

A validated stability indicating HPLC method for determination of sitagliptin

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ABSTRACT

A comparative and stability-indicating reversed phase high performance liquid chromatographic study have been developed and validated for sitagliptin phosphate. The liquid chromatographic determination was achieved isocratically on Poroshell 120 EC-C18 (100 × 4.6 mm, i.d.; particle size, 2.7 μm), Pursuit 5PPF (150 × 4.6 mm, i.d.; particle size, 5 μm) and Chromolith performance RP-18e (100 × 4.6 mm, i.d.; macropore diameter, 2 μm) columns using a mobile phase consisting of methanol:water:triethylamine:acetic acid (60:40:0.1:0.1; v:v:v:v), at a flow rate 0.5 mL/min and UV detection at 268 nm. The method was linear over the concentration range of 100-1000 μg/mL ($r = 0.9998$) with a limit of detection and quantitation of 10 and 30 μg/mL, respectively. All the validation parameters and stability indicating study were studied on Poroshell 120 EC-C18 column, which achieved the best separation. The proposed method has been found to have the required accuracy, selectivity, sensitivity, and precision to assay sitagliptin phosphate in bulk form and in a pharmaceutical dosage form. Degradation products resulting from the stress studies did not interfere with the detection of sitagliptin phosphate that indicates that the assay are stability-indicating assay.

1. Introduction

Sitagliptin phosphate (STG) [1-4] is 1,2,4-triazolo [4,3-a] pyrazine,7-[[[(3R)-3-amino-1-oxo-4-(2,4,5-trifluorophenyl) butyl]-5,6,7,8-tetra-hydro-3-(trifluoromethyl) phosphate (Figure 1). It is the first dipeptidylpeptidase-4 inhibitor to be used in the management of type 2 diabetes. It is widely used as an add-on therapy to ongoing management or as monotherapy when it is deemed necessary. It has been found to be beneficial in improving β-cell function and glycemic control. Moreover, in special circumstances like chronic kidney disease it is used with appropriate dose reductions [5].

Literature survey revealed that some LC-MS methods [6-9] were reported for the determination of sitagliptin phosphate in plasma and urine of humans, rats and dogs. Also, spectrophotometric techniques were used for the determination of sitagliptin and characterized by their simplicity, specificity and low cost [10,11]. In addition, some analytical methods were used for the validation and estimation of sitagliptin in bulk and pharmaceutical formulations [12-14]. This study presents a comparative determination of sitagliptin phosphate on three different stationary phases, Poroshell 120 EC-C18, Pursuit 5PPF and Chromolith Performance RP-18e column. All the validation parameters and stability-indicating study will be

studied on the column, which achieves the best determination of sitagliptin phosphate in bulk and pharmaceutical formulations.

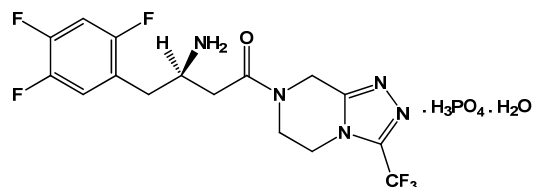


Figure 1. Sitagliptin phosphate.

2. Experimental

2.1. Chemicals and reagents

Sitagliptin phosphate standard bulk powder was obtained from Merck Sharp & Dohme Ltd., Cramlington Northumberland and was used as received, certified to be 99.80% pure. Januvia 100 mg, 50 mg and 25 mg (Labeled to contain 100 mg, 50 mg and 25 mg of sitagliptin phosphate per tablet) were obtained from the Merck Sharp & Dohme Ltd., Cramlington, UK

(Imported by SoficoPharm, Cairo, Egypt). Methanol, triethylamine, acetic acid were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade. Purified HPLC grade water was obtained by reverse osmosis and filtration through a Milli-Q system (Millipore, Milford, MA, USA) and was used as a mobile phase.

2.2. Instrumentation and conditions

The HPLC unit was an Agilent 1100 series apparatus equipped with a quaternary pump, a vacuum degasser, a column oven, a diode array UV-detector and HP Chemstation for LC 3D systems B.0301 [317] software. Three columns were used in this study namely (100 × 4.6 mm, i.d.; particle size, 2.7 μm), Pursuit 5PFP (150 × 4.6 mm, i.d.; particle size, 5 μm) were purchased from Agilent Technologies and (100 × 4.6 mm, i.d.; macropore diameter, 2 μm) was from Merck (Darmstadt, Germany). Separation was achieved using a mobile phase consisting of methanol:water:triethylamine:acetic acid (60:40:0.1:0.1; v:v:v:v), at a flow rate 0.5 mL/min. The column was maintained at temperature 20 °C and UV 268 nm and an injection volume of 5 μL was used. The mobile phase was filtered through 0.45 μm chromTech Nylon-66 filter and degassed in an ultrasonic bath. For analysis of forced degradation samples, the photodiode array detector was used in scan mode with a scan range of 200-500 nm.

2.3. Statistical analysis

Linear regression of the data was performed by using statistical package for social science (SPSS) program version 16 software (SPSS Inc., U.S.A.). The level of significance was set at $p \leq 0.05$.

2.4. Preparation of stock and standard solutions

A stock solution of sitagliptin phosphate (1 mg/mL) was prepared in HPLC-grade methanol. The stock solution was protected from light using aluminum foil and stored for 1 week at 4 °C, and was found to be stable during this period. In the HPLC assay, aliquots of the standard stock solution of sitagliptin phosphate were transferred using grade bulb pipettes into 10 mL volumetric flasks. The solutions were made up to volume with methanol to give final concentrations of 100, 150, 200, 300, 500, 800 and 1000 μg/mL, using external standard (1000 μg/mL).

2.5. Preparation of tablets for assay

Ten tablets were weighed and ground to a fine powder. A portion of the powdered tablets equivalent to 100 mg was accurately weighed into 100 mL-A grade volumetric flask and 30 mL methanol was added. The volumetric flask was sonicated for 20 min to ensure complete dissolution of the sitagliptin phosphate then the solution was made up to the volume with methanol. Suitable aliquots of solution were filtered through a 0.45 μm nylon filter and completed as previously mentioned under preparation of stock and standard solution for HPLC.

2.6. Forced degradation studies of authentic sitagliptin phosphate and its tablet formulation

In order to determine whether the proposed analytic method is stability-indicating, sitagliptin phosphate in its tablets pharmaceutical dosage form and powder was stressed under various conditions to conduct forced degradation studies. Regulatory guidance in ICH, Q2A, Q2B, Q3B and FDA 21 CFR section 211 require the development and validations of stability indicating potency assays [15].

2.6.1. Oxidation

Solutions for oxidation studies were prepared by dissolving 100 mg of standard sitagliptin phosphate and powdered tablets containing an equivalent to 100 mg of sitagliptin phosphate in methanol and 3% H₂O₂ (20:80%; v:v), protected from light and stored at room temperature for 3 days.

2.6.2. Acid degradation studies

Solutions for acid degradation studies were achieved by refluxing 100 mg of standard sitagliptin phosphate and powdered tablets containing an equivalent to 100 mg of sitagliptin phosphate in 2 M hydrochloric acid at 100 °C in an oil bath for 24 hr. The reaction mixture was neutralized with a~2.5 M sodium hydroxide and extracted with ethyl acetate, dried (Na₂SO₄ anhydrous) and evaporated under vacuum. The residue was dissolved in methanol (100 mL).

2.6.3. Alkali degradation studies

Solutions for alkali degradation studies were performed by refluxing 100 mg standard sitagliptin phosphate and powdered tablets containing an equivalent to 100 mg of sitagliptin phosphate in 1 M sodium hydroxide at 100 °C in an oil bath for 24 hr. The reaction mixture was then diluted with water and extracted with ethyl acetate, dried (Na₂SO₄ anhydrous) and evaporated under vacuum. The residue was dissolved in methanol (100 mL).

2.6.4. Neutral degradation studies

Solutions for neutral degradation studies were prepared by dissolving 100 mg standard sitagliptin phosphate and powdered tablets containing an equivalent to 100 mg of sitagliptin phosphate in methanol and water (20:80, v:v), protected from light, and stored at room temperature for 3 days.

2.6.5. Temperature stress studies

Standard sitagliptin phosphate and its powdered tablets were exposed to dry heat (90 °C) in an oven for 3 days.

2.6.6. Photostability degradation assay

Bulk sitagliptin phosphate powder, its powdered tablets, and their methanolic solutions were exposed to light to determine the effect of irradiation on the stability of sitagliptin phosphate in the solid state and in the solution form. Approximately, 100 mg of standard sitagliptin phosphate powders were spread on a glass dish in a layer that was less than 2 mm in thickness. Solutions of standard sitagliptin phosphate (1 mg/mL) were prepared in methanol. All samples for photostability testing were placed in a light cabinet (Thermolab, India) and exposed to light for 40 h resulting in an overall illumination ≥ 200 wh/m² at 25 °C with UV-radiation at 320-400 nm. Control samples, which were protected from light with aluminum foil, were also placed in the light cabinet and exposed concurrently following the removal from the light cabinet.

3. Results and discussion

3.1. HPLC method development and optimization

Poroshell 120 EC-C18 (100 × 4.6 mm, i.d.), Pursuit 5PFP (150 × 4.6 mm, i.d.) and Chromolith Performance RP-18e (100 × 4.6 mm) columns were used for the determination of sitagliptin phosphate.

Table 1 Comparative chromatographic and validation parameters of sitagliptin using different stationary phases

Parameter	Poroshell 120 EC-C18 Column	Chromolith performance RP-18e Column	Pursuit 5PFP Column
Regression equation, r ²	y = 0.0010x-0.008, 0.999	y = 0.0010x-0.010, 0.999	y = 0.0010x-0.014, 0.999
Capacity factor (k')	0.399	0.200	0.696
Time of analysis (min)	10.000	10.000	10.000
Retention time (min)	2.629	3.418	5.713
No. of theoretical plates (N)	8794.286	4673.200	19130.824
No. of theoretical plates/mm	87.94286	46.73200	12.75388
Tailing factor	2.5	1.4	2
Asymmetry factor	3	2	3
Linearity (µg/mL)	100-1000	100-1000	100-1000
Sensitivity (µg/mL)	100	100	100
Precision	1.23	-	-
Repeatability (Intraday)	0.99	-	-
Intermediate precision (Interday)	0.62	-	-
Accuracy	1.23	-	-
	0.82	-	-
	0.63	-	-
Selectivity	1.34	1.20	1.30
L.O.D. (µg/mL)	10	-	-
L.O.Q. (µg/mL)	30	-	-

Calibration curves, correlation coefficient, slope, intercept, capacity factor, retention time, number of theoretical plates, number of theoretical plates/mm, tailing factor and asymmetry factors were determined using the previously mentioned columns as shown in Table 1. It was that Poroshell 120 EC-C18 has achieved the best separation due to it has the highest number of theoretical plates/mm so it was chosen to study all the validation parameters. The method was validated for the determination of sitagliptin phosphate in bulk and in pharmaceutical dosage form. The stressed samples were initially analyzed using a mobile phase consisting of methanol: water: triethylamine:acetic acid (60:40:0.1:0.1; v:v:v:v) which achieved better separation without tailing at a flow rate of 0.5 mL/min and UV detection at 268 nm using 1000 µg/mL sitagliptin phosphate as an external standard.

3.2. Validation

The method was validated with respect to parameters including linearity, limit of quantitation (LOQ), Limit of detection (LOD), precision, accuracy, selectivity and recovery using Poroshell 120 EC-C18.

3.2.1. Linearity

The calibration curves constructed for sitagliptin phosphate were linear over the concentration range 100-1000 µg/mL. Peak areas of sitagliptin phosphate were plotted versus its concentration and linear regression analysis was performed on the resultant curve.

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

The LOQ and LOD were determined based on signal to noise ratios. The LOQ was found to be 30 µg/mL with a resultant %R.S.D of 0.4% (n = 5), where the LOD was found to be 10 µg/mL.

3.2.3. Precision

Precision of the assay was investigated with respect to both repeatability and reproducibility. Repeatability was investigated by injecting nine replicate samples of each of the 200, 400 and 600 µg/mL where the mean concentrations were found to be 199.81, 400.23 and 598.40 with associated relative standard deviation (% R.S.D.) values of 1.23, 0.99 and 0.62, respectively. Inter-day precision was assessed by injecting the

same three concentrations over 3 consecutive days, resulting in mean concentrations of sitagliptin phosphate of 200.30, 400.48 and 600.22 µg/mL and associated % R.S.D of 1.60, 1.20 and 0.99 %, respectively.

3.2.4. Accuracy

Accuracy of the assay was determined by interpolation of replicate (n = 6) peak area of three accuracy standards (200, 400 and 600 µg/mL) from a calibration curve prepared as previously described. In each case the percent relevant error was calculated. The resultant concentration, were 199.62±0.81 µg/mL, (mean±S.D.), 400.34±0.56 µg/mL and 600.52±0.41 µg/mL with percent relevant errors of 1.23, 0.82 and 0.63, respectively.

3.2.5. Selectivity

The results of stress testing studies were performed on Poroshell 120 EC-C18 and showed a high degree of selectivity of this method for sitagliptin phosphate. The degradation of sitagliptin phosphate was found to be similar for both the tablets and bulk powder. Typical chromatograms obtained following the assay of pure bulk sample and stressed samples are shown in Figure 2-4.

Figure 3 showed that sitagliptin was completely degraded by hydrogen peroxide with the appearance of two degradation products at retention time 1.987 and 7.429 min. Also, sitagliptin was partially degraded by alkaline condition and showed three degradation products at 1.921, 6.535 and 7.430 min, while the acidic condition led to partial degradation of sitagliptin with the formation of several degradation products.

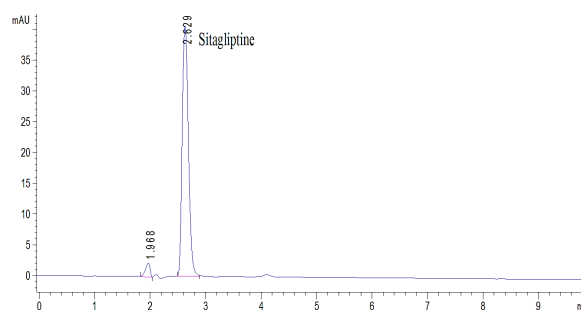


Figure 2. Typical chromatogram of sitagliptin phosphate on Poroshell 120 EC-C18 column.

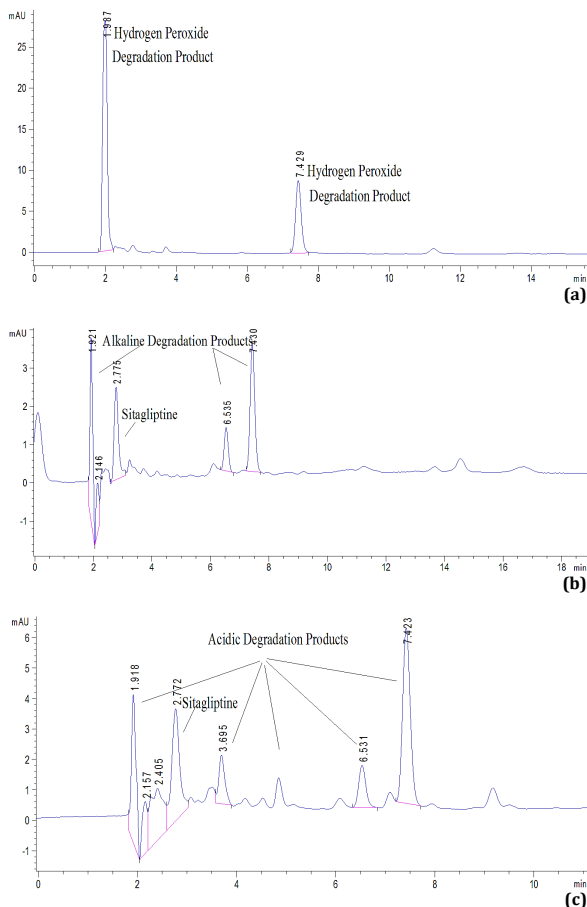


Figure 3. Degradation products of sitagliptin phosphate on Poroshell 120 EC-C18 column. (a) Hydrogen peroxide degradation products, (b) Alkaline degradation products, and (c) Acidic degradation products.

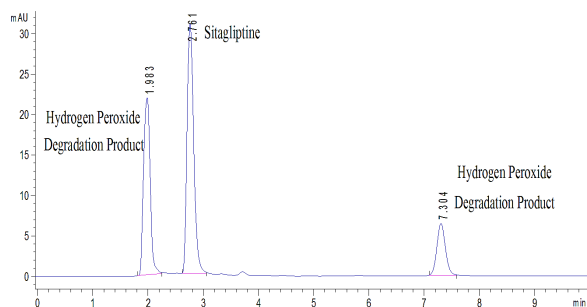


Figure 4. Lab mixture of sitagliptin and hydrogen peroxide degradation products on Poroshell 120 EC-C18 column.

3.2.6. Recovery

A known amount of sitagliptin phosphate standard bulk powder was added to aliquots ($n = 20$) of tablet contents, mixed, and the powder was extracted and diluted to yield a starting concentration. This solution was analyzed and the assay was repeated ($n = 9$) over 3 consecutive days to obtain intermediate precision data. The observed concentration of sitagliptin phosphate was found to be $300.00 \pm 0.42 \mu\text{g/mL}$ (mean \pm S.D.). The resultant % R.S.D. for this study was found to be 0.418% with a corresponding percentage recovery value of 100.44%.

3.3. Assay

The proposed method was applied for the determination of sitagliptin phosphate in the tablet formulations. The results of these assays yielded 100.60% (% R.S.D = 1.62%) of expected amount of the tablets. These results indicated that the method is selective for the assay of sitagliptin phosphates without interference the excipient used in those dosage forms.

3.4. Stability studies

All stressed samples in both solid states and in solution forms remained colorless. No decomposition was observed in neutral medium and on exposure of sitagliptin phosphate in solid states and in solution forms to light in a photo stability chamber at room temperature. On the other hand, it was found that sitagliptin had been extensively degraded on treating with 3% hydrogen peroxide for 3 days. The retention time R_t of hydrogen peroxide degradation products of sitagliptin phosphate were 1.987 and 7.429 min. Also 76 and 28% degradation was observed in alkaline and acidic media, respectively, as shown in Figure 3 and 4.

3.4.1. Stability-indicating method for analysis of sitagliptin phosphate in presence of its hydrogen peroxide -oxidative degraded products by HPLC

A simple HPLC method was adopted for the simultaneous determination of sitagliptin phosphate and its oxidative hydrogen peroxide degraded products in bulk powder. To optimize the proposed HPLC method, all of the experimental conditions were investigated. To optimize the mobile phases, different systems were tried for chromatographic separation of the two components by combining homogenous design and solvent polarity optimization. The best resolution was achieved using a mobile phase consisting of methanol:water:triethyl amine:acetic acid (60:40:0.1:0.1; v:v:v:v) which gave good resolution and sensitivity of all components. A linear relationship was obtained between peak area and the concentration of sitagliptin phosphate in the presence of its hydrogen peroxide oxidative degraded products in the range of 100-1000 $\mu\text{g/mL}$. The linear regression equation was computed as:

$$y = 0.0010x + 0.0033 \quad r = 0.9998 \quad (1)$$

where y is the area under the peak, x is the concentration in $\mu\text{g/mL}$, and r is a correlation coefficient.

The results obtained by applying the HPLC procedure showed that sitagliptin phosphate can be simultaneously analyzed in the presence of its hydrogen peroxide oxidative degraded with mean recoveries of $100.05 \pm 1.399\%$, Table 2.

The proposed method has been applied to the assay of sitagliptin phosphate in its tablet form without any interference from the excipients. The validity of the suggested procedure was further assessed by applying the standard addition technique with mean recoveries $100.48 \pm 0.692\%$, Table 3.

A statistical comparison of the results obtained by the proposed HPLC method and the Clarke procedure that depends on UV spectrophotometric determinations of sitagliptin phosphate [16] in a pure drug are shown in Table 4. The calculated t and F values were less than the tabulated values [17], which revealed that there is no significant difference with respect to accuracy and precision determined through assay of variables. Also, some statistical analysis was studied such as linear regression parameters as shown in Table 5 and 6.

Blind samples of pure sitagliptin phosphate of different concentrations were calculated from its regression equation.

Table 2. Determination of pure bulk sitagliptin phosphate by HPLC.

Mixture no	Concentration taken ($\mu\text{g/mL}$)	Found ($\mu\text{g/mL}$)	Found (%)
1	150.00	151.70	101.13
2	300.00	294.70	98.23
3	500.00	508.70	101.74
4	800.00	795.70	99.46
5	1000.00	996.70	99.67
Mean \pm S.D			100.05 \pm 1.399

Table 3. Application the standard addition technique to the analysis of sitagliptin phosphate by HPLC in its tablet dosage form.

Product	Found (%)	Pure added ($\mu\text{g/mL}$)	Found ($\mu\text{g/mL}$)	Recovery (%)
Januvia 100 mg Sitagliptin phosphate/Tablet	100.60 \pm 1.62	100.00	101.20	101.20
		200.00	199.10	99.55
		300.00	302.30	100.77
		400.00	403.60	100.90
		500.00	499.80	99.96
Mean \pm S.D				100.48 \pm 0.69

Table 4. Statistical analysis of the results obtained by applying the proposed UV spectrophotometric method [16] for the analysis of pure sitagliptin phosphate.

Parameter	Proposed HPLC method	UV spectrophotometric method
Mean \pm S.D	100.05 \pm 1.399	99.88 \pm 0.899
n ^a	5	5
t	0.251 (2.306) ^b	-
F	2.422 (6.390) ^b	-

^a n: Number of determinations.

^b The values in parentheses are corresponding to the theoretical values of t and F at ($p = 0.05$).

Table 5. Linear regression data, ANOVA^a of regression.

Model	Sum of squares	df	Mean square	F	Sigma	
Mixture no: 1	Regression	0.720	1	0.720	11335.665	0.000 ^b
	Residual	0.000	5	0.000		
	Total	0.720	6			

^a Dependent Variable: Area.

^b Predictors: (Constant), Conc. $\mu\text{g/mL}$.

Table 6. Linear regression data, coefficients^a.

Model	Unstandardized coefficients		Standardized coefficients	t	Sigma	95% Confidence interval for B	
	B	Standard error	Beta			Lower bound	Upper bound
Mixture no: 1	Constant	0.003	0.005	0.658	0.539	-0.010	0.016
	Conc. $\mu\text{g/mL}$	0.001	0.000	1.000	106.469	0.001	0.001

^a Dependent Variable: Area.

The degree of method repeatability (intra-day) and intermediate precision (inter-days) were measured by analysing each concentration of sitagliptin phosphate four times by the proposed method in the same day and for four days, then calculating %R.S.D. values which were 0.53 and 0.74, respectively. The results of the proposed method showed that it is accurate, precise, specific, and rugged according to the %R.S.D. values of intra-day and inter-day determinations.

4. Conclusion

A validated stability-indicating HPLC analytical method has been developed for the determination of sitagliptin phosphate in pure form and in tablet dosage formulation. All the validation parameters were studied on Poroshell 120 EC-C18 column which achieved the best separation. The results of stress testing were under taken according to the international conference on harmonization (ICH) guidelines, which revealed that the studied method is selective and stability-indicating. The proposed method has been found to have the required accuracy, selectivity, sensitivity, and precision to assay sitagliptin phosphate in bulk form and in a pharmaceutical dosage form. Degradation products resulting from the stress studies and the excipients found in the tablet dosage forms did not interfere with the detection of sitagliptin phosphate which indicates that the assay are stability-indicating assay. The achieved methods are suitable for the routine analysis of sitagliptin phosphate in either bulk powder or in pharmaceutical dosage forms. The simplicity of the applied method allows their application in quality control laboratories.

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References

- [1] Herman, G. A.; Stevens, C.; Dyck, K. V.; Bergman, A.; Yi, B.; De Smet, M.; Snyder, K.; Hilliard, D.; Tanen, M.; Tanaka, W.; Wang, A. Q.; Zeng, W.; Musson, D.; Winchell, G.; Davis, M. J.; Ramael, S.; Gottesdiener, K. M.; Wagner, J. *Clin. Pharmacol. Ther.* **2005**, *78*, 675-688.
- [2] Amori, R. E.; Lau, J.; Pittas, A. G. *JAMA* **2007**, *298*, 194-206.
- [3] Rosenstok, J.; Sankoh, S.; List, J. F. *Diabetes Obes. Metab.* **2008**, *10*, 376-386.
- [4] Herman, G. A.; Bergman, A.; Liu, F.; Stevens, C.; Wang, A. Q.; Zeng, W.; Chen, L.; Snyder, K.; Hilliard, D.; Tanen, M.; Tanaka, W.; Meehan, A. G.; Lasseter, K.; Dilzer, S.; Blum, R.; Wagner, J. A. *J. Clin. Pharmacol.* **2006**, *46*, 876-886.
- [5] Eligar, V. S.; Bain, S. C. *Drug Des. Dev. Ther.* **2013**, *7*, 893-903.
- [6] Vincent, S. H.; Reed, J. R.; Bergman, A. J.; Elmore, C. S.; Zhu, B.; Xu, S.; Ebel, D.; Larson, P.; Zeng, W.; Chen, L.; Dilzer, S.; Lasseter, K.; Gottesdiener, K.; Wagner, J. A.; Herman, G. A. *Drug Metab. Dispos.* **2007**, *35*, 533-538.
- [7] Wei, Z.; Donald, G. M.; Alison, L. F.; Li, C.; Michael, S. S.; Eric, J. W.; Amy, Q. W. *J. Pharmaceut. Biomed.* **2008**, *46*, 534-542.
- [8] Ramakrishna, N.; Vishwottam, K.; Koteshwara, M.; Prashanth, K.; Raghupathi, A.; Rajeshkumar, B. *Biomed. Chromatogr.* **2008**, *22*, 214-222.
- [9] Beconi, M. G.; Reed, J. R.; Teffera, Y.; Xia, Y. Q.; Kochansky, C. J.; Liu, D. Q.; Xu, S.; Elmore, C. S.; Cicco, S.; Hora, D. F.; Stearns, R. A.; Vincent, S. H. *Drug Metab. Dispos.* **2007**, *35*, 525-532.

- [10]. Sekaran, C. B.; Prameela, R. *Int. J. Pharm. Pharm. Sci.* **2010**, *2*, 138-142.
- [11]. Raja, T.; Rao, A. L. *Int. J. Pharm. Chem. Biol. Sci.* **2012**, *2*, 696-702.
- [12]. Maste, M. M.; Parate, A. N.; Bhat, A. R. *Asian J. Res. Chem.* **2011**, *4*, 1466-1468.
- [13]. Lavanya, R.; Yunoos, Md. *J. Adv. Pharm. Edu. Res.* **2013**, *3*, 475-479.
- [14]. Pathan, H.; Sreenivas, R. T.; Chandanam, S.; Ashok, R.; Swetha, K. *DHR Int. Pharm. Sci.* **2014**, *5*, 80-87.
- [15]. Moffat, A. C.; Osselten, M. D.; Widdop, B. *Clarke's Analysis of Drugs and Poisons in Pharmaceuticals, Body Fluids and Post Mortem Material*, 3rd Edn., Pharmaceuticals Press: London, 2004.
- [16]. Clark, E. G. C. *Clarke's Isolation and Identification of Drugs in Pharmaceuticals Body Fluids and post-Mortem Materials*, 2nd Edn. The Pharmaceutical Press: London, 1986.
- [17]. Hinchey, J. D. *Practical Statistics for Chemical Research*, 1st Edn. Methuen and Science Paperbacks, London, 1969.