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ICH Guidelines in Practice: Development and Validation of HPTLC Method for Simultaneous Estimation of Ketorolac Tromethamine and Ofloxacin in Ophthalmic Formulation.

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Research Article

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ABSTRACT

Ketorolac Tromethamine (KET) and Ofloxacin (OFL) from bulk and eye drop formulation were precisely and accurately determined using developed high performance thin layer chromatographic (HPTLC) method. Analysis was done on silica gel $60F_{254}$ as stationary phase with aluminum backed TLC plates. Dichloromethane: Methanol: Ammonia 25% (6:3:1) was used as a mobile phase. The two drugs were satisfactorily resolved with Rf 0.79± 0.03 and 0.41± 0.02 for KET and OFL respectively. The method performance was validated as per ICH guidelines. Linearity was found in the range of 25-75ng/band (r^2 = 0.999) for KET and 15-45ng/band (r^2 = 0.9998) for OFL. The assay results were found to be 99.14±0.413 %w/w and 98.11±0.232 % w/w for KET and OFL respectively. The % Recovery for KET was in the range of 99.74-99.95% w/w and that of OFL was 99.78-99.87% w/w. This validated method can be used for simultaneous estimation of KET and OFL from bulk and ophthalmic formulation.

INTRODUCTION

Ketorolac Tromethamine (Fig.1) is 5-benzoyl-2, 3-dihydro-1H-pyrrolizine-1-carboxylicacid, 2-(hydroxymethyl)-1, 3 propanediol[1]. It is a potent analgesic and with moderate anti-inflammatory activity.

Ofloxacin (Fig.2) chemically is 9-fluro-2, 3-dihydro-3-methyl-10-(4-methyl-1-piperizinyl)-7-oxo-7H-pyrido [1, 2, 3-de]- 1, 4- benzoxaine- 6 -carboxylicacid [1]. Ofloxacin is a synthetic chemotherapeutic antibiotic of the fluoroquinolone drug class considered to be a second-generation fluoroquinolone.

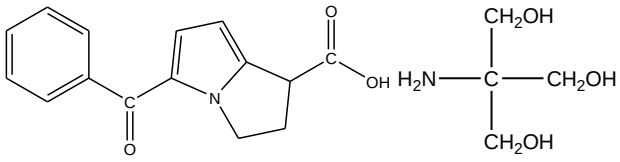

Figure 1: Ketorolac Tromethamine

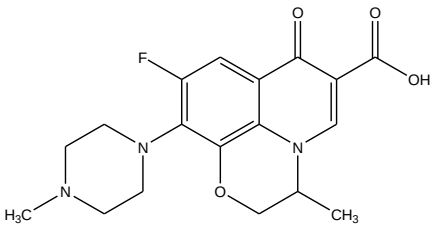

Figure 2: Ofloxacin

KET has been estimated by UV Spectroscopy [2], HPTLC [3] and HPLC [4,5,6,7,8]. OFL was estimated alone or in combination with other drugs using techniques like UV Spectrophotometry [9,10,11,12,13,14,15], HPLC [16-27] and electrochemical analysis [28]. KET (5 mg/ml) and OFL (3 mg/ml) combination is available in the market in the form

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of ophthalmic solution (KETLUR). This paper provides simple, economic and validated method for simultaneous estimation of KET and OFL in eye drop formulation.

EXPERIMENTAL

Chemicals and Reagents

Pure drugs of Ketorolac Tromethamine (Lupin Pharma Ltd, Pune) and Ofloxacin (Litaka Pharma Ltd., Pune) were obtained as a gift samples. All the other chemicals were of analytical grade and were purchased from Merck Chemicals.

Instrumentation and Chromatographic Conditions

Aluminum-backed TLC plates (20 cm × 10 cm), coated with 250 µm layer of silica gel 60 F_{254} (E. Merck, Darmstadt, Germany) were used. Plates were prewashed by methanol and activated at 110°C for 5 min. Samples were applied as 8 mm bands using 25 µl Linomat V applicator (Camag, Muttenz Switzerland) equipped with a Hamilton syringe (Bonaduz, Switzerland). A constant application rate of 1 µl/s was used and the space between two bands was 10 mm.

The optimized chamber saturation time for mobile phase was 10 min at room temperature (25°C±2) at 60±5% RH. Ascending development of the plates was performed with Dichloromethane: Methanol: Ammonia 25% (6:3:1) as mobile phase, in a Camag 20×10 cm twin trough glass chamber with chromatogram run of 8 cm. HPTLC plates after development were dried in current of air with the help of air dryer in a wooden chamber with adequate ventilation. A unidirectional air flow was maintained in the laboratory. The densitometric measurement was performed using a slit dimensions of 6.00×0.30 mm, the scanning speed of 20mm s^{-1} and monochromator bandwidth of 20 nm. Each track was scanned thrice using Camag HPTLC scanner III in the reflectance absorbance mode at 307 nm.

Preparation of standard solutions

An accurately weighed KET and OFL (50 mg of each) were dissolved separately in 20 ml methanol in 50 ml volumetric flask. The solutions were further diluted to the volume with methanol to the concentration of 1 mg ml^{-1} for both the drugs. Standard stock solutions were further diluted with methanol to concentration of 100 µg/ml for each drug.

Optimization of Mobile Phase

Various binary mobile phases as shown in Table 1 were used in initial few trials wherein OFL showed tailing and KET was found to have Retention factor close to 1. The ternary mobile phase were also tried to minimize the tailing factor of OFL.

Table 1: Optimization of Mobile phase for Simultaneous estimation of KET and OFL by HPTLC.

Sr. No.	Mobile Phase	Observations
1	Dichloromethane: methanol (9:1)	Both drugs have same Rf and exhibit tailing.
2	Dichloromethane: methanol: Acetic acid (7:3:0.5)	KET merged in solvent front and Rf of OFL was reduced.
3	Dichloromethane: methanol (5:5)	Resolution increased but KET merged in solvent front and OFL showed tailing.
4	Dichloromethane: Methanol: Acetic acid (5:5:0.5)	KET with Rf values close to one.
5	Chloroform: Ethyl acetate (3:8)	Poor resolution in drugs.
6	Chloroform: Methanol (9:1)	Both drugs have same Rf.
7	Dichloromethane: Methanol: Ammonia 25% (6:3:1)	Well resolved spots with good shape.

Analysis of Eye drop formulation

For analysis, 1 ml of the marketed formulation containing KET (5 mg) and OFL (3mg) was diluted to 10 ml with methanol to produce a concentration of 0.5 mg/ml for KET and 0.3 mg/ml for OFL. The solution was sonicated for 10 min. and was filtered through whatmann filter paper No. 41. Stock solution was diluted to get final concentration of 50ng/band for KET and 30ng/band for OFL. The plate was developed in an optimized mobile phase. The analysis was repeated in triplicate. The possibility of excipient interference with the analysis was examined.

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Method Validation

Validation of the TLC method was carried out as per ICH guidelines [29] with the following parameters:

Linearity and range

Calibration curves were prepared using a standard solution containing 50 µg/ ml and 30 µg/ ml KET and OFL respectively in methanol. Six concentrations were spotted on the TLC plate to obtain the final concentration in the range of 25-75 ng/band (KET) and 15-45 ng/band (OFL). Each solution was applied thrice on the plate and average peak area of each concentration was measured at 307 nm. It was plotted against the corresponding concentrations to obtain the calibration curves. Residuals for both the drugs were plotted against the concentration of analyte to confirm linearity of method.

Precision

System Precision was performed by six repeated applications of standard solutions containing 50 ng/band for KET and 30 ng/ band for OFL. The precision of the method was established in terms of repeatability, Intraday and Interday studies. Repeatability studies were performed by applying standard solutions containing 50 ng/band for KET and 30 ng/ band for OFL respectively (represents 100% of the test concentration) six times, to a TLC plate followed by development. Each solution was separately prepared.

The intra-day precision was assessed by analyzing standard drug solutions at 100% of the test concentration (50 ng/band for KET and 30ng/ band for OFL) three times on the same day. Each solution was applied six times. The Interday precision of the method was checked by repeating a study on three different days and % RSD of peak area was calculated.

Accuracy

Accuracy of the method was carried out by applying the method to the sample (KET and OFL combination in ophthalmic solution) to which a known amount of KET and OFL standard solution corresponding to 80, 100, and 120% of label claim has been added in the initial phase of sample preparation, mixed, and analyzed by running chromatogram in an optimized mobile phase. % Recovery at each level was calculated.

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD is the concentration of the analyte that yield signal-to-noise ratio of 3 and of 10 at LOQ. The LOD and LOQ were determined by measuring the magnitude of analytical background by spotting a blank and calculating the signal-to-noise ratio for KET and OFL by spotting a series of solutions until the S/N ratio of 3 was obtained for the LOD and 10 for the LOQ.

Specificity

The specificity of the method was ascertained by analysis of drug standards and samples. The identities of the bands for KET and OFL were confirmed by determining peak purity and also by comparing the Retention factor of the bands with those of standards.

Robustness

Small deliberate changes in the experimental parameters were done. These include time from spotting to chromatography and from chromatography to scanning were varied by $\pm$ 5 mins, Ultra-sonication time of the mixture was varied by $\pm$ 3 min. Plates from different lot number were used.

RESULTS AND DISCUSSION

The developed HPTLC method for simultaneous estimation of KET and OFL in eye drops was found to be simple and convenient for the routine analysis of two drugs.

Optimization of other chromatographic conditions

The proposed method uses silica gel 60 F_{254} as a stationary phase, sample application in the form of 8 mm band at the rate of 1 µl/s, 10 mm as a space between two bands, chamber saturation time of 10 min at room temperature, 8 cm chromatogram run, the scanning speed of 20mm s^{-1}, detection wavelength at 307nm and the average development time of 20 min.

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Preparation of standard solution

Both drugs exhibit same absorptivity at 307 nm as indicated in Fig 3 was selected as analytical wavelength.

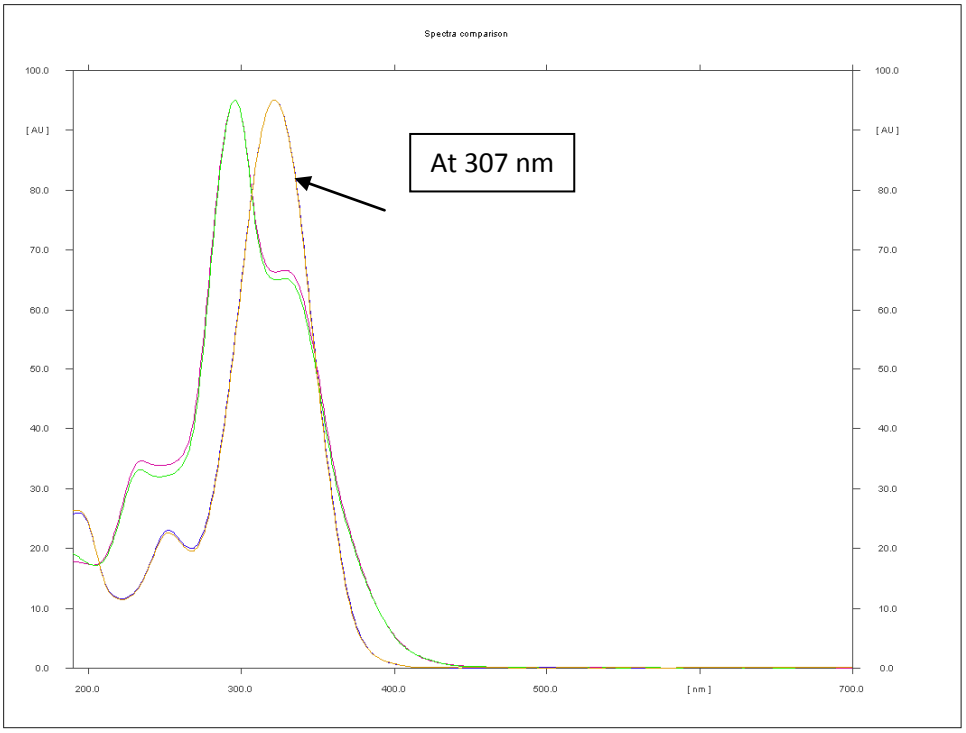

Figure 3: Overlay spectrum of KET and OFL

Optimization of mobile phase

The development of chromatogram in binary mobile phase like Chloroform: Ethyl acetate (3:8), Chloroform: Methanol (9:1) was unsuccessful due to tailing of OFL and Retention factor close to 1 for KET as shown in Table 1. Use of Dichloromethane: Methanol: Ammonia 25% (6:3:1) has resulted in a dense, compact and well resolved spots. Chromatogram is shown in Fig. 4.

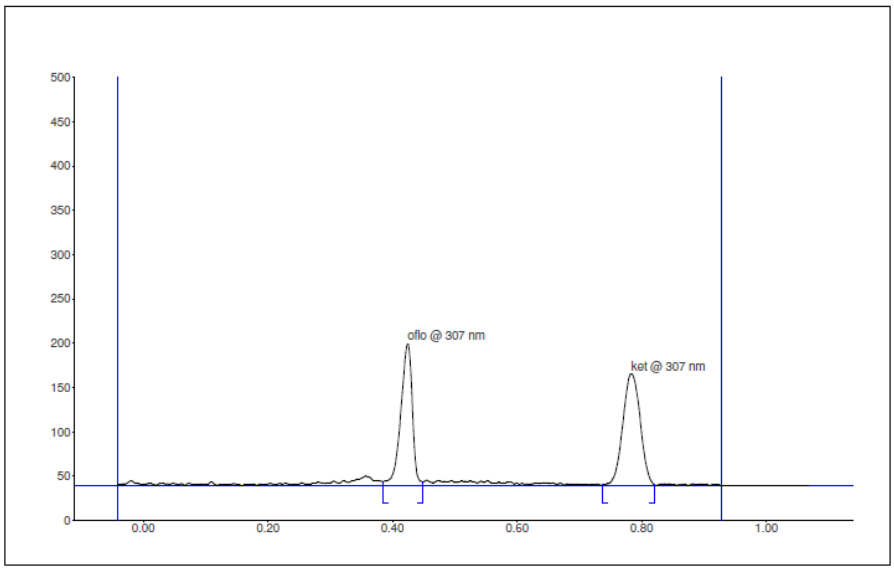

Figure 4: Chromatogram of KET and OFL at 307nm with Rf value of 0.79± 0.03 and 0.41± 0.02 respectively.

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Analysis of Marketed formulation

The assay results as shown in Table 2 were found to be 99.14±0.813 9 %w/w and 98.11±0.232 % w/w for KET and OFL respectively.

Table 2: It shows Assay results for KET and OFL in ophthalmic formulation

Drug	Label Claim (ng/spot)	Amount found* (ng/spot)	Assay value (%)*± S.D.	% RSD
KET	50	49.57	99.14 ± 0.8139	1.6279
OFL	30	29.43	98.11 ± 0.2325	0.9303

* Average of Three determinations

Linearity and Range

Linearity of the method was studied by spotting six concentrations of the drugs in the range of 25-75 ng/band and 15-45 ng/band for KET and OFL respectively. Calibration curves as found in Fig. 5 and 6 were found to be linear. Residuals do not show either of the positive or negative values and are randomly distributed around the regression function as shown in Fig 7. This proves the linearity of the method. The correlation coefficient of these drugs was found to be close to 1.00 indicates linearity of the method. Linearity data is presented in Table 3.

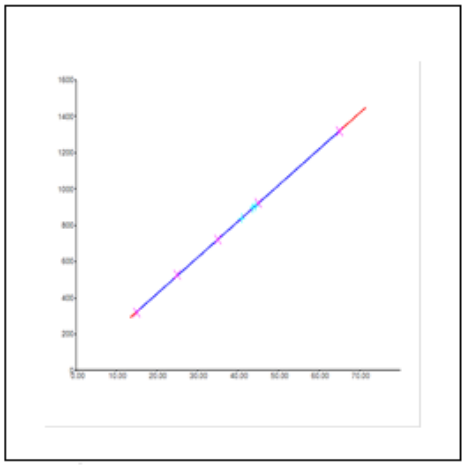


Figure 5: Calibration curve of KET

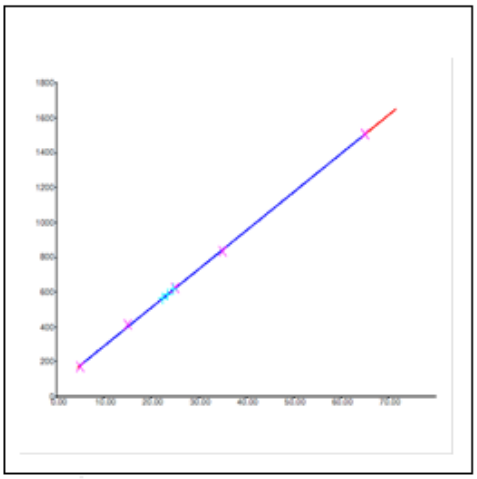
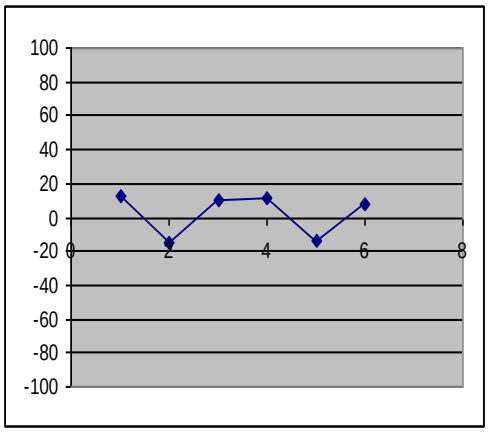
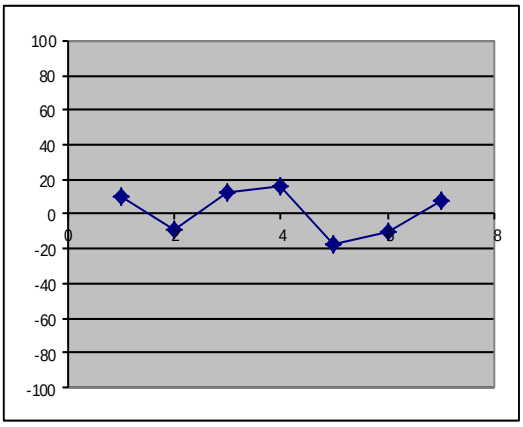


Figure 6: Calibration curve of OFL



KET



OFL

Figure 7: Residuals of KET and OFL obtained from regression data at 307 nm by plotting distance of response from regression line.

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Table 3: Linearity data of KET and OFL

Parameters	KET	OFL
Range as per Beers law	25-75ng/spot	15-45ng/spot
Régressions équation (y= mx + c)	y = 19.94 x + 23.41	y= 22.1 x + 68.05
Correlation Coefficient (r^2)	0.9999	0.9998
Residuals	Random	Random
Sample peak purity r (S, M)	0.9997	0.9995
r (M,E)	0.9996	0.9994

Precision

Results of System Precision, Repeatability, intra-day studies and inter-day precision studies are shown in Table 4-6. % RSD of peak area after six subsequent injections of the same standard solution for both the drugs was not more than 2% indicating system precision. Percentage RSD of the peak area after application of six independent solutions of the same concentration was less than 2%. This proves method precision.

Table 4: It shows statistical analysis for System Precision.

Drug	Area Band 1	Area Band 2	Area Band 3	Area Band 4	Area Band 5	Area Band 6	Mean ± S.D	% R.S.D.
KET (50ng/spot)	1035.1	1031.9	1020.8	1011.2	1010.0	1011.4	1020.1 ± 12.4	1.215
OFL (30ng/spot)	717.1	740.1	733.2	737.4	719.6	741.3	731.4 ± 9.65	1.319

Table 5: It shows method precision

Drug	Std 1 (peak area)	Std 2 (peak area)	Std 3 (peak area)	Std 4 (peak area)	Std 5 (peak area)	Std 6 (peak area)	Mean ± S.D	% R.S.D.
KET (50ng/spot)	1040.1	1015.6	1030.4	1008.7	1021.3	1022.7	1023.13 ± 10.3	1.006
OFL (30 ng/spot)	731.9	726.2	732.6	739.8	725.9	740.2	732.76 ± 12.7	1.731

Table 6: It shows data for Intraday and Interday Precision of the proposed method.

Drug	Intraday Precision * (peak Area)			Average peak Area ± S.D	% R.S.D.	Interday Precision * (peak Area)			Average peak Area ± S.D	% R.S.D.
	1	2	3			Day 1	Day 4	Day7		
KET (50ng/spot)	1025.3	1017.9	1018.6	1020.6 ± 11.9	1.166	1027.6	1016.9	1011.6	1018.7 ± 13.1	1.286
OFL (30ng/spot)	722.9	727.9	740.8	730.53 ± 13.8	1.8	723.8	731.8	739.1	731.56 ± 10.9	1.49

n= 6

Accuracy

Percent recovery of the proposed method when applied for marketed formulation after spiking with standard was found to be 99.74-99.95 % for both drugs (Table 7).

Table 7: It shows statistical recovery of the proposed method.

Drug (ng/spot)	Amount Spiked (%)	Amount spiked (ng/spot)	Mean concentration found (ng/spot)	% Mean Recovery ± S.D.	% RSD
KET (50)	80	40	39.98	99.95 ± 1.1	0.866
	100	50	49.87	99.74 ± 1.1	0.905
	120	60	59.91	99.85 ± 1.2	0.838
OFL (30)	80	24	23.94	99.78 ± 0.4	0.401
	100	30	29.96	99.87 ± 0.5	0.501
	120	36	35.93	99.83 ± 0.8	1.24

n= 6

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Sensitivity

Sensitivity of the method as shown in Table 8 was proved by low limit of detections 0.077/0.2165 ng/band (KET/OFL) and limit of quantitation 0.2357/0.656 ng/band (KET/OFL).

Table 8: It shows statistical analysis for LOD and LOQ of the proposed methods

Sr. No.	Parameter	KET(ng/band)	OFL(ng/band)
1.	LOD	0.077	0.2165
2.	LOQ	0.2357	0.656

Specificity and robustness

The low values of % RSD in peak area after doing deliberate minor changes in the method parameters indicates robustness of the method (Table 9). Values of r as shown in Table 1 are greater than 0.99 indicates peak purity of both analytes. From peak purity and absence of additional peaks other than drugs proves specificity of the method.

This method utilizes simple mobile phase. As compared to other reported chromatographic methods proposed method is economic. Hence it can be used for routine analysis of two drugs in combined dosage forms.

Table 9: Robustness study of KET and OFL

Parameter	SD of peak area (% RSD) KET	SD of peak area (% RSD) OFL
Time from spotting to chromatography	14.78 (1.08)	10.34 (0.67)
Time from chromatography to scanning	15.12 (1.12)	9.89 (0.71)
Ultrasonication time	11.65 (0.54)	8.11 (0.38)
Plate from different lot numbers	10.32 (0.39)	9.68 (0.43)

CONCLUSION

The developed TLC technique for simultaneous determination of KET and OFL was validated for precision, specificity, robustness, accuracy, Limit of quantitation and Limit of detection. It was found to comply with all validation parameters. Statistical analysis proves that the method is suitable for the analysis of KET and OFL from the bulk drug and ophthalmic formulations without any interference from the excipients.

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REFERENCES

1. Budavari S, O'Neill MJ, Smith A, Heckelman PE, Kinneary JF. The Merck Index: An Encyclopedia of Chemicals; Drugs and Biologicals; 13th ed. Merck and Co. Inc.; Whitehouse Station; 2001, pp 6800.
2. Prakash MS, Meena S. Fluorophotometric Determination of Ketorolac Tromethamine. Indian Drugs. 1996; 33: 149-151.
3. Devarajan PV, Gore SP, Chavan SV. HPTLC Determination of Ketorolac Tromethamine. J Pharm Biomed Anal. 2000; 22: 679-683.
4. Irene T, Frances C, Martin K, Marian P, Anne W. HPLC Method for the Determination of Ketorolac in Human Plasma; J of Liquid Chromatography & Related Technologies. 1996;19(6):957 – 967.
5. Chaudhary RS, Gangwal SS, Jindal KC, Khanna . Reversed-phase high-performance liquid chromatography of ketorolac and its application to bioequivalence studies in human serum. J Chromatography B: Biomedical Sciences and Applications. 1993; 614(1):180-184.
6. Guo-Ping Yang, Bin He, Shi-Kun Liu. Determination of the content of ketorolac tromethamine in injection by HPLC. Chinese J Hospital Pharm. 2003;12.
7. Reddy BP, Suryanarayana MV, Vemkatraman S, Krupadanam GL, Sastry CS. Purity Evaluation of Ketorolac Tromethamine by HPLC. Indian Drugs. 1993; 30:176-179.
8. Patri S, Patni AK, Iyer SS, Khuroo AH, Tausif M, Rana S. A Validated High-Performance Liquid Chromatography-Tandem Mass Spectrometric (LC-MS/MS) Method for Simultaneous Determination of

R(+)-Ketorolac and S(−)-Ketorolac in Human Plasma and Its Application to a Bioequivalence Study Chromatography Research International. 2011; 11.

9. Zhang XZ, Wen W, Jiang JY, Luo SD, Cai HS. First Order Derivative Spectrophotometry of Ofloxacin Gel. Chinese J Pharm. 1997; 28:314-315.
10. Liu JL. K-ratio and Differential Spectrophotometry of Ofloxacin and Metronidazole in Compound Ofloxacin Ear Drops. Chinese J Pharm. 1997; 28:514-515.
11. Jiang JY, Feng LZ, Li RL, Cai HS. Ultraviolet Spectrophotometry of Ofloxacin (OLX) Injection. Chinese J Pharm. 1996; 27:74-76.
12. Subrahmanyam CV, Reddy SE, Reddy MS. Analytical Method for Estimation of Ofloxacin in Urine. Indian Drugs. 1996; 33: 76-78.
13. Murugesan N, Mathur SC, Kumar Y, Rathore YK, Sethi PD. Spectrofluorometric Estimation of Ofloxacin in Tablet. Eastern Pharmacist. 1992; 35:117-118.
14. Mathur SC, Kumar Y, Murugesan N, Rathore V, Sethi PD. Spectrophotometric Determination of Ofloxacin in Pharmaceutical Formulations. Indian Drugs. 1992; 29:376-377.
15. Fabre D, Bressoll F, Kinowski JF, Bouvet O, Paganin F, Galtier M. A reproducible; simple and sensitive HPLC assay for determination of ofloxacin in plasma and lung tissue; Application in pharmacokinetic studies. J Pharm Biomed Anal. 1994; 12(11):1463-1469.
16. Fegade JD, Mehta HP, Chaudhari RY, Patil VR. Simultaneous Spectrophotometric Estimation of Ofloxacin and Ketorolac Tromethamine in Ophthalmic Dosage Form. International Journal of Chem Tech Research. 2009; 1(2):189-194.
17. Du YX, Luo D, Wang QF. Determination of Ofloxacin in Human Serum by Reversed Phase HPLC. J China Pharm Uni. 1994; 25:32-35.
18. Basci NE, Hanioglu-kargi S, Soysal S, Bozkurt A, Kayaalp SO. Determination of Ofloxacin in Human Aqueous Humor by High Performance Liquid Chromatography with Fluorescence Detection. J Pharm Biomed Anal. 15:663-666; 1997.
19. Siva Kumar R, Kumar Nallasivan P, Saravanakumar S, Kandasamy CS, Venkarayanan R. Simultaneous RP-HPLC Estimation of Nitazoxanide and Ofloxacin in Tablet Dosage Forms. Asian J Research Chem. 2009;2(1):43-45.
20. Shinde SR, Bhoir SI , Pawar NS, Yadav SB, Bhagwat AM. Simultaneous Estimation of Satranidazole and Ofloxacin in Tablet Dosage Form by High Performance Liquid Chromatography; E-J Chem. 2010; 7(1):198-202.
21. Khandagle KS, Gandhi SV, Deshpande PB, Gaikwad NV. A Simple and Sensitive RPHPLC Method for Simultaneous Estimation of Cefixime and Ofloxacin in Combined Tablet Dosage Form. International Journal of Pharmacy and Pharmaceutical Sciences. 2011; 3(1):46-48.
22. Premanand DC, Senthilkumar KL, Senthilkumar V, Saravanakumar M, Thirumurthy R. A Validated RP-HPLC Method for Simultaneous Estimation of Nitazoxanide and Ofloxacin in Pharmaceutical Formulation. Der Chemica Sinica. 2010; 1(2):1-5.
23. Dhandapani B, Thirumoorthy V, Shaikh HR, Rama kotaiah M, Anjaneyalu N. Method Development and Validation for the Simultaneous Estimation of Ofloxacin and Ornidazole in Tablet Dosage Form by RP-HPLC. International Journal of Pharma Sciences and Research. 2010; 1(1):78-83.
24. Singha R, Maithani1a M, Saraf K, Saraf S, Ram C. Simultaneous Estimation of Ciprofloxacin Hydrochloride; Ofloxacin; Tinidazole and Ornidazole by RP HPLC. Eu J Anal Chem. 2009; 4(2):161-167.
25. Dharuman J, Vasudevan M, Somasekaran KN, Dhandapania B, Ghodea PD, Thiagarajana M. RP-HPLC Method Development and Validation for the Simultaneous Estimation of Ofloxacin and Tinidazole in Tablets. International Journal of Pharm Tech Research. 2009; 1(2):121-124.
26. Puranik M, Bhawsar DV, Rathi P, Yeole PG. Simultaneous determination of ofloxacin and ornidazole in solid dosage form by RP-HPLC and HPTLC techniques. Indian J Pharm Sci. 2010; 72 (4):513-517.
27. Kraas E, Hirrle A. Determination of ofloxacin in biological fluids using HPLC with fluorimetric detection; Fresenius' J Anal Chem. 1986; 324(3-4):354.
28. Tuncel M, Atkosar Z. Determination of Ofloxacin in Tablet by Potentiometry and Conductometry; Pharmazie. 1992;47:642-643.
29. ICH Harmonised Tripartite Guidelines; Validation of Analytical Procedures; Text and Methodology; Q2 (R1); Geneva; November 2005, 1-8.