Journal of Reports in Pharmaceutical Sciences **2014, 3(2), 184-192**

Reversed Phase High Performance Liquid Chromatographic Method for Simultaneous Estimation of Pregabalin and Aceclofenac in Tablet Formulation (Acenac-N)

Shantaram Gajanan Khanage[a*], **Dnyaneshwar Santram Kale**[b], **Popat Baban Mohite**[a], **Vinayak Kashinath Deshmukh**[b]

[a]Department of Pharmaceutical chemistry and PG studies, M.E.S. College of Pharmacy, Sonai, Tq-Newasa, Dist.-Ahmednagar, Maharashtra, India-414105.
[b]Department of Quality assurance technique and PG studies, M.E.S. College of Pharmacy, Sonai, Tq-Newasa, Dist.-Ahmednagar, Maharashtra, India-414105.

ARTICLE INFO

Article Type:
Research Article

Article History:
Received: 2014-09-23
Revised: 2014-10-25
Accepted: 2014-11-20
ePublished:2014-11-25

***Keywords*:**
Pregabalin
Aceclofenac
RP-HPLC
Acenac-N
Formulation

A B S T R A C T

Pregabalin (PRG) is a new antiepileptic drug and Aceclofenac (ACE) is a potent non-steroidal anti-inflammatory drug. These drugs in combination are used for treatment of partial seizures and neuropathic pains. A simple and precise assay method by RP-HPLC was developed and validated for estimation of PRG and ACE in Acenac-N tablet. Analyses of commercial tablet, Acenac-N were performed using JASCO Isocratic HPLC system at 248 nm on a reverse phase column HiQ sil C18HS (4.6 × 250 mm, 5µm), a mobile phase; methanol: phosphate buffer (70:30 v/v, PH 3). The validation aspects were selectivity, linearity, precision, accuracy and quantification limit. Linearity, 5-25 µg/mL for PRG and ACE respectively, provided determination coefficients (R^2) of 0.998 and 0.994 respectively, for PRG and ACE and proved precise since the RSD% was less than 2%. The recoveries obtained ranged from 99.10% to 100.90% for both of the drugs with RSD% less than 2%. The LOD for PRG and ACE was found to be 0.270 µg/mL and 0.039 µg/mL respectively. The LOQ was found at 0.818 µg/mL and 0.120 µg/mL respectively for PRG and ACE. In this study, the optimization of mobile phase, flow rate, injection volume and wavelength were achieved. The retention time for PRG and ACE was 3.241 and 6.581 min respectively. Drug content of the Acenac-N tablet was found to be 99.53% and 100.12% respectively for PRG and ACE. The method was validated as per the ICH guidelines. This method is precise, accurate and easy to analysis PRG and ACE in tablets.

[*]**Corresponding Author:** Shantaram Gajanan Khanage, **E-mail:** shantaram1982@gmail.com

Introduction

Pregabalin (PRG) is a novel antiepileptic drug. PRG is chemically (S) -3-(aminomethyl) -5-methylhexanoic acid [1] and it is approved in the US and Europe for adjunctive therapy of partial seizures in adults, and also has been approved for the treatment of pain from diabetic neuropathy or post-herpetic neuralgia in adults. Recently, it has been approved for treatment of anxiety disorders in Europe. Pregabalin is structurally related to the antiepileptic drug Gabapentin and the site of action of both drugs is similar, the alpha2-delta (alpha2-delta) protein, an auxiliary subunit of voltage-gated calcium channels [2,3]. The structure of the PRG is presented in Fig. 1. According to the literature survey carried out, PRG is not official in any pharmacopoeia.

Aceclofenac (ACE) is chemically 2 [(2,6-dichlorophenyl) amino] phenyl acetoxy acetic acid (Fig. 2). ACE is a non-steroidal anti-inflammatory drug which has analgesic and anti-inflammatory activity. ACE is an analog of Diclofenac and it is a potent cyclooxygenase-2 inhibitor [4,5]. ACE is official in IP, BP and USP.

The pain management is always a trouble for a physician and searching for a safe and effective alternative is still on. The combination of the PRG (75 mg) and ACE (100 mg) is used for the relief of pain and inflammation in rheumatoid arthritis, osteoarthritis and ankylosing spondylitis. This combination is also used as an adjunctive in the treatment of partial seizures, epilepsy, fibromyalgia and neuropathic pain.

The literature survey reveals that UV spectrophotometric, HPLC and LC-MS-MS methods were reported for estimation of PRG individually in different pharmaceutical dosage forms and in human plasma [6-10]. Simultaneous estimation of PRG and Methylcobalamine by HPLC method has also reported [11]. In case of Aceclofenac alone or in combination with the other drugs, it is reported to be estimated by densitometry, UV spectrophotometry, HPLC, Voltametric, Spectrofluorimetric, HPTLC and LC-MS [12-21].

According to literature survey, there was not any developed analytical method which has been reported for simultaneous estimation of PRG and ACE in combined dosage form. So an attempt was being made to a developed simple, accurate, precise, economical and reproducible chromatographic method for simultaneous estimation of PRG and ACE in tablet dosage form. The developed method was validated in accordance with ICH guideline [22,23] and successfully employed in the assay of PRG and ACE in combined tablet dosage form.

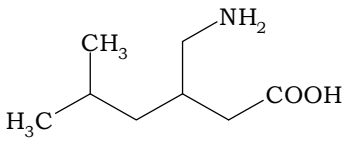

Fig. 1. Chemical structure of PRG.

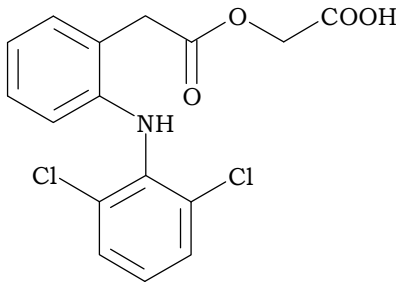

Fig. 2. Chemical structure of ACE.

Materials and Methods

Standard and chemical reagents

The standard drug Pregabalin was obtained from Sun Pharmaceuticals Ltd., Ahmednagar, India. Aceclofenac was obtained from Wockhardt Ltd., Aurangabad, India. Deionised distilled water (DIW) used was obtained from Loba Chemie Mumbai, India. HPLC grade methanol Merck Ltd., India, HPLC-grade acetonitrile, Merck Ltd., India. Buffering agent's ortho phosphate, tri ethylamine was procured from Fisher scientific, Mumbai. India. Ortho phosphoric acid was obtained from SD fine, Mumbai. India.

Chromatographic conditions

Liquid chromatography was performed on JASCO Isocratic HPLC system model LC-NET II/ADC (JASCO Corporation, Japan). The system built with UV-2070 as UV-VIS detector and HiQ sil C18HS (4.6 × 250 mm, 5µm) column with a 20 µL manual sample injector. The HPLC system

was equipped with Chrom-NAV software for data processing.

All compounds were eluted off the column with a mobile phase consisting of methanol: phosphate buffer (70:30 v/v, PH 3) at a flow rate of 1.0 mL/min in isocratic mode. The mobile phase was filtered through a 0.45 µm nylon filter and then ultrasonicated for 30 min. The injection volume was 20 µL and the eluent was detected at 248 nm, which was selected as wavelength for further analysis. The retention time of PRG and ACE was around 3.241 and 6.581 min, respectively and the total run was 10 min (Table 2).

The method was validated in accordance with the International Conference on Harmonization guidelines for validation of analytical procedures [22,23].

Specificity and selectivity

These parameters were determined by comparing the chromatograms of the PRG and ACE standard, tablet drug Acenac-N and mobile phase as a solvent.

Linearity

The linearity of an analytical procedure is its ability within a given range to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample [22,23]. The linearity was tested for PRG and ACE in the concentration range value of 5-25 µg/mL.

Accuracy

To check the degree of accuracy of the method, recovery studies were performed in triplicate by the standard addition method at 50%, 100% and 150%. Known amounts of standard PRG and ACE were added to the pre-analyzed samples and were subjected to the proposed HPLC method.

Precision

The precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day). The repeatability was calculated by the relative standard deviation with three replications and three different concentrations during the same day. Intermediate precision was studied by comparing the assays on two different days.

Limit of Detection

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. Limit of detection can be calculated using the following equation as per ICH guidelines [22,23].

$LOD = 3.3 \times N/S$

Where, N is the standard deviation of the peak area of the drug and S is the slope of the corresponding calibration curve.

Limit of Quantification

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which 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. Limit of quantification can be calculated using the following equation as per ICH guidelines [22,23].

$LOQ = 10 \times N/S$

Where, N is the standard deviation of the peak area of the drug and S is the slope of the corresponding calibration curve.

Sample preparation

A sample solution was prepared by taking accurately weighed twenty tablets (Acenac-N) and finely powdered. A precisely weighed portion of the powder equivalent to 7.5 mg of PRG and 10 mg of ACE were extracted with the mobile phase. The extract was transferred to a 100 mL volumetric flask and volume made up to the mark with the mobile phase. The solution was filtered through 0.45 µm nylon filter to remove particulate matter, if any. Then sample solution was ultrasonicated for 15 min. The tablet extract was appropriately diluted with mobile phase to obtain a concentrations 5-25 µg/mL. The amount of drug present in the sample solution was calculated by using the calibration curve. The chromatogram was hold up to 10 min. The chromatogram obtained is shown in Figure 3(D) and the area obtained in each chromatogram of five replicates was

correlated with regression equation and the amount of PRG and ACE was calculated, which was within the limit of label claim as mentioned in Table 1.

Table 1. Analysis of Acenac-N tablet formulation

Formulation	Label claim (mg)		Amount fount (mg) ±SD, n=5		% Amount found ±SD, n=5	
	PRG	ACE	PRG	ACE	PRG	ACE
Acenac-N	75	100	74.65±0.142	100.12±0.241	99.53±0.453	100.12±0.169

Table 2. Optimal chromatographic conditions of tablet formulation

Aspect	Description
Mobile phase	Methanol: phosphate buffer (70:30 v/v, PH 3)
HPLC Column	HiQ sil C18HS (4.6 × 250 mm, 5µm)
Flow rate	1.0 mL/min
Injection volume	20 µL
Retention time	for PRG 3.241 min and for ACE 6.581 min
Runtime	10 min

Method optimization

Four parameters were optimized to get better separation. These parameters were mobile phase, flow rate, wavelength and injection volume.

Results and Discussion

Analytical method development

The optimization of mobile phase, flow rate, wavelength and injection volume is considered very important to achieve good separation and peak area. In proposed method, the estimation these four parameters were optimized individually for PRG and ACE then optimized for in combination. In this study, we observed no significant difference in the results obtained with the mobile phase Methanol: phosphate buffer (70:30 v/v, PH 3). The mobile phase made up of 100% methanol produced too late peak with an area lower than last mobile phase, may be this is attributed buffer effect.

In case of these three mobile phases (methanol/acetonitrile, 50:50; methanol/acetonitrile, 60:40; methanol/acetonitrile/phosphate buffer, 70:30:10) less resolution and late elution peak was obtained. Different trials (methanol: phosphate buffer; 70:30 v/v) were conducted at varying of PH range (2-6) of phosphate buffer with satisfactory results, but non-symmetrical peak and smaller number of theoretical plates were observed. The mobile phase chosen for analytical method validation was methanol: phosphate buffer (70:30 v/v) at PH 3, presented a mobile phase holdup time of 3.241 min for PRG and 6.581 min for ACE and giving good separation, well defined peak with more number of theoretical plates.

The flow rate was optimized with (0.8, 1.0, 1.5 and 2 mL/min). At 0.8 mL/min, there is no peak appeared in the chromatogram with 3 replications. This is attributed to the insufficient flow rate to elute PRG and ACE through the column. However, a significant difference was observed among all the rest flow rates. Based on the results obtained, 1 mL/min showed the best results in terms of peak area and retention time. An optimization on the flow rates PRG and ACE analysis shown in Table 3.

Table 3. The optimization of flow rate on PRG and ACE analysis

Effect of flow rate				
Flow rate (mL/min)	**RT (min) ± SD, n = 3**		**Peak area (µV/S) ± SD***	
	PRG	**ACE**	**PRG**	**ACE**
0.8	No Peak	No Peak	No Peak	No Peak
1.0	3.241±0.035	6.581±0.014	125489±478.282	242447±658.234
1.5	2.316±0.012	4.873±0.045	227896±712.781	458963±478.453
2.0	1.750±0.024	3.434±0.015	464781±964.413	784568±367.631

*average of three determinations

In case of wavelength, there is no significant difference among the three wavelengths as shown in Table 4. While, injection volume caused significant difference in peak area between 5 and 20 µL, which is related to the amount of analyte passed through the column. Table 5 presents the results of optimization of injection volume on PRG and ACE analysis.

By appropriate dilution of each standard stock solution with mobile phase, various concentrations of PRG and ACE were prepared separately. Each solution was scanned at the UV range of 200 nm to 400 nm and their overlain spectra were taken. The wavelength selected for the analysis was 248 nm, at which both the drugs showed significant absorbance.

Table 4. The optimization of wavelength on PRG and ACE analysis

Effect of wavelength				
Wavelength (nm)	**RT (min) ± SD**		**Peak area (µV/S) ± SD***	
	PRG	**ACE**	**PRG**	**ACE**
243	3.242±0.018	6.584±0.038	121647 ±345.264	241489±557.221
248	3.241±0.041	6.581±0.046	121322 ±748.581	241779±996.053
253	3.248±0.022	6.588±0.027	121489 ±627.380	241257±211.673

*average of three determinations

Table 5. The optimization of injection volume on PRG and ACE analysis

Effect of injection volume				
Injection volume (µL)	**RT (min) ± SD**		**Peak area (µV/S) ± SD***	
	PRG	**ACE**	**PRG**	**ACE**
5	3.244±0.034	6.588±0.039	46892±447.744	119021±549.895
10	3.245±0.049	6.589±0.056	115687±789.124	214587±787.430
20	3.240±0.055	6.583±0.067	287841±658.393	451489±886.252

*average of three determinations

Analytical method validation

Linearity

The linearity of the method was determined by constructing calibration curves. Tablet solution of the PRG and ACE of different concentrations at the range of (5-25 µg/mL) were used for this purpose. Each measurement was carried out in five replicates and the peak areas of the chromatograms were plotted against the concentrations to obtain the calibration curves and correlation coefficients which are presented in Table 6.

Table 6. Linearity data for PRG and ACE

Concentration (µg/mL)	Peak Area (µV/S)*	
	PRG	ACE
5	58489	106155
10	121916	241483
15	184296	351255
20	246490	469002
25	296523	556115
Slope	12086	22907
Intercept	483	916
Correlation Coefficient	0.998	0.994
RSD%	1.045	0.963

*average of five determinations

Accuracy

To check the degree of accuracy of the method, recovery studies were performed in triplicate by the standard addition method at 50%, 100% and 150%. Known amounts of standard PRG and ACE were added to the pre-analyzed samples and were subjected to the proposed HPLC method. Tablet solution of Acenac-N presented good recoveries and agreement with the standards of method validation [22,23] as shown in Table 7.

Table 7. Results of recovery study by standard addition procedure

Drug	Amount taken (µg/mL)	Amount added (µg/mL)	Amount found (µg/mL)	Percent recovery ± SD*	RSD%
PRG	5.0	2.5	7.568	100.92±0.57	0.571
	5.0	5.0	9.987	99.87±0.15	0.164
	5.0	10.0	14.895	99.30±0.87	0.898
ACE	5.0	2.5	7.433	99.10±0.69	0.614
	5.0	5.0	9.884	98.84±0.42	0.312
	5.0	10.0	15.114	100.76±0.27	0.275

*average of three determinations

Precision

The precision of the method was evaluated based on the results of the analysis of three samples with three replications for each one at day 1 and the results from intermediate precision from other three samples at day 2. The values were compared with the standards [22,23], thus all values demonstrated good results as shown in Table 8.

Table 8. Precision of method development on PRG and ACE analysis

Drug	Repeatability				Intermediate Precision		
	conc. µg/mL	Rt ±SD	Peak area ±SD*	RSD %	Rt ±SD	Peak area ±SD*	RSD %
PRG	5	3.244±0.071	58539 ±568.312	0.886	3.240±0.041	58685 ±352.412	0.488
	10	3.249±0.056	121751±647.413	0.623	3.242±0.016	121534±417.263	0.895
	15	3.245±0.033	184241±437.061	0.348	3.246±0.074	184333±147.251	0.744
ACE	5	6.588±0.041	106146±141.524	0.462	6.577±0.022	106632±434.470	0.965
	10	6.584±0.025	241517±724.743	0.779	6.586±0.047	241334±278.945	0.716
	15	6.587±0.068	351270±666.452	0.397	6.582±0.038	351114±896.554	1.042

*average of three determinations

Limits of quantification (LOQ) and detection (LOD)

The LOD and LOQ were calculated using signal-to noise ratio method according to the guidance of ICH guidelines of method validation [22,23]. LOD was taken as the concentration of the analyte where the signal to-noise ratio was 3, and for PRG and ACE it was found to be 0.270 µg/mL and 0.039 µg/mL respectively. LOQ defined as the analyte concentration at a signal-to-noise ratio of 10 and it was 0.818 µg/mL and 0.120 µg/mL respectively for PRG and ACE.

Selectivity

Comparison of the chromatograms obtained from the mobile phase (blank), PRG, ACE standard and the tablet revealed no significant interference, using same chromatographic conditions for all samples. Fig. 3 refers to the selective method for the analyte concerned.

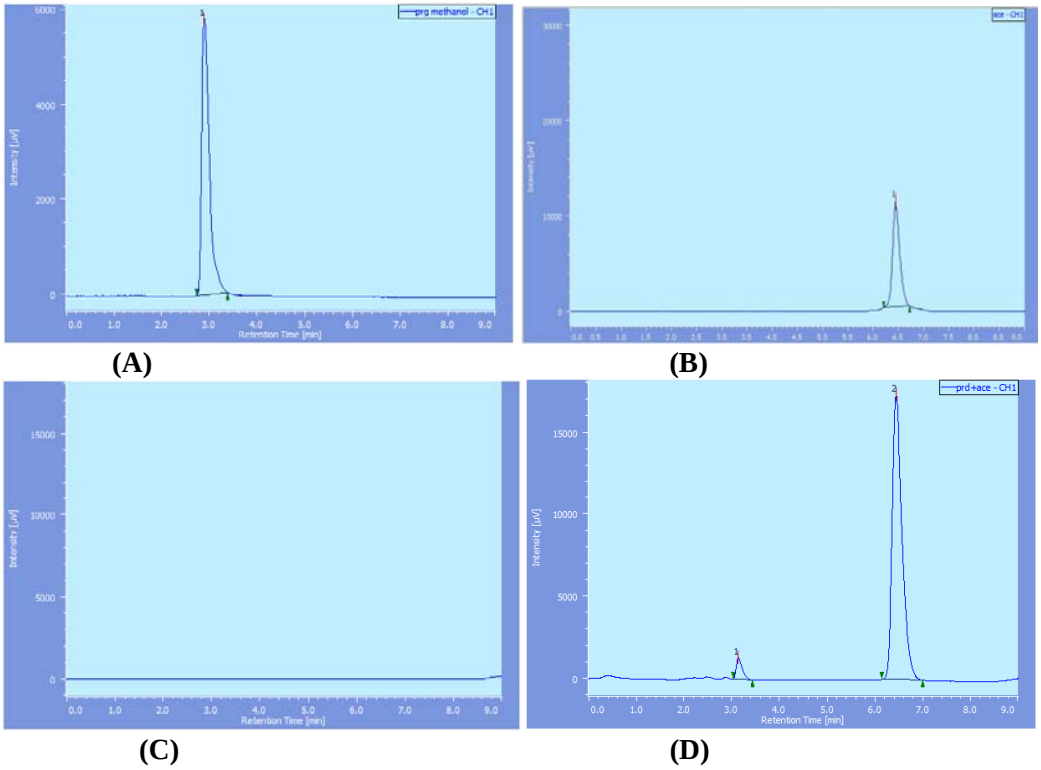

Fig. 3. Selective method of PRG and ACE analysis
(A: Standard solution of PRG 5 µg/mL B: Standard solution of ACE 5 µg/mL
C: Blank D: Tablet solution PRG 7.5 µg/mL and ACE 10 µg/mL)

Conclusion

The results show that the HPLC method presented here can be considered suitable for the analytical determination of PRG and ACE in tablet dosage form. The proposed method is being linear in the concentration range used, high selectivity and specificity, high precision and adequate accuracy at the concentrations studied. The proposed method uses a simple mobile phase compared to the multi-component mobile phase in many reported methods. The separation and determination were achieved at an ambient temperature. Thus, it offers the advantages of low column back pressure, good peak shape, improved column efficiency, higher theoretical plates and consistent retention time. The developed method suggested no interference of formulation excipients in the estimation. Hence it can be easily and conveniently adopted for routine analysis.

Acknowledgements

Authors are heartily thankful to Sun Pharmaceuticals Ltd., Ahmednagar, India and

Wockhardt Ltd., Aurangabad, India for providing pure standard drug samples. The authors also express gratitude to the management and the Principal of M.E.S.'s. College of pharmacy Sonai, India for providing amenities to carry out this work.

Conflict of interest

The authors certify that no actual or potential conflict of interest in relation to this article exists.

References

[1] Tassone DM, Boyce E, Guyer J, Nuzum D. Pregabalin: A novel gamma aminobutyric acid analogue in the treatment of neuropathic pain, partial-onset seizures, and anxiety disorders. Clin Ter. 2007; 29(1):26-48.

[2] Taylor CP, Angelotti T, Fauman E. Pharmacology and mechanism of action of Pregabalin: The calcium channel alpha2-delta (alpha2-delta) subunit as a target for antiepileptic drug discovery. Epilepsy Res. 2007; 73(2):137-50.

[3] Hamandi K, Sander JW. Pregabalin: A new antiepileptic drug for refractory epilepsy. Seizure. 2006; 15(2):73-78.

[4] Budawari S. The Merck Index- An encyclopedia of chemical, drugs and biologicals. 13th ed. Merck and Co. Inc.: NJ; 2003.

[5] Sweetman SC. Martindale-The complete Drug Reference. 33rd ed. Pharmaceutical Press: UK; 2002.

[6] Alka B, Prateek G. A novel method for spectrophotometric determination of Pregabalin in pure form and in capsules. Chem Cen J. 2011; 5(59):1-7.

[7] Jadhav AS, Pathare DB, Shingare MS. Validated enentioselective LC method with precolumn derivatisation with Marfey's reagent for analysis of Antiepileptic drug Pregabalin in bulk drug sample. Chromatographia. 2007; 65(3-4):253-256.

[8] Vermeij TA, Edelbroek PM. Simultaneous high-performance liquid chromatographic analysis of Pregabalin, Gabapentin and Vigabatrin in human serum by precolumn derivatisation with o-phtaldialdehyde and fluorescence detection. J Chromatogr B Analyt Technol Biomed Life Sci. 2004; 810(2):297-303.

[9] Mandal U, Sarkar AK, Gowda DV, Agarwal S, Bose A, Bhaumik U, et al. Determination of Pregabalin in Human Plasma by Using LC-MS-MS. Chromatographia. 2008; 67(3-4):237-243.

[10] Zhang Y, Holliman C, Tang D, Fast D, Michael S. Development and validation of a direct enantiomeric separation of Pregabalin to support isolated perfused rat kidney studies. J Chromatogr B Analyt Technol Biomed Life Sci. 2008; 875(1):148-153.

[11] Kannapan N, Nayak SP, Venkatachalam T, Prabhakaran V. Analytical RP-HPLC method for development and validation of Pregabalin and Methylcobalamine in combined capsule formulation. J App Chem Res. 2010; 13:85-89.

[12] El-Saharty YS, Refaat M, El-Khateeb SZ. Stability indicating spectrophotometric and densitometric methods for determination of Aceclofenac. Drug Dev Indian Pharm. 2002; 28(5):571-582.

[13] Hasan NY, Abdel-Elkawy M, Elzeany BE, Wagieh NE. Stability indicating methods for the determination of Aceclofenac. Farmaco II. 2003; 58(2): 91-99.

[14] Zawilla NH, Mohammad M, Abdul Azim, El Kousy NM, EI-Moghazy Aly SM. Determination of Aceclofenac in bulk and pharmaceutical formulation. J Pharm Biomed Anal. 2002; 27(1):243-251.

[15] Raja RK, Sankar GG, Rao AL, Seshagiri RJ. Development and Validation of RP HPLC method for the estimation of Aceclofenac in Tablet Dosage form. Indian Drugs. 2005; 42(10):693-695.

[16] Srinivasan KK, Shirwaikar A, Joseph A, Jacob S, Prabu SL. Simultaneous estimation of Aceclofenac and Paracetamol in solid dosage form by Ultraviolet spectrophotometry. Indian Drugs. 2006; 43(2): 141-145.

[17] Posac JR, Vazquez MD, Tascon ML, Acuna JA, Fuente C, Velasco E, et al. Determination of Aceclofenac using adsorptive stripping voltametric techniques on conventional and surfactant chemically modified carbon paste electrodes. Talanta. 1995; 42(2):293-304.

[18] Sawant RL, Hadawale SD, Tajane PS. Spectrophotometric methods for simultaneous estimation of Rabeprazole sodium and Aceclofenac from the combined capsule dosage form. Pharm Methods. 2011; 2(3):193-197.

[19] El Kousy NM. Spectrophotometric and Spectrofluorimetric determination of Etodolac and Aceclofenac. J Pharm Biomed Anal. 1999; 20(1-2):185-194.

[20] Patil ST, Bhusari VK, Dhaneshwar SR. Validated HPTLC method for simultaneous estimation of Thiocolchicoside and Aceclofenac in bulk drug and formulation. Int J Pharma Bio Sci. 2011; 2(2):482-490.

[21] Eunyoung K, Byoungki A, Keumhan N, Wonku K, Hye sun G. Quantitative determination of Aceclofenac and its three major metabolites in rat plasma by HPLC-MS/MS. J Separation Sci. 2012; 35(17):2219-2222.

[22] International Conference on Harmonization; Guideline on Validation of Analytical Procedures: Definitions and Terminology; Availability, Federal Register/Notice/Vol. 60/ Pub. P. No 11260 (1995).
[23] International Conference on Harmonization; Guideline on Validation of Analytical Procedures: Definitions and Terminology; Availability, Federal Register/Notice/Vol. 62/ Pub. P. No 27463 (1997).

