Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 37 (2005) 27–38
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Application of HPLC and HPTLC for the simultaneous determination of tizanidine and rofecoxib in pharmaceutical dosage form

Neeraj Kaul, S.R. Dhaneshwar*, Himani Agrawal, Abhijit Kakad, Bharat Patil

Department of Quality Assurance Techniques, Poona College of Pharmacy, Bharati Vidyapeeth Deemed University, Erandwane, Pune 411038, Maharashtra, India

Received 19 March 2004; received in revised form 19 August 2004; accepted 13 September 2004
Available online 14 November 2004

Abstract

Two methods are described for the simultaneous determination of tizanidine and rofecoxib in binary mixture. The first method was based on HPTLC separation of the two drugs followed by densitometric measurements of their spots at 311 nm. The separation was carried out on Merck HPTLC aluminium sheets of silica gel 60 F_{254} using toluene:methanol:acetone (7.5:2.5:1.0, v/v/v) as mobile phase. The linear regression analysis data was used for the regression line in the range of 10–100 and 100–1500 ng/spot for tizanidine and rofecoxib, respectively. The second method was based on HPLC separation of the two drugs on the reversed phase kromasil column [C_{18} (5 μm, 25 cm × 4.6 mm, i.d.)] at ambient temperature using a mobile phase consisting of phosphate buffer pH 5.5 and methanol (45:55, v/v). Flow rate was 1.0 ml/min with an average operating pressure of 180 kg/cm^2. Quantitation was achieved with UV detection at 235 nm based on peak area with linear calibration curves at concentration ranges 10–200 and 100–2000 μg/ml for tizanidine and rofecoxib, respectively. Both methods have been successively applied to pharmaceutical formulation. No chromatographic interference from the tablet excipients was found. Both methods were validated in terms of precision, robustness, recovery and limits of detection and quantitation. The analysis of variance (ANOVA) and Student's *t*-test were applied to correlate the results of tizanidine and rofecoxib determination in dosage form by means of HPTLC and HPLC method.

Keywords: Tizanidine; Rofecoxib; HPTLC; Reversed phase HPLC; Method validation; Quantitative analysis; ANOVA; Student's *t*-test

1. Introduction

Tizanidine 5-chloro-4-(2-imidazolin-2-ylamino)-2,1,3-benzothiadiazole (Fig. 1) is α_2 – adrenergic agonist and centrally active myotonolytic skeletal muscle relaxant with a chemical structure unrelated to other muscle relaxants [1,2]. It reduces spasticity by increasing presynaptic inhibition of motor neurons. The effects of tizanidine are greatest on polysynaptic pathways. The overall effect of these actions is thought to reduce facilitation of spinal motor neurons. It also reduces increased muscle tone associated with spasticity in patients with multiple sclerosis or spinal cord injury. The plasma concentration of tizanidine after oral administration is presumed to be several nanograms [3]. In the literature, a radioimmunoassay method for the quantification of tizanidine hydrochloride has been widely used [4]. Also determination of tizanidine in human plasma by gas chromatography–mass spectrometry has been reported [5]. Tizanidine, which contains a cyclic guanidine moiety, can exist as two tautomers [6]. There are very few reports on analytical methods for estimation of tizanidine in bulk and its dosage form. A RP–HPLC method for estimation of tizanidine hydrochloride in combination with nimesulide has been reported by Raman and Patil [7]. Qi et al. [8] have reported stability indicating HPLC method for tizanidine. Mahadik et al. [9] have reported stability indicating HPTLC method for tizanidine hydrochloride.

Rofecoxib chemically 4-(4-methanesulfonylphenyl)-3-phenyl-5H-furan-2-one (Fig. 2) is a new generation

* Corresponding author. Tel.: +91 20 25437237; fax: +91 20 25439383.
E-mail address: srdhaneshwar@hotmail.com (S.R. Dhaneshwar).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2004.09.036

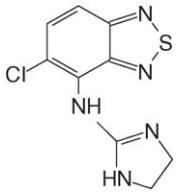

Fig. 1. Structure of tizanidine.

non-steroidal anti-inflammatory agent (NSAID) that exhibits promising anti-inflammatory, analgesic and antipyretic activity. It selectively inhibits cyclo-oxygenase II (COX-2) isoenzyme in a dose dependent manner in man [10–13]. COX-2 is found in elevated levels in inflammatory exudates [14,15]. Rofecoxib (a specific COX-2 inhibitor) selectively targets the prostaglandins involved in pain and inflammation. Several methods for quantitative estimation of rofecoxib in pharmaceutical dosage form and in biological fluids have been reported in the literature. Woolf et al. [16] has reported HPLC method for rofecoxib in plasma with post column photochemical derivitization and fluorescence detection. Matthews et al. [17] have described LC method after solid phase extraction with fluorescence detection. Several LC–MS methods for determination of rofecoxib in human plasma have been reported [18–20]. Simple reverse phase HPLC method for quantitative estimation of rofecoxib in pharmaceutical formulation [21] and from human plasma [22–24] has been reported. Mao et al. [25] has reported stability indicating HPLC method for rofecoxib. Isolation and characterization of process related impurities in rofecoxib have been reported [26].

The present work presents two new methods for simultaneous determination of tizanidine and rofecoxib in tablets using HPTLC-densitometry and reverse phase HPLC. The two methods are simple, reduce the duration of the analysis and suitable for routine determination of two drugs.

2. Experimental

2.1. Materials

Pharmaceutical grade of tizanidine (batch no.: TNZ/QA/0038) and rofecoxib (batch no.: RXB/FP/01) were kindly supplied as a gift sample by Sun Pharma Ltd., Gujarat, India, used without further purification and certified to contain 99.75% (w/w) and 99.52% (w/w), respectively on dried basis. All chemicals and reagents used were of HPLC grade and were purchased from Merck Chemicals, India.

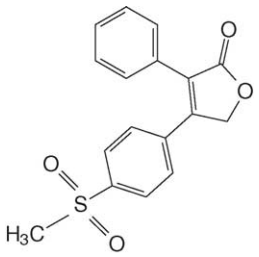

Fig. 2. Structure of rofecoxib.

2.2. Instrumentation and chromatographic conditions

2.2.1. For TLC densitometry

The samples were spotted in the form of bands of width 6 mm with a Camag 100 μl sample (Hamilton, Bonaduz, Switzerland) syringe on precoated silica gel aluminium Plate 60 F-254 (20 cm × 10 cm) with 250 μm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, (Mumbai) using a Camag Linomat IV (Switzerland). The plates were prewashed by methanol and activated at 110 °C for 5 min prior to chromatography. A constant application rate of 0.1 μl/s was employed and space between two bands was 5 mm. The slit dimension was kept at 5 mm × 0.45 mm and 10 mm/s scanning speed was employed. The monochromator bandwidth was set at 20 nm with K 320 cut off filter, each track was scanned thrice and baseline correction was used. The mobile phase consisted of toluene–methanol–acetone (7.5:2.5:1.0, v/v/v) and 15 ml of mobile phase was used per chromatography. Linear ascending development was carried out in 20 cm × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland). Dimensions: length × width × height = 12 cm × 4.7 cm × 12.5 cm. It was saturated (lined on the two bigger sides with filter paper that had been soaked thoroughly with the mobile phase) and the chromatoplate development was carried out in dark with the mobile phase. The optimized chamber saturation time for mobile phase was 30 min at room temperature (25 °C ± 2) at relative humidity of 60% ± 5. The length of chromatogram run was 9 cm and approximately 30 min. Subsequent to the development, TLC plates were dried in a current of air with the help of an air dryer in wooden chamber with adequate ventilation. The flow of air in the laboratory was maintained unidirectional (laminar flow, towards exhaust). Densitometric scanning was performed on Camag TLC scanner III in the reflectance-absorbance mode at 311 nm for all measurements and operated by CATS software (V 3.15, Camag). The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. Evaluation was via peak areas with linear regression.

2.2.2. For HPLC method

The HPLC system consisted of a pump (model Jasco PU 1580, intelligent HPLC pump) with auto injecting facility (AS-1555 sampler) programmed at 20 μl capacity per injection was used. The detector consisted of a UV–vis (Jasco UV 1575) model operated at a wavelength of 235 nm. The software used was Jasco borwin version 1.5, LC-Net II/ADC system. The columns used were Kromasil C-18 (250 mm × 4.6 mm, 5.0 μm) Flexit Jour Laborarories Pvt. Ltd. Pune, India and Finepak SIL-5, C-18

(250 mm × 4.6 mm, 5.0 μ) Jasco Corporation, Japan. Different mobile phases were tested in order to find the best conditions for separating both the drugs simultaneously. The optimal composition of the mobile phase was determined to be phosphate buffer pH 5.5:methanol (45:55, v/v). Phosphate buffer pH 5.5 was prepared as per the procedure given in Indian Pharmacopoeia [27]. The flow rate was set to 1.0 ml/min and UV detection was carried out at 235 nm.

2.3. Standard solutions and calibration graphs

Stock standard solution was prepared by dissolving 0.10 g of tizanidine and 1.25 g of rofecoxib in 100 ml acetonitrile. The stock solution was protected from direct light by using amber colored volumetric flasks and keeping the solution at room temperature [22]. All work with stock solution was performed under controlled light conditions in order to prevent rofecoxib from undergoing photocyclization reaction.

2.3.1. For HPTLC–densitometric method

The standard solutions were prepared by dilution of the stock solution with methanol to reach a concentration range 10–100 and 100–1500 ng/μl for tizanidine and rofecoxib respectively. One micro liter from each standard solution was spotted on the TLC plate to obtain final concentration 10–100 and 100–1500 ng/spot for tizanidine and rofecoxib respectively. Each concentration was spotted six times on the TLC plate. The plate was developed on previously described mobile phase. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs.

2.3.2. For HPLC method

The standard solutions were prepared by dilution of the stock solution with methanol to reach a concentration range 10–200 and 100–2000 μg/ml for tizanidine and rofecoxib, respectively. Triplicate 20 μl injections were made six times for each concentration for tizanidine and rofecoxib, respectively and chromatographed under the conditions described above. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs.

2.4. Sample preparation

To determine the content of tizanidine and rofecoxib simultaneously in conventional tablets (label claim: 2 mg tizanidine and 25 mg rofecoxib per tablet, combination tablet containing both analytes), the twenty tablets were weighed, their mean weight determined and they were finely powdered and powder equivalent to 2 mg tizanidine and 25 mg rofecoxib was weighed. Then equivalent weight of the drug was transferred into a 100 ml volumetric flask containing 50 ml acetonitrile, sonicated for 30 min and diluted to 100 ml with acetonitrile. The resulting solution was centrifuged at 3000 rpm for 5 min. Supernatant containing 20 μg/ml of tizanidine and 250 μg/ml of rofecoxib was taken and filtered using 0.45 μm filter (Millipore, milford, MA).

2.4.1. For HPTLC–densitometric method

Different microlitres (2, 3 and 4 μl) of sample solution were applied six times to the HPTLC plate to give concentration 40, 60, 80 ng/spot and 500, 750, 1000 ng/spot for tizanidine and rofecoxib, respectively. The plate was developed in the previously described chromatographic conditions. The peak area of the spots were measured at 311 nm for tizanidine and rofecoxib, respectively and their concentrations in the samples were determined using multilevel calibration developed on the same plate under the same conditions using linear regression equation.

2.4.2. For HPLC method

A 20 μl volume of sample solution (20 and 250 μg/ml of tizanidine and rofecoxib, respectively) was injected into HPLC, six times, under the conditions described above. The peak area of the spots were measured at 235 nm for tizanidine and rofecoxib, respectively and their concentrations in the samples were determined using multilevel calibration developed on the same HPLC system under the same conditions using linear regression equation.

2.5. Method validation

Both methods were validated in compliance with ICH guidelines [28,29]. The following parameters were validated.

2.5.1. Precision

Precision of the method was determined with the product. An amount of the product powder equivalent to 100% of the label claim of tizanidine and rofecoxib was accurately weighed and assayed. System repeatability was determined by six replicate applications and six times measurement of a sample solution at the analytical concentration. The repeatability of sample application and measurement of peak area for active compound were expressed in terms of relative standard deviation (%R.S.D.) and standard error (S.E.). Method repeatability was obtained from R.S.D. value by repeating the assay six times in same day for intra-day precision. Intermediate precision was assessed by the assay of two, six sample sets on different days (inter-day precision). The intra-day and inter-day variation for determination of tizanidine and rofecoxib was carried out at three different concentration levels 30, 50, 80 ng/spot, 375, 625, 1000 ng/spot and 50, 100, 150 μg/ml and 625, 1250, 1875 μg/ml for HPTLC and HPLC, respectively.

2.5.2. Robustness of the method

2.5.2.1. For HPTLC–densitometric method. By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different

composition like toluene–methanol–acetone (7.0:3.0:1.0, v/v/v), toluene–methanol–acetone (7.0:2.5:1.5, v/v/v), toluene–methanol–acetone (8.0:2.0:1.0, v/v/v), toluene–methanol–acetone (7.5:2.0:1.5, v/v/v), toluene–methanol–acetone (8.0:2.5:0.5, v/v/v) and toluene–methanol–acetone (7.5:3.0:0.5, v/v/v) were tried and chromatograms were run. The amount of mobile phase, temperature and relative humidity was varied in the range of ±5%. The plates were prewashed by methanol and activated at 60 °C ± 5 for 2, 5, 7 min, respectively prior to chromatography. Time from spotting to chromatography and from chromatography to scanning was varied from 0, 20, 40 and 60 min. Robustness of the method was done at three different concentration levels 30, 50, 80 ng/spot and 375, 625, 1000 ng/spot for tizanidine and rofecoxib, respectively.

2.5.2.2. For HPLC method. To evaluate HPLC method robustness a few parameters were deliberately varied. The parameters included variation of C_{18} columns from different manufacturers, pH of the buffer, flow rate, percentage of methanol in the mobile phase, column temperature and methanol of different lots. Two analytical columns, One (Kromasil C 18 column) from Pune, India and the other (Finepak C 18 column) from Japan, were used during the experiment. Robustness of the method was done at three different concentration levels 50, 100, 150 ng/spot and 625, 1250, 1875 ng/spot for tizanidine and rofecoxib, respectively.

2.5.3. Limit of detection and limit of quantitation

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

2.5.3.1. For HPTLC–densitometric method. In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was spotted six times following the same method as explained above. The signal to noise ratio (S/N) of 3 and 10 was determined for six replicate determinations.

2.5.3.2. For HPLC method. The limit of detection (LOD) and limit of quantitation (LOQ) were separately determined at a signal to noise ratio (S/N) of 3 and 10. LOD and LOQ were experimentally verified by diluting known concentrations of tizanidine and rofecoxib until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations.

2.5.4. Specificity

2.5.4.1. For HPTLC–densitometric method. The specificity of the method was ascertained by analyzing standard drug and sample. The spot for tizanidine and rofecoxib in sample was confirmed by comparing the R_f and spectra of the spot with that of standard. The peak purity of tizanidine and rofecoxib was assessed by comparing the spectra at three different levels, i.e., peak start (*S*), peak apex (*M*) and peak end (*E*) positions of the spot.

2.5.4.2. For HPLC method. The specificity of the HPLC method was determined by the complete separation of tizanidine and rofecoxib along with other parameters like retention time (t_r), capacity factor (k), tailing or asymmetrical factor (T), etc.

2.5.5. Recovery studies

For both methods recovery studies was carried out by applying the method to drug sample to which known amount of tizanidine and rofecoxib corresponding to 80, 100 and 120% of label claim had been added (standard addition method). At each level of the amount six determinations were performed and the results obtained were compared with expected results.

3. Result and discussion

3.1. Optimization of procedures

3.1.1. Optimization of HPTLC–densitometric method

Initially toluene and methanol in the ratio of 5:5 (v/v) was tried for both drugs simultaneously. The spots were not developed properly and dragging was observed. Then toluene and methanol in the ratio of 3:7 (v/v) was tried. The developed spots were diffused and R_f was near to solvent front. Then the reverse ratio of same mobile phase was tried. The distance travelled by developed spots was less and dragging was observed. To the above mobile phase carbon tetrachloride and acetonitrile in different ratios were added but the developed spots lack compactness and were less persistent. Also the R_f values of tizanidine and rofecoxib were not satisfactory because of less resolution between them. Then 0.1 ml of ammonia was added to toluene, methanol in the ratio of 7:3 (v/v). Total dragging of the spots from the point of sample application was observed. Finally 0.1 ml of ammonia was replaced by 1.0 ml of acetone. The spots developed were dense, compact and typical peak nature for both tizanidine and rofecoxib was observed but resolution between them was less. To improve the resolution, the volume of toluene was increased by 0.5 ml and that of methanol was reduced by 0.5 ml. Ultimately mobile phase consisting of toluene:methanol:acetone (7.5:2.5:1.0, v/v/v) gave good resolution. Both the peaks were symmetrical in nature and no tailing was observed when plates were scanned at 311 nm (Fig. 3). Well-defined spots were obtained when plate was activated at 110 °C for 5 min

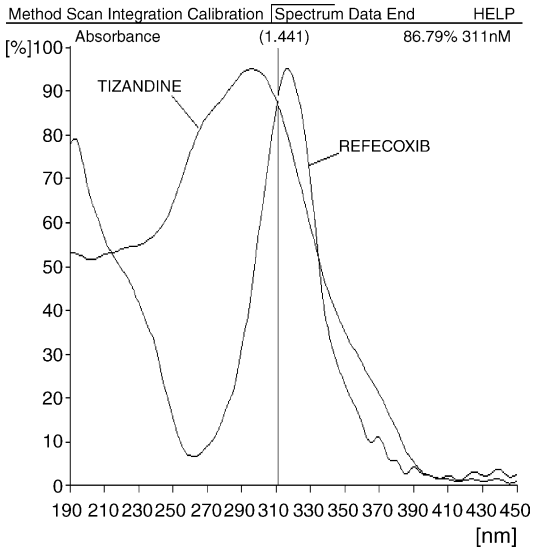

Fig. 3. In situ overlain spectra of tizanidine and refecoxib measured from 190 to 450 nm.

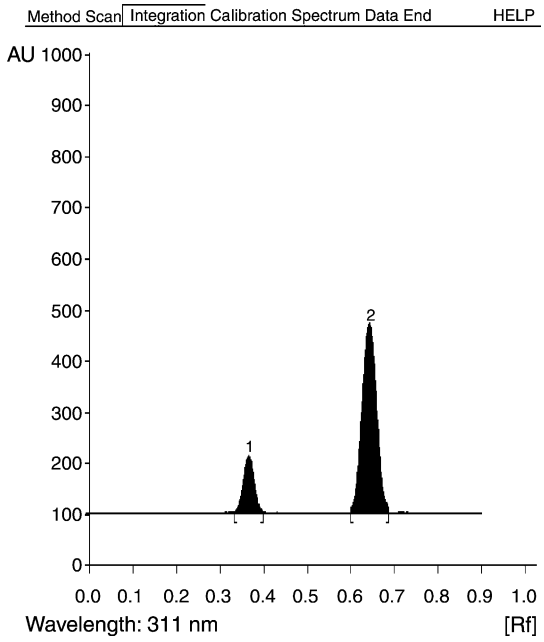

Fig. 4. Densitogram of standard tizanidine (60 ng/spot); peak 1 (R_f: 0.36 ± 0.02) and rofecoxib (750 ng/spot); peak 2 (R_f: 0.65 ± 0.02), in ratio of (1:12.5) measured at 311 nm, mobile phase toluene–methanol–acetone (7.5:2.5:1.0, v/v/v).

(Table 1) and the chamber was saturated with the mobile phase for 30 min at room temperature (Fig. 4).

3.1.2. Optimization of HPLC method

Initially methanol and water was tried in the ratio of 80:20 (v/v) for each drug individually. Rofecoxib showed good peak nature but for tizanidine negative absorbance was observed in the chromatogram. Then methanol was replaced by acetonitrile in the same ratio. Splitting was observed for both peaks. Then acetonitrile, methanol and water were tried 60:30:10 (v/v/v). Again the peaks for both drugs showed splitting. Then above mobile phase in different ratios were tried along with change in pH from 3.0 to 5.0 with the help of ortho phosphoric acid. Still the splitting was observed. Therefore, acetonitrile was completely removed and methanol:water in the ratio of 50:50 (v/v) pH 5.0 was tried. But the peak for tizanidine showed slight negative absorbance. To rectify it water was replaced by phosphate buffer pH 5.5. Both drugs showed typical peak nature and peaks were symmetrical at 235 nm (Fig. 5). Tailing factor for both peaks was less than 2% but the resolution was not satisfactory. To improve the resolution of two peaks ratio of methanol and phosphate buffer pH 5.5 was finally adjusted to 55:45 (v/v) and this ratio was selected for validation purpose (Fig. 6).

Table 1
Reproducibility of run time[a]

Plate condition[b]	Run time[c] (min)	S.D.	R.S.D. (%)
Blank plate	30.14	0.44	1.69
Plate spotted with standards	30.45	0.52	1.43

[a] $n = 6$.
[b] Plates pre-treated with methanol and activated at 110 °C.
[c] Development was performed in the ascending direction at constant run length of 9 cm.

3.2. Linearity

Tizanidine showed good correlation coefficient in concentration range of 10–100 ng/spot ($r = 0.9996 \pm 1.15$) and 10–200 μg/ml ($r = 0.9997 \pm 1.02$) where as rofecoxib in the concentration range of 100–1500 ng/spot ($r = 0.9995 \pm 1.25$) and 100–2000 μg/ml ($r = 0.9992 \pm 1.52$) for HPTLC and HPLC, respectively. Linearity was evaluated by determining six standard working solutions containing 30–80 ng/spot, 375–1000 ng/spot and 50–150 μg/ml and 625–1875 μg/ml of tizanidine and rofecoxib twice in triplicate for HPTLC and

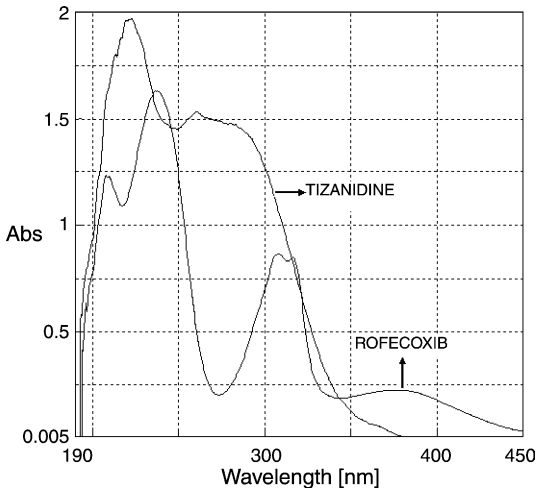

Fig. 5. Overlain spectra of tizanidine and rofecoxib (each 10 μg/ml in methanol) taken on UV–vis spectrophotometer (V 530 series).

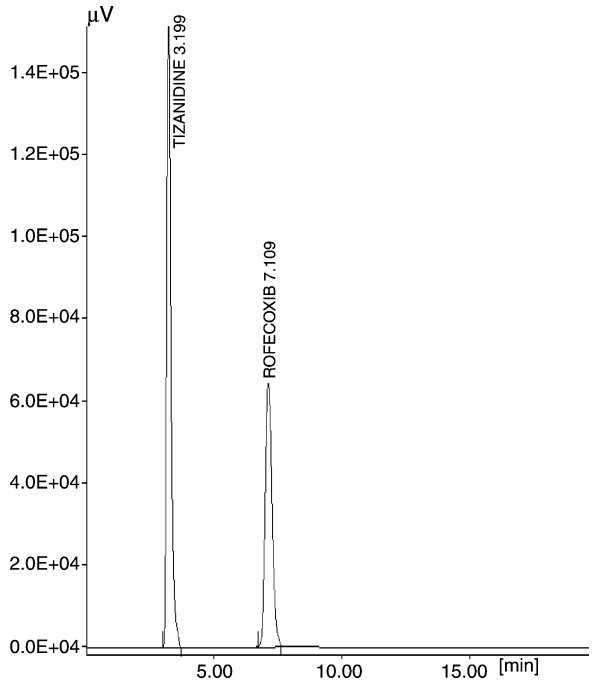

Fig. 6. Chromatogram of standard tizanidine (50 μg/ml); (R_t: 3.199 ± 0.05) and rofecoxib (625 μg/ml); (R_t: 7.109 ± 0.07), in ratio of (1:12.5) measured at 235 nm, mobile phase phosphate buffer pH 5.5 and methanol (45:55, v/v).

HPLC, respectively (Table 2). For both methods the linearity of calibration graphs and adherence of the system to Beer's law was validated by high value of correlation coefficient and the S.D. for intercept value was less than 2%. No significant difference was observed in the slopes of standard curves (ANOVA; $P < 0.05$).

3.3. Precision

3.3.1. For HPTLC–densitometric method

The repeatability of sample application and measurement of peak area were expressed in terms of %R.S.D. and were found to be 1.89, 1.26 and 0.48, 0.67 for tizanidine and rofecoxib, respectively. The %R.S.D. values depicted in Table 3a shows that proposed method provides acceptable intra-day and inter-day variation of tizanidine and rofecoxib.

3.3.2. For HPLC method

The within-run precision and between-run precision of the proposed HPLC method were determined by assaying the tablets in six times per day for consecutive six days and expressed as %R.S.D. The intra-day and inter-day precision has been depicted in Table 3b.

3.4. Robustness of the method

3.4.1. For HPTLC–densitometric method

The standard deviation of peak areas was calculated for each parameter and %R.S.D. was found to be less than 2%. The low values of %R.S.D. as shown in Table 4a indicated robustness of the method.

3.4.2. For HPLC method

Each factor selected (except columns from different manufacturers and solvents of different lots) to examine were charged at three levels (−1, 0 and 1). One factor at the time was changed to estimate the effect. Thus, replicate injections ($n = 6$) of mixed standard solution at three concentration levels were performed under small changes of six chromatographic parameters (factors). Results, presented in Table 4b indicate that the selected factors remained unaffected by small variations of these parameters. The results from the two columns indicated that there is no significant difference between the results from the two columns. It was also found that methanol of different lots from the same manufacturer had no significant influence on the determination. Insignificant differences in peak areas and less variability in retention time were observed.

3.5. LOD and LOQ

3.5.1. For HPTLC–densitometric method

The signal/noise ratios 3:1 and 10:1 were considered as LOD and LOQ, respectively. The LOD and LOQ were found to be 10, 20 ng/spot and 25, 40 ng/spot, respectively for tizanidine and rofecoxib.

3.5.2. For HPLC method

The LOD and LOQ were found to be 0.01, 0.10 μg/ml and 0.05, 0.15 μg/ml, respectively for tizanidine and rofecoxib.

Table 2
Linear regression data for calibration curves ($n = 6$)

Parameters	TLC densitometry		HPLC	
	Tizanidine	Rofecoxib	Tizanidine	Rofecoxib
Linearity range	30–80 (ng/spot)	375–1000 (ng/spot)	50–150 (μg/ml)	625–1875 (μg/ml)
$r \pm$ S.D.	0.9992 ± 2.3	0.9998 ± 1.60	0.9999 ± 1.44	0.9998 ± 1.85
Slope ± S.D.	1.23 ± 0.58	0.07 ± 0.01	1.65 ± 0.87	1.13 ± 0.84
Intercept ± S.D.	29.50 ± 2.35	49.83 ± 2.05	2.16 ± 1.42	2.55 ± 1.21
Confidence limit of slope[a]	0.765–1.695	0.062–0.078	0.95–2.34	0.46–1.80
Confidence limit of intercept[a]	27.62–31.38	48.18–51.47	1.02–3.29	1.58–3.52
S.E. of estimation	1.63	1.78	1.49	1.53

[a] 95% confidence limit.

Table 3
Intra- and inter-day precision of Tizanidine (a) and Rofecoxib (b) ($n = 6$)

TLC densitometry						HPLC					
Intra-day precision			Inter-day precision			Intra-day precision			Inter-day precision		
S.D. of areas	%R.S.D.	S.E.	S.D. of areas	%R.S.D.	S.E.	S.D. of areas	%R.S.D.	S.E.	S.D. of areas	%R.S.D.	S.E.
(a) Tizanidine[a] ($n = 6$)											
1.94	1.65	0.79	2.24	1.85	1.02	1.39	1.23	0.46	1.86	1.68	0.89
(b) Rofecoxib[b] ($n = 6$)											
1.87	1.21	0.71	2.18	1.34	0.88	1.64	1.45	0.59	1.37	1.98	0.45

[a] Average of three concentrations 30, 50, 80 ng/spot and 50, 100, 150 μg/ml for HPTLC and HPLC, respectively.
[b] Average of three concentrations 375, 625, 1000 ng/spot and 625, 1250, 1875 μg/ml for HPTLC and HPLC, respectively.

3.6. Specificity

3.6.1. For HPTLC–densitometric method

The peak purity of tizanidine and rofecoxib was assessed by comparing their respective spectra at peak start, peak apex and peak end positions of the spot, i.e., r (S, M) = 0.9995, 0.9997 and r (M, E) = 0.9992, 0.9996. Good correlation ($r = 0.9998$ and $r = 0.9997$) was also obtained between standard and sample spectra of tizanidine and rofecoxib, respectively.

3.6.2. For HPLC method

The specificity of the HPLC method is illustrated in Fig. 7 where complete separation of tizanidine and rofecoxib was noticed in presence of tablet excipients. The average retention time ±standard deviation for tizanidine and rofecoxib were found to be 3.19 ± 0.05 and 7.11 ± 0.07 min, respectively, for six replicates. The peaks obtained were sharp and have clear baseline separation.

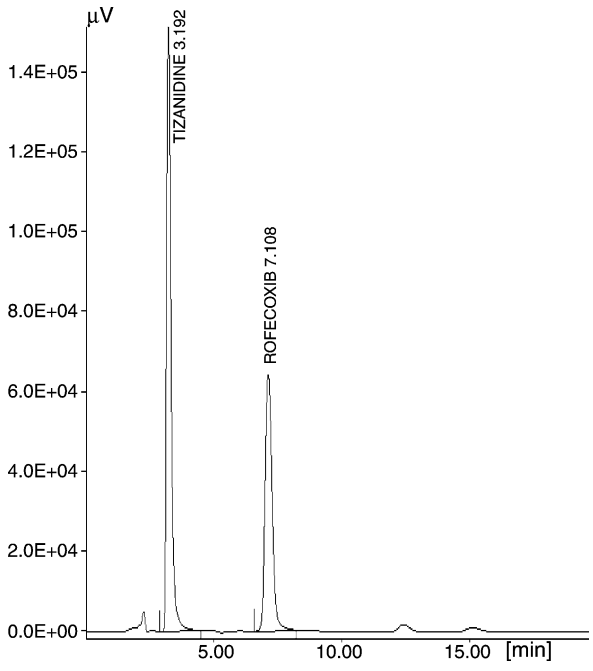

Fig. 7. Chromatogram of sample tizanidine (50 μg/ml); (R_t: 3.192 ± 0.05) and rofecoxib (625 μg/ml); (R_t: 7.108 ± 0.07), in ratio of (1:12.5) measured at 235 nm, mobile phase phosphate buffer pH 5.5 and methanol (45:55, v/v).

3.7. Recovery studies

Both the proposed methods when used for extraction and subsequent estimation of tizanidine and rofecoxib from pharmaceutical dosage form after spiking with additional drug afforded recovery of 98–102% and mean recovery for tizanidine and rofecoxib from the marketed formulation are listed in Table 5a and b.

The data of summary of validation parameters are listed in Table 6.

3.8. Stability in sample solution

3.8.1. For HPTLC–densitometric method

Solutions of two different concentrations (30 and 80 ng/spot for tizanidine) and (375 and 1000 ng/spot for rofecoxib) were prepared from sample solution and stored at room temperature for 0.5, 1.0, 2.0, 4.0 and 24 h respectively. They were then applied on the same TLC plate, after development the densitogram was evaluated as listed in Table 7 for additional spots if any. There was no indication of compound instability in the sample solution.

3.8.1.1. Spot stability. The time the sample is left to stand on the solvent prior to chromatographic development can influence the stability of separated spots and are required to be investigated for validation [30]. Two-dimensional chromatography using same solvent system was used to find out any decomposition occurring during spotting and development. In case, if decomposition occurs during development, peak(s) of decomposition product(s) shall be obtained for the analyte both in the first and second direction of the run. No decomposition was observed during spotting and development.

3.8.2. For HPLC method

Two different concentrations of tizanidine (50 and 100 μg/ml) and rofecoxib (625 and 1875 μg/ml) were prepared from sample solution and stored at room temperature for 3 days. They were then injected into the HPLC system and no additional peak was found in the chromatogram indicating the stability of tizanidine and rofecoxib in the sample solution (Table 7).

Table 4
Robustness (a) testing of HPTLC–densitometric method and (b) evaluation of the HPLC method ($n = 6$)

Parameter	Tizanidine		Rofecoxib	
	S.D.[b] of peak area	%R.S.D.[b]	S.D.[b] of peak area	%R.S.D.[b]
(a) Testing[a] of HPTLC–densitometric method				
Mobile phase composition	1.78	1.36	1.92	1.58
Amount of mobile phase	1.61	1.28	1.87	1.46
Temperature	1.18	0.84	1.27	0.95
Relative humidity	1.88	1.45	1.71	1.30
Plate pretreatment	0.78	0.52	0.91	0.78
Time from spotting to chromatography	0.67	0.43	0.79	0.51
Time from chromatography to scanning	0.51	0.36	0.68	0.46

Chromatographic changes		Tizanidine			Rofecoxib		
Factor[d]	Level	t_r[e]	k[f]	T[g]	t_r	k	T
(b) Robustness evaluation[c] of the HPLC method ($n = 6$)							
A: pH of the buffer							
5.40	−1	3.12	2.30	1.36	7.09	2.16	1.41
5.50	0	3.19	2.28	1.38	7.11	2.15	1.43
5.60	1	3.22	2.29	1.39	7.13	2.14	1.44
Mean ± S.D. ($n = 6$)		3.18 ± 0.05	2.29 ± 0.01	1.38 ± 0.02	7.11 ± 0.02	2.15 ± 0.01	1.43 ± 0.02
B: Flow rate (ml/min)							
0.90	−1	3.30	2.26	1.40	7.13	2.14	1.45
1.00	0	3.19	2.28	1.38	7.11	2.15	1.43
1.10	1	3.10	2.30	1.36	7.08	2.16	1.42
Mean ± S.D. ($n = 6$)		3.20 ± 0.10	2.28 ± 0.02	1.38 ± 0.02	7.10 ± 0.02	2.15 ± 0.01	1.43 ± 0.02
C: Percentage of methanol in the mobile phase (v/v)							
54	−1	3.26	2.25	1.39	7.12	2.13	1.44
55	0	3.19	2.28	1.38	7.11	2.15	1.43
56	−1	3.12	2.31	1.37	7.09	2.17	1.41
Mean ± S.D. ($n = 6$)		3.19 ± 0.07	2.28 ± 0.03	1.38 ± 0.01	7.11 ± 0.01	2.15 ± 0.02	1.43 ± 0.02
D: Temperature							
24	−1	3.22	2.29	1.39	7.14	2.16	1.45
25	0	3.19	2.28	1.38	7.11	2.15	1.43
26	1	3.14	2.27	1.37	7.07	2.13	1.41
Mean ± S.D. ($n = 6$)		3.18 ± 0.04	2.28 ± 0.01	1.38 ± 0.01	7.11 ± 0.03	2.15 ± 0.02	1.43 ± 0.02
E: Columns from different manufacturers							
Kromasil	3.19	2.28	1.38	7.11	2.15	1.43	
Finepak	3.20	2.30	1.39	7.13	2.16	1.42	
Mean ± S.D. ($n = 6$)		3.19 ± 0.007	2.29 ± 0.01	1.39 ± 0.007	7.12 ± 0.01	2.16 ± 0.07	1.43 ± 0.007
F: Solvents of different lots							
First lot	3.19	2.28	1.38	7.11	2.15	1.43	
Second Lot	3.16	2.29	1.37	7.12	2.14	1.44	
Mean ± S.D. ($n = 6$)		3.18 ± 0.02	2.29 ± 0.007	1.38 ± 0.007	7.12 ± 0.007	2.15 ± 0.007	1.44 ± 0.007

[a] $n = 6$.
[b] Average of three concentrations 30, 50, 80 ng/spot and 375, 625, 1000 ng/spot for tizanidine and rofecoxib, respectively.
[c] Average of three concentrations 50, 100, 150 μg/ml and 625, 1250, 1875 μg/ml for tizanidine and rofecoxib, respectively.
[d] Four factors were slightly changed at three levels (1, 0, −1); each time a factor was changed from level (0) the other factors remained at level (0).
[e] Retention time.
[f] Capacity factor.
[g] Tailing factor.

3.9. Analysis of the marketed formulation

3.9.1. For HPTLC–densitometric method

The spots at R_f 0.36 (for tizanidine) and 0.65 (for rofecoxib) were observed in the densitogram of the drug samples extracted from tablets. There was no interference from the excipients commonly present in the tablets. The drug content was found to be 99.40% ± 1.56 (%R.S.D. of 0.58) and 99.63% ± 1.68 (%R.S.D. of 0.64) for tizanidine and rofecoxib, respectively. It may therefore be inferred that degradation of tizanidine and rofecoxib had not occurred in the marketed formulations that were analyzed by this method as shown in Table 8. The low %R.S.D. value indicated the suitability of this method for routine analy-

Table 5
Standard addition technique for determination of tizanidine (a) and rofecoxib (b) by TLC densitometry and HPLC ($n = 6$)

TLC densitometry					HPLC				
Excess drug added to the analyte (%)	Theoretical content (ng)	Recovery (%)	%R.S.D.	S.E.	Excess drug added to the analyte (%)	Theoretical content (μg)	Recovery (%)	%R.S.D.	S.E.
(a) Tizanidine									
0	30	99.65	2.50	1.01	0	60	100.26	1.56	1.11
80	54	98.56	1.84	0.98	80	108	99.41	2.42	1.23
100	60	99.25	1.71	1.23	100	120	99.88	1.65	0.98
120	66	100.04	1.95	1.56	120	132	100.57	1.44	1.95
(b) Rofecoxib									
0	375	99.62	2.63	1.94	0	750	99.52	1.68	1.14
80	675	100.25	2.14	1.38	80	1375	100.47	1.65	1.32
100	750	100.85	1.56	1.05	100	1500	101.98	2.01	1.75
120	825	101.21	1.35	0.96	120	1650	101.77	1.46	1.69

Table 6
Summary of validation parameters: Statistical data for the calibration graphs of tizanidine and rofecoxib by TLC densitometric and HPLC method ($n = 6$)

Parameter	HPTLC densitometric		HPLC	
	Tizanidine	Rofecoxib	Tizanidine	Rofecoxib
Linearity range	10–100 ng/spot	100–1500 ng/spot	10–200 μg/ml	100–2000 μg/ml
Correlation coefficient	0.9996 ± 1.15	0.9995 ± 1.25	0.9997 ± 1.02	0.9992 ± 1.52
Limit of detection	10 ng/spot	25 ng/spot	0.01 μg/ml	0.05 μg/ml
Limit of quantitation	20 ng/spot	40 ng/spot	0.10 μg/ml	0.15 μg/ml
Recovery ($n = 6$)	99.38 ± 0.63	100.48 ± 0.70	100.03 ± 0.50	100.94 ± 1.16
Precision (%R.S.D.)				
Repeatability of application[a]	1.89	1.26	–	–
Repeatability of measurement[a]	0.48	0.67	–	–
Inter-day ($n = 6$)	1.85	1.34	1.68	1.98
Intra-day ($n = 6$)	1.65	1.21	1.23	1.45
Robustness	Robust	Robust	Robust	Robust
Specificity	0.9998	0.9997	0.05	0.07

[a] $n = 7$.

Table 7
Stability of tizanidine and rofecoxib in sample solutions ($n = 6$)

Parameter	HPTLC densitometry[a]		HPLC[b]	
	Tizanidine	Rofecoxib	Tizanidine	Rofecoxib
Area mean	1854.90	4943.60	426,241.68	288,7984.36
Area range	1821.64–1875.36	4911.07–4978.51	426,008.31–426,911.82	288,7421.78–288,7998.58
%R.S.D.	1.25	1.58	0.81	0.12
S.E.	1.14	1.26	0.04	0.09

[a] Average of three concentrations 30, 50, 80 ng/spot and 375, 625, 1000 ng/spot for tizanidine and rofecoxib, respectively.
[b] Average of three concentrations 50, 100, 150 μg/ml and 625, 1250, 1875 μg/ml for tizanidine and rofecoxib, respectively.

Table 8
Applicability of the proposed methods for the determination of tizanidine and rofecoxib in commercial tablets ($n = 6$)

Parameters	HPTLC densitometry		HPLC	
	Tizanidine	Rofecoxib	Tizanidine	Rofecoxib
Label claim (mg)	2	25	2	25
Drug content (%) ± S.D.	99.40 ± 1.56	99.63 ± 1.68	99.91 ± 1.62	100.16 ± 1.35
%R.S.D.	0.58	0.64	0.68	0.71
S.E.	0.23	0.26	0.36	0.40
t-value[a]	0.063	0.105	0.315	0.254
F-value[a]	1.093	1.263	1.255	1.278

[a] The theoretical values for *t*- and *F*-values are equal to 2.57 and 5.05, respectively ($P = 0.05$).

Table 9
Two-way ANOVA test of tizanidine (a) and rofecoxib (b) determination in six independent samples in duplicate by HPTLC and HPLC

Sample	HPTLC[a]		HPLC[a]	
	First sampling	Second sampling	First sampling	Second sampling
(a) Two-way ANOVA test of tizanidine determination				
1	98.31	98.48	99.88	98.16
2	98.24	98.94	99.64	99.39
3	99.20	99.91	98.41	98.32
4	98.51	99.26	98.24	98.27
5	100.48	100.31	101.57	101.11
6	98.45	98.66	98.74	98.95

Summary	HPTLC	HPLC	
ANOVA: two-factor with replication			
Count	6	6	12
Sum	593.19	596.48	1189.67
Average	98.865	99.41333333	99.13916667
Variance	0.74347	1.551426667	1.125135606
Count	6	6	12
Sum	595.56	594.2	1189.76
Average	99.26	99.03333333	99.14666667
Variance	0.51916	1.260186667	0.822806061
Count	12		12
Sum	1188.75		1190.68
Average	99.0625		99.22333333
Variance	0.616475		1.317387879

Source of variation	SS	d.f.	MS	F[b]	$P-value$	$Fcrit$
ANOVA						
Sample	0.0003375	1	0.0003375	0.00033135	0.98565727	4.351250027
Columns	0.155204167	1	0.155204167	0.152375942	0.700401589	4.351250027
Interaction	0.9009375	1	0.9009375	0.884520071	0.358188118	4.351250027
Within	20.37121667	20	1.018560833			
Total	21.42769583	23				

Sample	HPTLC[c]		HPLC[c]	
	First sampling	Second sampling	First sampling	Second sampling
(b) Two-way ANOVA test of rofecoxib determination in six independent samples in duplicate by HPTLC and HPLC				
1	99.42	99.74	100.35	99.77
2	99.61	99.94	99.86	99.96
3	100.21	100.45	99.48	100.78
4	100.58	101.30	100.71	100.84
5	101.30	100.81	101.89	101.62
6	99.91	100.18	100.20	100.87

Summary	HPTLC	HPLC	
ANOVA: Two-factor with replication			
Count	6	6	12
Sum	601.03	602.49	1203.52
Average	100.1716667	100.415	100.2933333
Variance	0.478056667	0.69907	0.551206061
Count	6	6	12
Sum	602.42	603.84	1206.26
Average	100.4033333	100.64	100.5216667
Variance	0.335226667	0.45844	0.376033333
Count	12		12
Sum	1203.45		1206.33
Average	100.2875		100.5275
Variance	0.384311364		0.539947727

Table 9 (*Continued*)

Source of variation	SS	d.f.	MS	F[d]	P-value	F crit
ANOVA						
Sample	0.312816667	1	0.312816667	0.634905064	0.434919409	4.351250027
Columns	0.3456	1	0.3456	0.701443412	0.412196085	4.351250027
Interaction	6.66666E-05	1	6.66666E-05	0.000135309	0.990834274	4.351250027
Within	9.853966667	20	0.492698333			
Total	10.51245	23				

[a] The results are presented as [%] of declared amount of tizanidine per tablet.
[b] $F_{stat} < F_{crit}$.
[c] The results are presented as [%] of declared amount of rofecoxib per tablet.
[d] $F_{stat} < F_{crit}$.

Table 10
Average results of tizanidine (a) and rofecoxib (b) determination by HPTLC and HPLC and their correlation by paired *t*-test

Sample	HPTLC[a]	HPLC[a]
(a) Tizanidine		
1	98.40	99.02
2	98.59	99.52
3	99.56	98.37
4	98.89	98.26
5	100.40	101.34
6	98.56	98.85
Average	99.07	99.23

	Variable 1	Variable 2
t-Test: paired two sample for means		
Mean	99.06666667	99.22666667
Variance	0.596146667	1.281026667
Observations	6	6
Pearson correlation	0.630454741	
Hypothesized mean difference	0	
d.f.	5	
t Stat	**−0.44510894**	
P ($T \leq t$) one-tail	0.337424859	
t Critical one-tail	2.015049176	
P ($T \leq t$) two-tail	0.674849718	
t Critical two-tail	**2.570577635**	
t Stat < *t* critical		

Sample	HPTLC[b]	HPLC[b]
(b) Rofecoxib		
1	99.58	100.06
2	99.78	99.91
3	100.33	100.13
4	100.70	100.78
5	101.30	101.76
6	100.05	100.54
Average	100.29	100.53

	Variable 1	Variable 2
t-Test: paired two sample for means		
Mean	100.2875	100.5275
Variance	0.4030075	0.4651875
Observations	6	6
Pearson Correlation	0.910988973	
Hypothesized Mean Difference	0	
d.f.	5	
t Stat	−2.087482734	
P ($T \leq t$) one-tail	0.045595459	
t Critical one-tail	2.015049176	
P ($T \leq t$) two-tail	0.091190918	
t Critical two-tail	2.570577635	
t Stat < *t* critical		

[a] The results are presented as [%] of declared amount of tizanidine per tablet.
[b] The results are presented as [%] of declared amount of rofecoxib per tablet.

sis of tizanidine and rofecoxib in pharmaceutical dosage form.

3.9.2. For HPLC method

The peaks at t_r 3.19 (for tizanidine) and 7.11 min (for rofecoxib) were observed in the chromatogram of the drug samples extracted from tablets (Fig. 7). Experimental results of the amount of tizanidine and rofecoxib in tablets, expressed as percentage of label claim were in good agreement with the label claims, thereby suggesting that there is no interference from any excipients, which are normally present in tablets. The drug content was found to be 99.91% ± 1.62 (%R.S.D. of 0.68) and 100.16% ± 1.35 (%R.S.D. of 0.71) for tizanidine and rofecoxib, respectively. Statistical evaluation was performed using Student's *t*-test and the *F*-ratio at 95% confidence level as shown in Table 8.

3.10. HPTLC versus HPLC

Six different samples taken during in process control of tablet manufacturing were determined simultaneously by HPTLC and HPLC methods. Each sample was analyzed in duplicate. To test differences between the proposed HPTLC and HPLC method statistical tests were performed for the level of confidence 95% ($P = 0.05$). Two way ANOVA was applied to test both method–sample interactions (interaction variation) and differences in the method precision (column variation). Since the within cell variation (residual variation) is greater than interaction variation as well as column variations, the method–sample interaction and the differences between the methods are not significant. To test means (averages) a paired *t*-test was applied. The test removes any variations between samples [31]. The obtained value of t_{stat} is

lower than two tail t_{crit}, which leads to the conclusion that there is no significant difference between the means. The results of two way ANOVA and paired *t*-test are given in Tables 9a, b and 10a, b, respectively.

4. Conclusion

The proposed HPTLC and HPLC methods provide simple, accurate and reproducible quantitative analysis for simultaneous determination of tizanidine and rofecoxib in tablets. Both the methods were validated as per ICH guidelines. Six real samples of tablets were determined simultaneously by HPTLC and HPLC methods and the results were correlated. Statistical tests indicate that the proposed HPTLC and HPLC methods reduce the duration of analysis and appear to be equally suitable for routine determination of tizanidine and rofecoxib simultaneously in pharmaceutical formulation.

Acknowledgement

The authors thank Sun Pharma India Ltd. for gift sample of tizanidine and rofecoxib. Authors are also thankful to Dr. Dilip Charegaonkar (MD, Anchrom, HPTLC Specialists, Mumbai, India) and Mr. Shailesh Bawaskar (GM-Service, Anatek Services Pvt. Ltd. Jasco Co., Mumbai, India) for providing the facilities for completion of the project.

References

[1] P. Koch, D.R. Hirst, B.R. von Wartburg, Xenobiotica 19 (1989) 1255–1260.
[2] F.L.S. Tse, J.M. Jaffe, S. Bhuta, Fundam. Clin. Pharmacol. 1 (1987) 479–485.
[3] M.K. Shellenberger, L. Groves, J. Shah, G.D. Novak, Drug Metab. Dispos. 27 (1999) 201–205.
[4] V. Healzlewood, P. Symoniw, P. Maruff, M.J. Eadie, Eur. J. Clin. Pharmacol. 25 (1983) 65–72.
[5] J. Lee, J.H. Seo, D.H. Kim, Analyst 127 (2002) 917–920.
[6] L.J. Jackman, T. Jen, J. Am. Chem. Soc. 97 (1975) 2818.
[7] B. Raman, D. Patil, Indian Drugs 39 (2002) 392–394.
[8] M.L. Qi, P. Wang, L. Wang, Anal. Chim. Acta 478 (2003) 171–177.
[9] K.R. Mahadik, A.R. Paradkar, H. Agrawal, N. Kaul, J. Pharm. Biomed. Anal. 33 (2003) 545–552.
[10] J.R. Vane, R.M. Botting, Inflamm. Res. 44 (1995) 1–10.
[11] H.R. Herschman, Biochim. Biophys. Acta 1299 (1996) 140–155.
[12] D.E. Griswold, J.L. Adams, Med. Res. Rev. 16 (1996) 181–206.
[13] M.C. Allison, A.G. Howatson, C.J. Torrance, F.D. Lee, R.I. Russell, N. Engl. J. Med. 327 (1992) 749–754.
[14] Y. Harada, K. Hatanaka, M. Kawamura, M. Saito, M. Ogino, M. Majima, T. Ohno, K. Ogino, K. Yamamoto, Y. Taketani, Prostaglandins 51 (1994) 19–333.
[15] P. Prasit, Z. Wang, C. Brideau, C.C. Chan, S. Charleson, Bioorg. Med. Chem. Lett. 9 (1999) 1773–1778.
[16] E. Woolf, I. Fu, B. Matuszewski, J. Chromatogr. B Biomed. Sci. Appl. 730 (1999) 221–227.
[17] C.Z. Matthews, E.J. Woolf, B.K. Matuszewski, J. Chromatogr. A 949 (2002) 83–89.
[18] C.M. Chavez-Eng, M.L. Constanzer, B.K. Matuszewski, J. Chromatogr. B Biomed. Sci Appl. 748 (2000) 31–39.
[19] U. Werner, D. Werner, R. Mundkowski, M. Gillich, K. Brune, J. Chromatogr. B Biomed. Sci Appl. 760 (2001) 83–90.
[20] C.M. Chavez-Eng, M.L. Constanzer, B.K. Matuszewski, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 767 (2002) 117–129.
[21] T. Radhakrishna, D. Sreenivas Rao, G. Om Reddy, J. Pharm. Biomed. Anal. 26 (2001) 617–628.
[22] S. Sattari, F. Jamali, J. Pharm. Pharmaceut. Sci. 3 (2000) 312–316.
[23] P.T. Vallano, R.S. Mazeno, E.J. Woolf, B.K. Matuszewski, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 779 (2002) 249–257.
[24] M.K. Aravind, R. Prescilla, J.P. Ofenstein, J. Chromatogr. Sci. 40 (2002) 26–28.
[25] B. Mao, A. Abrahim, Z. Ge, D.K. Ellison, R. Hartman, S.V. Prabhu, R.A. Reamer, J. Wyvratt, J. Pharm. Biomed. Anal. 28 (2002) 1101–1113.
[26] K.V.K. Reddy, J.M. Babu, P.K. Dubey, B.C. Sekhar, G. Om Reddy, K.J. Vyas, J. Pharm. Biomed. Anal. 29 (2002) 355–360.
[27] Indian Pharmacopoeia, Government of India, Ministry of Health and Family Welfare, vol-II, appendix 13, A-147, 1996.
[28] ICH, Q2A, Harmonised tripartite guideline, text on validation of analytical procedures, IFPMA, in: Proceedings of the International Conference on Harmonization, Geneva, March 1994, pp. 1–5.
[29] ICH, Q2B, Harmonised tripartite guideline, validation of analytical procedure: methodology, IFPMA, in: Proceedings of the International Conference on Harmonization, Geneva, March 1996, pp. 1–8.
[30] P.D. Sethi, High Performance Thin Layer Chromatography, Quantitative Analysis of Pharmaceutical Formulations, CBS Publishers and Distributors, New Delhi, 1996.
[31] J.C. Miller, J.N. Miller, Statistics for Analytical Chemistry, second ed., Ellis Horwood, New York, 1992.