Original Article

Development and validation of analytical method for simultaneous estimation of lutein, lycopene, and beta-carotene using reversed-phase high-performance liquid chromatography

Harmandeep Singh[1], Gurpreet Singh[1], Rohit Bhatia[2]*

[1]Department of Pharmaceutical Analysis, BIS College of Pharmacy, Moga, Punjab, India, [2]Department of Pharmaceutical Chemistry, ISF College of Pharmacy, Moga, Punjab, India

Correspondence:
Rohit Bhatia, Department of Pharmaceutical Chemistry, ISF College of Pharmacy, Moga, Punjab, India.
E-mail: bhatiarohit5678@gmail.com

How to cite this article: Singh H, Singh G, Bhatia R. Development and validation of analytical method for simultaneous estimation of lutein, lycopene, and beta-carotene using reversed-phase high-performance liquid chromatography. Pharmaspire 2020;12(2):55-60.

Source of Support: Nil,

Conflicts of Interest: None declared.

ABSTRACT

Background: Lutein, lycopene, and beta-carotene (MiNOXiV) drug combination is used for the treatment of eye diseases including age-related macular degeneration, cataracts, retinitis, retinitis pigmentosa, cancer of the prostate, breast, lung, bladder, ovaries, colon, and pancreas. **Objective:** The objective of the study was to develop and validate a high-performance liquid chromatographic method for the simultaneous determination of lutein, lycopene, and beta-carotene in tablet dosage forms. **Materials and Methods:** A Waters C_{18} column (50 mm × 4.6 mm, 5 µm) with mobile phase consisting of acetonitrile:butanee-1-ol:dichloromethane (70:30:5 [v/v]) was used. The flow rate was 1.0 ml/min and effluents were monitored at 472 nm. **Results:** The retention time of lutein, lycopene, and beta-carotene in tablet formulation were found to be 7.2 min, 6.0 min, and 10.1 min, respectively. The method was validated according to the ICH guidelines for specificity, limit of detection, limit of quantitation, precision, accuracy, linearity, ruggedness, and robustness. **Conclusion:** The method showed good reproducibility and recovery with % reflex sympathetic dystrophy <2. Hence, the proposed method was found to be simple, specific, precise, accurate, and linear. Hence, it can be applied for routine analysis of lutein, lycopene, and beta-carotene in pharmaceutical combined dosage forms.

Keywords: Lutein, lycopene, beta-carotene, validation

INTRODUCTION

Nowadays, combined dosage forms are mostly preferred when compared to single dosage forms. Lutein, lycopene, and beta-carotene are available in combined pharmaceutical dosage form. The brand name is MiNOXiV tab containing 3.2 mg lutein, 2 mg of lycopene, and 10 mg of Beta carotene used in the treatment of cancer of the prostate, breast, lung, bladder, ovaries, colon, and pancreas.[1-3] High-performance liquid chromatography (HPLC) is the well-established and advanced analytical technique for the separation of mixtures with great efficiency.[4-10] Literature reveals that various analytical methods have been reported for only single dosage forms and not for the combination.[11-13] The present paper aims to report a simple, accurate, precise, and reverse-phase HPLC (RP-HPLC) method for the estimation of lutein, lycopene, and beta-carotene in combined dosage forms.

Access this article online	
Website: www.isfcppharmaspire.com	**P-ISSN:** 2321-4732 **E-ISSN:** XXXX-XXXX

Drug profile of lutein

It is trans-lutein; 4-[18-(4-hydroxy-2,6,6-trimethyl-1-cyclohexenyl)-3,7,12,16-tetramethyl octadeca-1,3,5,7,9,11,13,15,17-nonaenyl]-3,5,5-trimethyl-cyclohex-2-en-1-ol. It acts as antioxidant and is orange-red powder which is soluble in methanol [Figure 1].

Drug profile of lycopene

It is a (6E,8E,10E,12E,14E,16E,18E,20E,22E,24E,26E)-2,6,10,14,19,23,27,31-octamethyldotriaconta-

2,6,8,10,12,14,16,18,20,22,24,26,30-tridecaene. It acts as an anticancer drug for the treatment of prostate, breast, lung, bladder, ovaries, colon, and pancreas cancer. It is deep red colored powder which is soluble in dimethylformamide and insoluble in water [Figure 2].

Drug profile of beta-carotene

It is a 1,3,3-trimethyl-2-[(1Z,3E,5E,7E,9E,11Z,13E,15Z,17E)-3,7,12,16-tetramethyl-18-(2,6,6-trimethylcyclohexen-1-yl) octadeca-1,3,5,7,9,11,13,15,17-nonaenyl]cyclohexene. It belongs to dietary antioxidants and vitamin precursor category. It is red-orange colored powder and is soluble in dimethylformamide [Figure 3].

MATERIALS AND METHODS

Chemicals and reagents

Lutein, lycopene, and beta-carotene were received as gift sample from ISF Analytical Laboratories Ltd., Moga, Punjab, India. The pharmaceutical preparations of combination of lutein, lycopene, and beta-carotene in the form of MiNOXiV tablet (Optica Pharmaceuticals Pvt. Ltd.) contain 3.2 mg lutein, 2 mg of lycopene, and 10 mg of beta-carotene which were procured from local market. The solvents which were used during the method development and validation were methanol analytical reagent grade, HPLC grade methanol (S.D Fine Chemicals Ltd., Mumbai, India), HPLC grade acetonitrile (ACN) and water for HPLC (Finar Chemicals Ltd., Mumbai, India), butane-1-ol (Sigma-Aldrich), dimethylformamide (Sigma-Aldrich), and dichloromethane (Gujarat Alkalies and Chemicals Limited) which were used to prepare the mobile phase which is filtered through a nylon 0.45 μm membrane filter paper (Gelman Laboratories, Mumbai, India).

RP-HPLC method

HPLC system of WATERS (Milford, USA) composed of 515 HPLC pump as a solvent delivery system equipped with Waters 2707 autosampler valve with a 1–100 μL loop. The chromatographic separation was performed with a WATERS, 2998 separation module HPLC instrument equipped with DAD detector and Empower software, version 2.0. The Waters XBridge stainless steel C_{18} column (250 mm ×4.6 mm, 5 μm) packed with ODS chemically bounded porous silica particles was used as stationary phase for analysis. BL-220H analytical balance (Shimadzu Corporation, Japan), an ultrasonic cleaner (Frontline FS 4, Mumbai, India), and digital pH meter (LI 612 pH analyzer, Elico Ltd., Ahmadabad) were used in the study.

Chromatographic condition

The optimal composition of the mobile phase was determined to be ACN:butanee-1-ol:dichloromethane (70:30:5 (v/v)). The mobile phase was filtered through nylon 0.22 μm membrane filters and was degassed to remove the air before use (30 min). Stock solution was prepared by dissolving lutein, lycopene, and beta-carotene (50 mg each) that were weighed accurately and separately transferred into 50 ml volumetric flasks. All the drugs were dissolved in 25 ml of diluent (methanol or dimethylformamide) to prepare standard stock solutions. After the immediate dissolution, sonicate the solution and make up the volume up to the mark with diluent (methanol or dimethylformamide). These standard stock solutions were observed to contain l mg/ml of lutein, lycopene, and beta-carotene. Appropriate volume from this solution was further diluted to get appropriate concentration levels according to the requirement. From the above stock solutions, dilutions were made in the concentration range of 54, 59, 64, 69, and 74 μg/mL for lutein; 30, 35, 40, 45, and 50 μg/mL for lycopene; and 190, 195, 200, 205, and 210 μg/mL for beta-carotene drugs. A volume of 20 μL of each sample was injected into column.

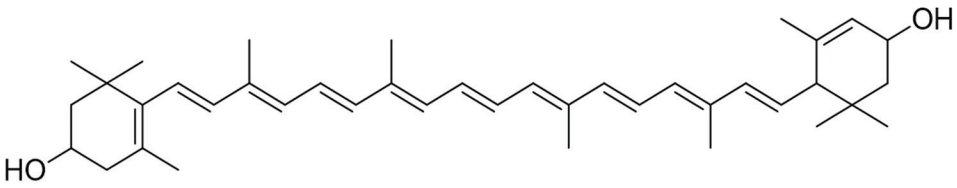

Figure 1: Structure of lutein [29]

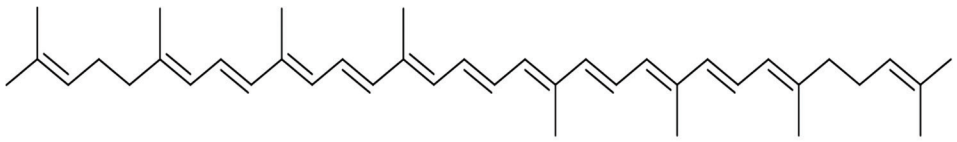

Figure 2: Structure of lycopene

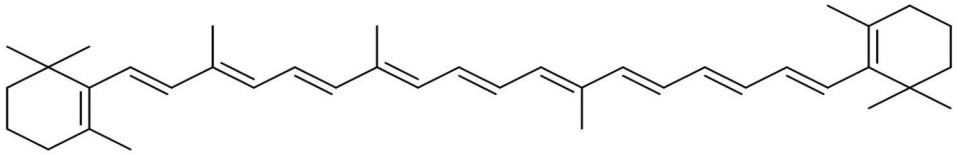

Figure 3: Structure of beta-carotene

Preparation of mobile phase

Mobile phase was prepared by mixing ACN (HPLC grade):butane-1-ol (HPLC Grade):dichloromethane (HPLC grade) (70:30:5 v/v). Mixture was shaken vigorously and sonicated for 30 min before use.

Preparation of stock solutions

Stock solutions and sample solutions of lutein, lycopene, and beta-carotene and its tertiary mixture were prepared by adding accurately weighed 25 mg lutein, lycopene, and beta-carotene separately in 25 mL volumetric flask containing 15 mL of diluent (methanol or dimethylformamide). The flasks were then sonicated for 10 min and the volume was made up to the mark using diluent (methanol or dimethylformamide). These standard stock solutions were containing 1000 μg/mL of lutein, lycopene, and beta-carotene. Stock solutions were used to prepare desired concentration range as per sample calibration range.

Preparation of sample solutions

Twenty tablets were weighed and emptied. The equivalent weight was calculated and according to average weight, required drug was taken in volumetric flask to obtain 3.2 mg of lutein, 2 mg of lycopene, and 10 mg of beta-carotene in single dilution, i.e., according to the label claim of the formulation. The dilutions were first sonicated and then filtered through 0.22 μ filter paper.

Preparation of calibration curve

The test concentrations for lutein, lycopene, and beta-carotene in HPLC method development were found to be 64 μg/mL, 40 μg/mL, and 200 μg/mL, respectively. The calibration curve was prepared by injecting the concentrations of 57.6–76.8 μg/mL of lutein, 32–44 μg/mL of lycopene, and 160–240 μg/mL of beta-carotene in tertiary mixture solution manually in triplicate to the HPLC system at detection wavelength of 472 nm. Mean of $n = 5$ determinations was plotted as the standard curve. The calibration curve was tested by validating it with interday and intraday measurements. Linearity, accuracy, and precision were determined for both interday and intraday measurement.

Method validation

Linearity

The methods were validated according to the International Conference on Harmonization Q2B guidelines (2005) for validation of analytical procedures to determine the linearity, sensitivity, precision, and accuracy for each analyte.[14] Calibration curves were generated with appropriate volumes of working standard solutions for HPLC.

For HPLC assay method validation, the test concentrations were found to be 64 μg/ml of lutein, 40 μg/ml of lycopene, and 200 μg/ml of beta-carotene. Linearity of the proposed method was carried out by the preparation of 80%, 90%, 100%, 110%, and 120% of lutein, lycopene, and beta-carotene test concentration, i.e., 51.2, 57.6, 64, 70.4, and 76.8 μg/mL for lutein and 32, 36, 40, 44, and 60 μg/mL for lycopene and 160, 180, 200, 220, and 240 μg/mL for beta-carotene. The linearity was evaluated by the least square regression method using unweight data, as shown in Table 1.

Precision and accuracy

Both precision and accuracy were determined with standard quality control samples (in addition to calibration standards) prepared in triplicates at different concentration levels covering the entire linearity range. Precision is the degree of repeatability of an analytical method under normal operational conditions. The precision of the assay was determined by repeatability (intraday) and intermediate precision (interday) and reported as % relative standard deviation (R.S.D.) for a statistically significant number of replicate measurements. The intermediate precision was studied by comparing the assays on three different days and the results documented as standard deviation and % R.S.D.[15]

Accuracy is the percent of analyte recovered by assay from a known added amount. For the measurement of accuracy, data from nine

Table 1: Summary of the HPLC method validation

S. No.	Validation parameter	Lutein	Beta-carotene	Lycopene
1.	Absorption maxima, λ_{max} (nm)	472	472	472
2.	Linearity range (μg/mL)	51.2–76.8	160–240	36–44
3.	Coefficient of determination (R^2)	0.996	0.999	0.998
4.	Regression equation (y)	y=13,270x−16,263	y=27,877x−44,916	y=40,975x−46,808
5.	Slope (b)	13,270	27,877	40,975
6.	Intercept (a)	16,263	44,916	46,808
7.	Limit of detection (μg/mL)	3.56	3.10	2.32
8.	Limit of quantification (μg/mL)	10.78	9.41	6.97
9.	Precision (%RSD)	Intraday=0.410 Interday=0.693	Intraday=1.26 Interday=1.28	Intraday=0.524 Interday=0.540
10.	Solution stability >12 h	Stable up to 24 h %RSD=0.653	Stable up to 24 h %RSD=0.756	Stable up to 24 h %RSD=0.645
11.	Robustness (%RSD)	Complies	Complies	Complies
	Flow plus	0.640	0.733	0.460
	Flow minus	0.454	0.809	0.315
	Organic plus	0.598	0.527	0.665
	Organic minus	0.775	0.336	0.669
	Wavelength plus	0.488	0.393	0.201
	Wavelength minus	0.566	0.482	0.315

RSD: Reflex sympathetic dystrophy, HPLC: High-performance liquid chromatography

determinations over three concentration levels covering the specified range were determined. The samples containing 80%, 100%, and 120% of test concentrations were spiked with 100% of standard solution in HPLC and percentage recovery was calculated, as shown in Table 2.

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

The LOD is defined as the lowest concentration of an analyte that an analytical process can reliably differentiate from background levels. The LOQ is defined as the lowest concentration of the standard curve that can be measured with acceptable accuracy, precision, and variability.[16]

The LOD and LOQ were calculated as

$LOD = 3.3\ \sigma/S.$
$LOQ = 10\ \sigma/S.$

Where, σ is the standard deviation of the lowest standard concentration and S is the slope of the standard curve, as shown in Table 1.

Stability

The stability of lutein, lycopene, and beta-carotene in diluent (methanol or dimethylformamide) was assessed by analyzing the sample concentrations at 1 h, 4 h, 9 h, 24 h, 48 h, and 60 h. The concentrations used in this study were 64 µg/mL for lutein, 40 µg/mL for lycopene, and 200 µg/mL for beta-carotene prepared from stock solution of pure form and powdered tablet sample, respectively. Six replicate samples for each concentration were assayed at each time point. The stability was tested over a period of 3 days at room temperature.

Robustness

The effect of intentional variation in analytical conditions such as detection wavelength, mobile phase flow rate, and diluent (methanol or dimethylformamide) composition on the retention time and peak area was investigated one by one in Table 1.

Table 2: Results of recovery study by HPLC methods

Method	Drug	Amt. present (µg/mL)	Amt. spiked (µg/mL)	%Recovery*
HPLC method	Lutein	51.2	64	102.82
		64	64	99.98
		76.8	64	99.93
	Lycopene	32	40	100.31
		40	40	99.79
		60	40	99.72
	Beta-carotene	160	200	100.47
		200	200	99.21
		240	200	103.47

*Average of six determinations, HPLC: High-performance liquid chromatography

Analysis of marketed tablet formulation by HPLC

For the assay, estimation of marketed formulation by HPLC weighed 10 tablets. Accurately weighed powder sample equivalent to 3.2 mg of lutein, 2 mg of lycopene, and 10 mg of beta-carotene was dissolved in a 50 mL volumetric flask containing diluent (methanol or dimethylformamide). The solution was kept for sonication for 20 min, filtered through Whatman filter paper No. 41. Aliquot of this solution was the concentration of 64 µg/mL of lutein, 40 µg/mL for lycopene, and 200 µg/mL for beta-carotene (n= 6).

RESULTS AND DISCUSSION

RP-HPLC – method validation

RP-HPLC spectrophotometric methods were developed for lutein, lycopene, and beta-carotene which can be conveniently employed for routine analysis in pharmaceutical dosage forms and will eliminate unnecessary tedious sample preparations. The chromatographic conditions were optimized to provide a good performance of the assay. The retention time of lutein, lycopene, and beta-carotene in tablet formulation were found to be 7.2 min, 6.0 min, and 10.1 min, respectively. The chromatograms are shown in Figure 4a. Various system suitability parameters are shown in Table 3. A five-point calibration curve was constructed with working standards and was found linear ($r^2 \geq 0.998$) for each of the analyte over their calibration ranges. The slopes were calculated using the plot of drug concentration versus area of the chromatogram. The developed HPLC method was accurate, precise, reproducible, and very sensitive, as shown in Figure 4.

All the method validation parameters are well within the limits as specified in the ICH Q2B guidelines, as shown in Tables 1 and 2 lists the percent recovery (content uniformity) of all the drugs in the commercial formulations by HPLC methods. Moreover, the %R.S.D. (less variation) shows good precision of both developed methods. The calculated LOQ and LOD concentrations confirmed that the methods were sufficiently sensitive. The methods were suitably employed for assaying all the drugs in commercial marketed formulation [Table 4].

Table 3: System suitability studies of lutein, lycopene, and beta-carotene in optimized RP-HPLC compared with USP pharmacopoeia limits

S. No.	Parameters	USP limit	Lutein	Lycopene	Beta-carotene
1.	Conc. (%)	-	100	100	100
2.	Retention time	-	7.261	6.011	10.103
3.	Average peak area of six injections	-	1,051,242	5,759,661	3,517,422
4.	%RSD of peak area of six injections	≤2.0	1.020	0.676	0.0225
5.	Peak asymmetry (at 10% peak height)	≤1.5	1.73	1.52	1.69
6.	Mean number of theoretical plate	≥2000	2289	2434	2218
7.	Final retention time		7.212	6.019	10.108

RSD: Reflex sympathetic dystrophy, HPLC: High-performance liquid chromatography

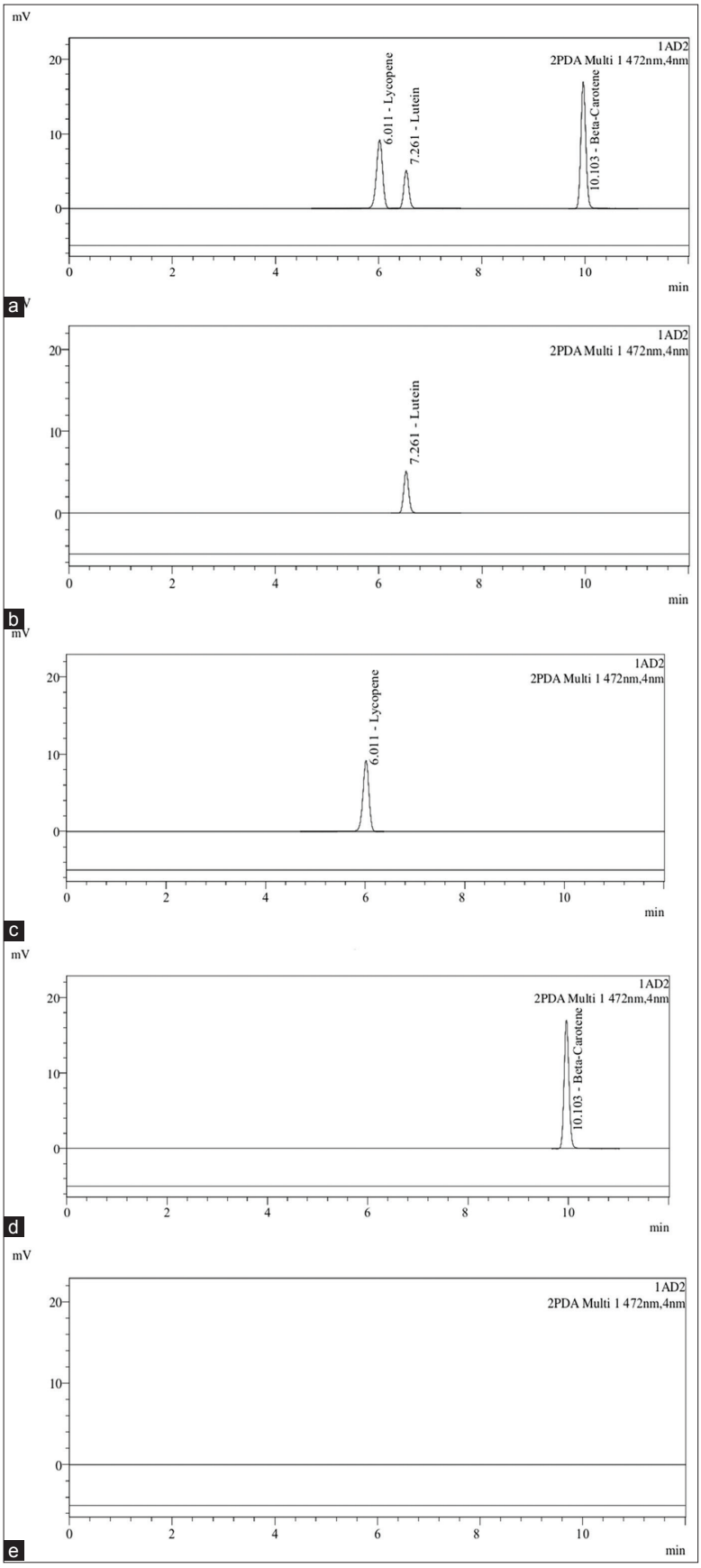

Figure 4: (a) Chromatograms of lutein, lycopene, and beta-carotene, (b) chromatogram of lutein at test concentration, (c) chromatogram of lycopene at test concentration, (d) chromatogram of beta-carotene at test concentration, (e) chromatogram of blank

Table 4: Assay of marketed formulation MiNOXiV by HPLC methods

Drug	Label claim (mg)	Amount found (mg)	Assay (%)
Lutein	3.2	3.2016	100.05
Lycopene	2	2.0024	100.12
Beta-carotene	10	9.9820	99.82

HPLC: High-performance liquid chromatography

CONCLUSION

Simple, rapid, accurate, and precise RP-HPLC methods have been developed and validated for the routine analysis of lutein, lycopene, and beta-carotene in active pharmaceutical ingredient and tablet dosage forms. The methods are suitable for the simultaneous determination of lutein, lycopene, and beta-carotene in multicomponent formulations without interference of each other. The developed methods are suggested for routine and quality control analysis of the investigated drugs in tertiary component pharmaceutical preparations. The amount found from the proposed methods was in good conformity with the label claim of the formulation. Furthermore, the value of standard deviation and coefficient of variation calculated were acceptably low, indicating the suitability of the proposed methods for the routine estimation of tablet dosage forms.

CONFLICTS OF INTEREST

The authors confirm that this article content has no conflicts of interest.

REFERENCES

1. Analysis B. HPLC method for the simultaneous determination of beta-carotene, retinol and alpha-tocopherol in serum. J Pharm Biomed Anal 1988;6:853-7.
2. Thürmann PA, Schalch W, Aebischer JC, Tenter U, Cohn W. Plasma kinetics of lutein, zeaxanthin, and 3' -dehydro-lutein after multiple oral doses of a lutein supplement. Am J Clin Nutr 2005;82:88-97.
3. Chandra-Hioe MV, Rahman HH, Arcot J. Lutein and β-carotene in selected asian leafy vegetables. J Food Chem Nanotechnol 2017;3:93-7.
4. Aguilar MI. Reversed-phase high-performance liquid chromatography. HPLC Pept Proteins 2004;251:9-22.
5. Ahuja S. 1-Overview of modern pharmaceutical analysis. Sep Sci Technol 2011;10:1-9.
6. Behera S, Ghanty S, Ahmad F, Santra S, Banerjee S. UV-visible spectrophotometric method development and validation of assay of paracetamol tablet formulation. Int J Pharm Sci Res 2012;3:4945-53.
7. Bose A. HPLC calibration process parameters in terms of system suitability test. Austin Chromatogr 2014;1:1-4.
8. Chan CC. Analytical Method Validation and Instrument Performance Verification. Hoboken: John Wiley & Sons; 2004.
9. Chatrabhuji P, Pandya C, Patel M. HPLC Method for Determination of APIs in Pharmaceutical Formulation. Morrisville: Lulu Press; 2002.
10. Chatwal G, Anand S. Instrumental Methods of Chemical Analysis: Analytical Chemistry. Bengaluru: Himalaya Publishing House; 1986.
11. Huber L. Validation of Analytical Methods. Germany: Agilent Technologies; 2010.
12. Bhatnagar-panwar M, Bhatnagar-Mathur P, Bhaaskarla VV, Dumbala SR, Sharma KK. Rapid, accurate and routine HPLC method for large-scale screening of pro-Vitamin A carotenoids in oilseeds. J Plant Biochem Biotechnol 2015;24:84-92.
13. Pathak A, Rajput SJ. Simultaneous derivative spectrophotometric analysis of doxylamine succinate, pyridoxine hydrochloride and folic acid in combined dosage forms. Indian J Pharm Sci 2008;70:513-7.
14. ICH. ICH Topic Q2 (R1) Validation of analytical procedures: Text and methodology. In: International Conference on Harmonization. Geneva: ICH; 2005. p. 17.
15. ICH. Guidance for industry: Q2B validation of analytical procedures: Methodology. In: International Conference on Harmonisation of Technical Requirements for Registration Tripartite Guideline. Geneva: ICH; 1996. p. 13.
16. Siddiqui MR, AlOthman ZA, Rahman, N. Analytical techniques in pharmaceutical analysis: A review. Arab J Chem 2017;10:S1409-21.