In HPTLC, 18 spot can be applied on one plate at once so less time consuming.

Conclusion

The proposed HPTLC method for the simultaneous estimation of gatifloxacin and ornidazole in human plasma is selective and sensitive. Sensitivity of the method is suitable for handling various plasma levels of the drug.In future these method can be used for bioequivalence study.

Acknowledgement

The authors wish to thank the management and Principal Dr. Rajendra S. Bhambar of M.G.V.'s Pharmacy College, Nashik for providing necessary facilities. The authors are also thankful to Arpan Blood Bank, Nashik for providing human plasma, Gatifloxacin sesquihydrate (Piramal Healthcare Limited, Pithampur, Madhya Pradesh,India), Ornidazole and Diclofenac sodium (Kirti Pharmachem, Sinner, Maharashtra, India) as gift sample for project work.

References

1. Budavari S (2001) The Merck Index Merck Research Lab, Division of Merck and co. Inc., Whitehouse Station.
2. Sweetman SC (2009) Martindale: The Complete Drug Reference. Pharmaceutical Press, London.
3. Indian Pharmacopoeia (2007) Government of Indian, The Controller of Publications, Delhi 1,2.
4. Boopathy D, Mathew B, Prakash M, Sureshkumar S, Perumal P (2010) Simultaneous estimation and method validation of Gatifloxacin and Ambroxol Hydrochloride in tablet dosage form by RP-HPLC. Der Pharmacia Lettre 2: 346-349.
5. Patel AB, Shah NJ, Patel NM (2009) Development and Validation of HPLC Method for the Simultaneous Estimation of Satranidazole and Gatifloxacin in Tablet Dosage Form. International J Chem Tech Res1: 587-590.
6. Tasso L, Costa TD (2007) High performance liquid chromatography for quantification of gatifloxacin in rat plasma following automated on-line solid phase extraction. J Pharm Biomed Anal 44: 205-210.
7. Vishwanathan K, Bartlett MG, Stewart JT (2001) Determination of gatifloxacin in human plasma by liquid chromatography/electrospray tandem mass spectrometry. Rapid Commun Mass Spectrom 15: 915-919.
8. Nanda RK, Gaikwad J, Prakash A, (2009) Simultaneous Spectrophotometric Estimation of Cefixime And Ornidazole In Tablet Dosage Form. Int J Pharm Tech Res 1: 488-491.
9. Chepurwar SB, Shirkhedkar AA, Bari SB (2007) Validated HPTLC method for simultaneous estimation of levofloxacin hemihydrate and ornidazole in pharmaceutical dosage form. J Chromatogr Sci 45: 531-536.
10. Ranjane PN, Gandhi SV, Kaduka SS (2008) Simultaneous determination of cefuroxime axetil and ornidazole in tablet dosage form using reversed-phase high performance liquid chromatography. Se Pu 26: 763-765.
11. Guidance for Industry Bioanalytical Method Validation (2001) US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), BP.