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Research Article

DEVELOPMENT AND VALIDATION OF UV SPECTROPHOTOMETRIC AND REVERSED PHASE-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY - PDA METHODS FOR THE ESTIMATION OF ALOGLIPTIN BENZOATE

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ABSTRACT

Objective: To develop and validate simple, rapid, precise, accurate, and economical UV spectrophotometric and reverse phase high performance liquid chromatographic methods for the estimation of alogliptin benzoate (AGP).

Methods: UV spectrophotometric method was performed using UV/Vis double beam spectrophotometer with a spectral bandwidth of 1 nm and 1.0 cm matched quartz cells. The maximum absorbance of AGP was observed at 276 nm using methanol as solvent. Reversed phase-high performance liquid chromatography (RP-HPLC) method was carried out on a Unisol reverse phase C18 column (150 mm × 4.6 mm, 3 µm) with a mobile phase composed of methanol and 10 mM ammonium acetate buffer (adjusted to pH 5.0 with glacial acetic acid) in the ratio of 80:20 v/v with a flow rate of 0.8 ml/minutes.

Results: The linearity of methods was found to be in the range of 5-35 µg/ml (UV) and 20-100 µg/ml (RP-HPLC) and the correlation coefficient was 0.999 for both the methods. The regression equations were y = 0.028x + 0.023 (UV) and y = 28,58,942x - 4,33,647 (HPLC). The retention time of AGP was 2.37 minutes.

Conclusion: The proposed methods were validated in terms of linearity, precision, accuracy, specificity, robustness, limit of detection, and limit of quantitation as per International Conference on Harmonization Q2 R1 guidelines. Thus, the proposed methods are novel, sensitive, and reliable and can be successfully used for the quantitation of AGP.

Keywords: Alogliptin benzoate, UV-visible spectrophotometer, Reversed phase-high performance liquid chromatography, International Conference on Harmonization guidelines.

INTRODUCTION [1-4]

Alogliptin is a selective, orally bioavailable inhibitor of dipeptidylpeptidase-4 (DPP-4) enzyme. It is used in the treatment of Type 2 diabetes. Type 2 diabetes is a chronic and progressive disease usually identified by the resistance of insulin and dysfunction of β-cells. DPP-4 inhibitors potentiate the effect of incretin hormones. Incretin hormones are secreted from the gastrointestinal tract into the bloodstream in response to food intake. The two most well-characterized incretin hormones are the glucagon-like peptide-1 (GLP-1) and glucose-dependent insulin tropic polypeptide, also known as gastric inhibitory peptide (GIP). DPP-4 is an enzyme, which could inactivate endogenous GLP-1, an insulin tropic hormone playing an important role in promoting insulin secretion, inhibiting glucagon secretion. Particularly, the GLP-1 hormone was found to be responsible for the majority of the incretin effects on the pancreatic β - cell function. Chemically, Alogliptin is synthesized as a benzoate salt, which is 2-([6-[(3R)-3-aminopiperidin-1-yl]-3-methyl-2, 4-dioxo-3, 4-dihydropyrimidin- 1(2H)-yl] methyl) benzonitrile monobenzoate. Its molecular formula is $C_{18}H_{21}N_5O_2 \bullet C_7H_6O_2$.

Very few analytical methods were reported in the literature for the determination of alogliptin benzoate (AGP) by UV spectrophotometry. However, there were several methods sited in the literature for the determination of AGP by reversed phase-high performance liquid chromatography (RP-HPLC), but they are having drawbacks such as high flow rate and high retention time, and the aqueous phase is not compatible to liquid chromatography-mass spectrometry (LC-MS) analysis. So far, there was no reported HPLC method for the determination of AGP using ammonium acetate buffer as the aqueous phase. Hence, an attempt has been made to develop a new UV and RP-HPLC method for AGP. The proposed HPLC method yielded better retention time, very sharp, and symmetrical peak shapes and is compatible with LC-MS analysis. The HPLC method can be used in the quality control of drugs because of its sensitivity, reproducibility, and specificity.

The present work represents sensitive, reliable, precise, and simple UV and RP-HPLC methods for the estimation of AGP. These methods were validated according to International Conference on Harmonization (ICH) guidelines.

METHODS

Instruments

An Agilent Infinity 1260 HPLC system equipped with quaternary pumps G1311C, degasser G4225A, autosampler G1329B, thermostated column compartment G1316A, and PDA detector G4212B was used. The software used for data acquisition was open LAB CDS EZChrom A.04.05. The chromatographic analysis was performed on Unisol reverse phase C18 column (150 × 4.6 mm, 3 µm). Lab India - T60 UV/Vis double beam spectrophotometer with a spectral bandwidth of 1 nm and 1.0 cm matched quartz cells was used for UV Spectrophotometry. The software used for data acquisition was UV Win. Eutech pH 700 pHmeter and Mettler Toledo ME 204 weighing balance were used.

CHEMICALS AND SOLVENTS

AGP reference standard was kindly supplied by Active Pharma Labs Pvt. Ltd. (Hyderabad, India). AR grade Methanol (Thermo Fisher scientific Ind. Pvt. Ltd. Mumbai, India) was used to prepare all

solutions for the UV method. HPLC grade chemicals and reagents include Glacial acetic acid, Ammonium acetate, Methanol and Water, which were obtained from Merck Specialities Pvt. Ltd., Mumbai, India. Double distilled water was used for the preparation of buffer and cleaning of glassware.

Selection of solvent and detection wavelength

From the literature survey, it was clear that the drug is freely soluble in methanol, and moreover, the drug showed higher absorbance in methanol and hence, methanol was used as a solvent for further preparation of solutions. Drug solution of 20 µg/ml was prepared and scanned over the range of 200-400 nm in UV/VIS Spectrophotometer and the maximum absorbance was found at 276 nm using methanol as blank. Hence, methanol was used as solvent and 276 nm as the detection wavelength.

Chromatographic conditions

The mobile phase consisted of methanol and 10 mM ammonium acetate buffer (pH 5.0 adjusted with glacial acetic acid) in the ratio 80:20 v/v. Freshly prepared mobile phase was filtered through a Millipore vacuum filter system equipped with 0.45 µm filter and degassed by sonicator for 10 minutes before use. Chromatography was performed at ambient temperature by pumping the mobile phase at a flow rate of 0.8 ml/minutes and an injection volume of 10 µL. The column effluent was monitored at 276 nm.

Preparation of diluent

The mobile phase was used as the diluent, i.e., methanol: 10 mM ammonium acetate buffer (60:40).

Preparation of standard and working standard solutions

For UV method

Accurately 10 mg of standard AGP was weighed and transferred into 100 ml volumetric flask and was dissolved in AR grade methanol. The solution was sonicated, and the volume was made up with methanol to obtain the final concentration of 100 µg/ml. Appropriate dilutions were made into 10 ml volumetric flasks with methanol to produce working solutions in the concentrations range 5-35 µg/ml. The absorbances for each of these solutions were measured.

For HPLC method

Accurately 10 mg of standard AGP was weighed and transferred into 100 ml volumetric flask and was dissolved in HPLC grade methanol. The solution was sonicated, and the volume was made up with methanol to obtain the final concentration of 100 µg/ml. Appropriate dilutions were made into 10 ml volumetric flasks with methanol to produce working solutions in the concentration range 20-100 µg/ml. Each of these drug solutions (10 µl) was injected into the chromatographic system, and peak areas were measured.

Method validation [6]

The proposed methods were validated for following parameters: Linearity, precision, accuracy, specificity, robustness, limit of detection (LOD), and limit of quantitation (LOQ) according to an international conference on harmonization guidelines for validation of analytical procedures.

Linearity

The calibration curve was obtained with seven concentrations of the standard solution (5-35 µg/ml for UV method) and five concentrations of the standard solution (20-100 µg/ml for HPLC method). The solutions were prepared in triplicate. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method.

Precision

The precision of the assay was determined by repeatability (intraday) and intermediate precision (interday). Repeatability was evaluated by assaying samples, at same concentration on the same day. The intermediate precision was studied by comparing the assays on consecutive days. Six sample solutions (n=6) of fixed concentration (15 µg/ml, for UV method and 60 µg/ml, for HPLC method) were prepared and assayed.

Accuracy

The accuracy was determined by recovery studies at three concentration levels (80%, 100%, and 120%), i.e., 12, 15, and 18 µg/ml solutions in UV and (50%, 100%, and 150%), i.e., 20, 40, and 60 µg/ml solutions in HPLC using standard addition method and each concentration was injected three times. The accuracy of an analytical method should be established across its range. The percentage recovery of added AGP standard was calculated.

Specificity

The specificity was determined for the HPLC method. It was established by injecting the 10 µl solutions of standard and blank individually to investigate interference from the representative chromatograms.

Robustness

The robustness of the UV method was determined by changing the wavelength (±1 nm). The effect on absorbance was studied (15 µg/ml). For the HPLC method, robustness was determined by analysis of samples under a variety of conditions such as small changes in the flow rate (±0.2 ml/minutes) and in the percentage of methanol

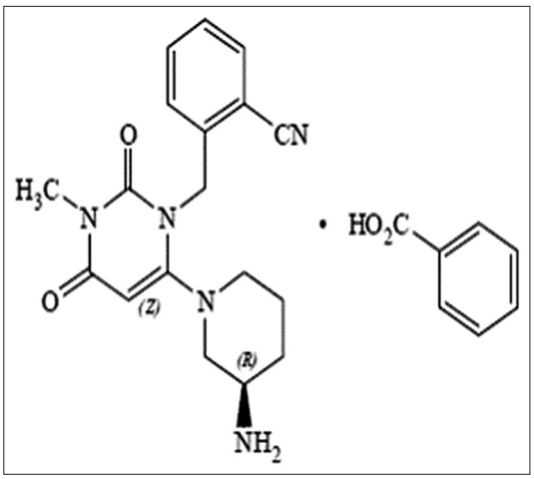

Fig. 1: Structure of alogliptin benzoate [5]

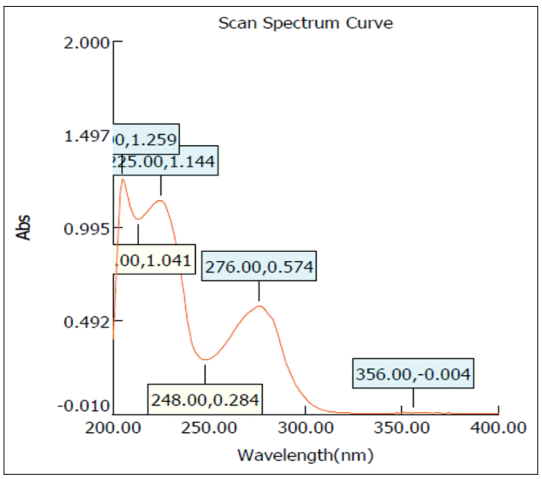

Fig. 2: UV spectrum of alogliptin benzoate reference standard in methanol (20 µg/ml)

(±5%) in the mobile phase and changing the pH (±0.2). The effect on retention time and peak parameters were studied.

LOD and LOQ

The parameters LOD and LOQ were determined on the basis of response and slope of the regression equation for the UV method. For the HPLC method, LOD and LOQ were determined by injecting progressively low concentrations of the standard solution using the developed RP-HPLC method.

RESULTS AND DISCUSSION

HPLC method development

Different trials were carried out by varying the ratio of methanol and ammonium acetate buffer (v/v) and optimizing the chromatographic conditions on a Unisol reverse phase C18 column (150 mm × 4.6 mm, 3 µm). The mobile phase consisting of methanol: Ammonium acetate buffer in the ratio 80:20 v/v, pH 5.0 with a flow rate of 0.8 ml/minutes, injection volume of 10 µl and run time of 5 minutes was optimized as the best chromatographic response for the entire study where AGP was eluted forming symmetrical peak, well separated from the solvent front. The retention time for AGP was determined as 2.37 minutes which allows a rapid determination of the drug, which is important for routine analysis (Fig. 3).

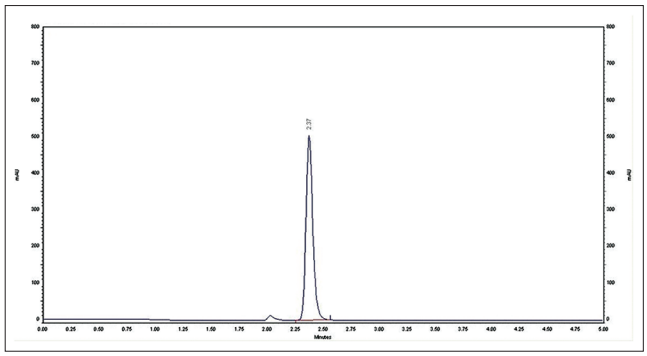

Fig. 3: Chromatogram of alogliptin benzoate standard – 100 µg/ml

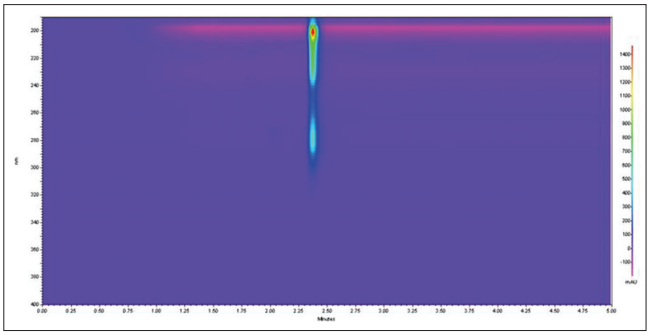

Fig. 4: Counterplot of alogliptin benzoate standard - 100 µg/ml

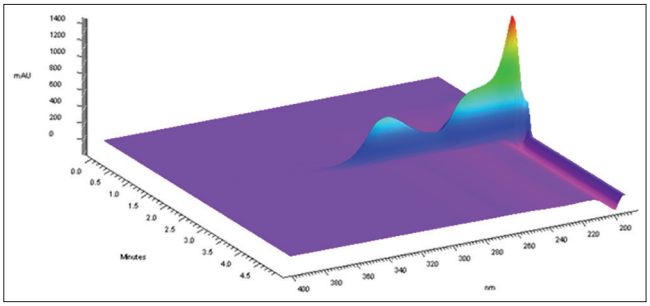

Fig. 5: Three dimensional view of chromatogram of alogliptin benzoate standard - 100 µg/mL

Method validation

For HPLC method

System suitability

System suitability studies were carried out by injecting six times a 100 µg/ml standard concentration of AGP at 10 µl injection volume. The relative standard deviation (RSD) values for system suitability test parameters, such as retention time, peak area, tailing factor, and theoretical plate number, were <2% indicating the present conditions were suitable for the analysis of AGP. The data were given in Table 2.

Linearity

The calibration curve for AGP was constructed by plotting concentration versus peak area in the concentration range 20-100 µg/ml. The representative linear equation was y = 28,58,942x - 4,33,647, with a correlation coefficient (r^2= 0.999) highly significant for the method (Fig. 6)

The results show that an excellent correlation exists between the peak area and concentration of the drug. Table 3 lists the linearity parameters of the calibration curve for AGP.

Precision

The precision of the method was determined by repeatability (intraday) and intermediate precision (interday) and was expressed as RSD (%) of a series of measurement. The experimental values obtained for the determination of AGP in samples are present in Table 4. The result obtained shows RSD of 0.18% indicating good intraday precision. Interday variability was calculated from assays on consecutive days

Table 1: Results of regression analysis data for the quantitation of AGP by RP-HPLC method

Parameters	Results
Concentration range (µg/ml)	20-100
Correlation coefficient (r^2)	0.999
Slope (m)	2858942
Intercept (c)	−433647
LOD (µg/ml)	0.42
LOQ (µg/ml)	1.30

AGP: Alogliptin benzoate, RP-HPLC: Reversed phase-high performance liquid chromatography, LOD: Limit of detection, LOQ: Limit of quantitation

Table 2: System suitability testing of AGP - HPLC method

S. No	Retention time (minutes)	Peak area	Tailing factor	Theoretical plates
1	2.371	287676902	0.98	7508
2	2.370	288353953	1.02	7393
3	2.374	289391365	0.99	7403
4	2.370	289396933	0.99	7488
5	2.371	288216951	1.00	7381
6	2.373	289755760	1.01	7471
Mean	2.371	288798644	0.998	7440.66
SD	0.0016	827014.01	0.0147	54.67
% RSD	0.07%	0.29%	1.47%	0.73%

AGP: Alogliptin benzoate, RP-HPLC: Reversed phase-high performance liquid chromatography, RSD: Relative standard deviation, SD: Standard deviation

Table 3: Linearity data of AGP - HPLC method

S. No	Concentration (µg/ml)	Peak area
1	20	56626235
2	40	114440220
3	60	172643752
4	80	224127341
5	100	287676902

AGP: Alogliptin benzoate, RP-HPLC: Reversed phase-high performance liquid chromatography

and shows RSD of 0.30%. From the result obtained, the proposed HPLC method was found to be precise.

Accuracy

Standard solution of the accuracy of 50%, 100%, and 150% solutions, i.e., 20, 40, and 60 µg/ml were injected into the chromatographic system. Table 5 represents the high percent recovery values indicating that the proposed method is accurate and reproducible.

Specificity

Specificity was established by injecting the 10 µl solutions of standard and blank individually to investigate interference from the representative chromatograms in Figs. 7 and 8. It can be inferred that there were no co-eluting peaks at the retention time of AGP, this shows that peak of analyte was pure and did not interfere with the analysis.

Robustness

The method remained unaffected by deliberate small changes in parameters such as flow rate, mobile phase composition, and pH. Below tabulated values indicate that the method is robust in terms of changed flow rate, mobile phase, and pH. The data were presented in Table 6.

LOD and LOQ

The LOD and LOQ of AGP were found to be 0.42 and 1.30 µg/ml, respectively.

For UV method

Linearity

The calibration curve for AGP was constructed by plotting concentration versus absorbance in the concentration range 5-35 µg/ ml. The representative linear equation was y = 0.028x + 0.023, with a correlation coefficient (r^2= 0.999) highly significant for the method (Fig. 9).

The results show that an excellent correlation exists between the absorbance and concentration of the drug. Table 8 lists the linearity parameters of the calibration curve for AGP.

Table 4: Result of precision study of AGP - HPLC method

S. No (n=6)	Peak area	
	Intraday	Interday
1	172464631	172428564
2	172351689	171852869
3	172245768	172893751
4	172554891	173256971
5	173108925	172997648
6	172685797	173069759
Mean	172568616.833	172749927
SD	305926.335	519504.455
% RSD	0.18%	0.30%

AGP: Alogliptin benzoate, RP-HPLC: Reversed phase-high performance liquid chromatography, RSD: Relative standard deviation, SD: Standard deviation

Precision

The precision of the method was determined by repeatability (intraday) and intermediate precision (interday) and was expressed as RSD (%)

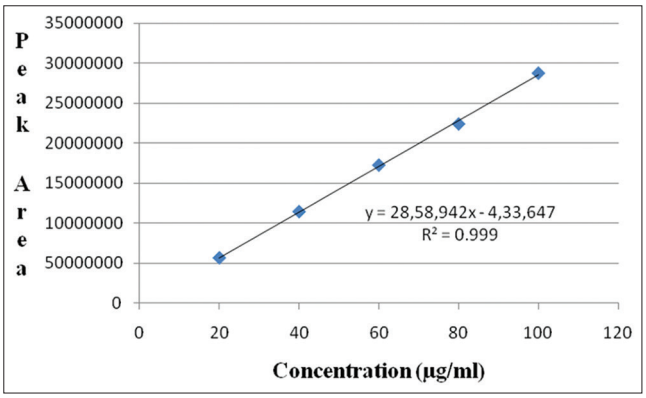

Fig. 6: Standard calibration curve of alogliptin benzoate – high performance liquid chromatography method

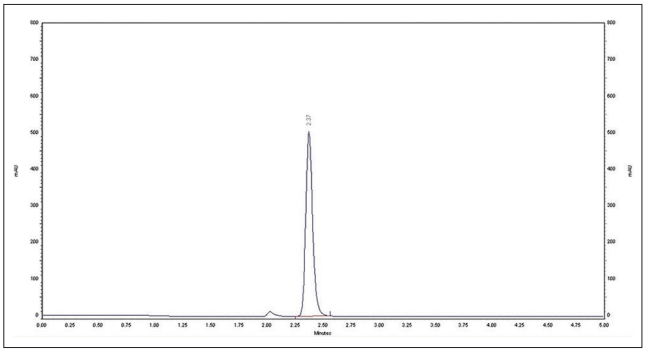

Fig. 7: Representative chromatogram of alogliptin benzoate standard

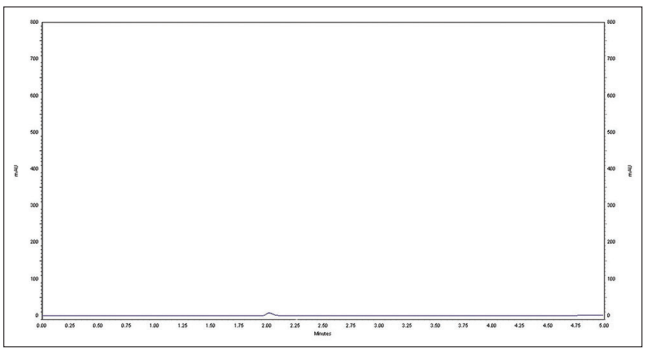

Fig. 8: Representative chromatogram of blank

Table 5: Result for recovery studies - HPLC method

Sample Id no	% spike level	Amount added (µg/ml)		Amount found (µg/ml)	% recovery	Statistical parameters
		Standard drug	Sample			
1	50	20.0	40.0	59.70	99.50	Mean=99.71
2		20.0	40.0	59.99	99.98	SD=0.244
3		20.0	40.0	59.80	99.66	% RSD=0.25%
4	100	40.0	40.0	80.02	100.02	Mean=100.01
5		40.0	40.0	79.98	99.97	SD=0.040
6		40.0	40.0	80.04	100.05	% RSD=0.04%
7	150	60.0	40.0	99.85	99.85	Mean=99.92
8		60.0	40.0	99.88	99.88	SD=0.102
9		60.0	40.0	100.04	100.04	% RSD=0.10%

HPLC: High performance liquid chromatography, RSD: Relative standard deviation, SD: Standard deviation

of a series of measurement. The experimental values obtained for the determination of AGP in samples are present in Table 9. The result obtained shows RSD of 0.42% indicating good intraday precision. Interday variability was calculated from assays on consecutive days and shows RSD of 0.93%. From the result obtained, the proposed UV method was found to be precise.

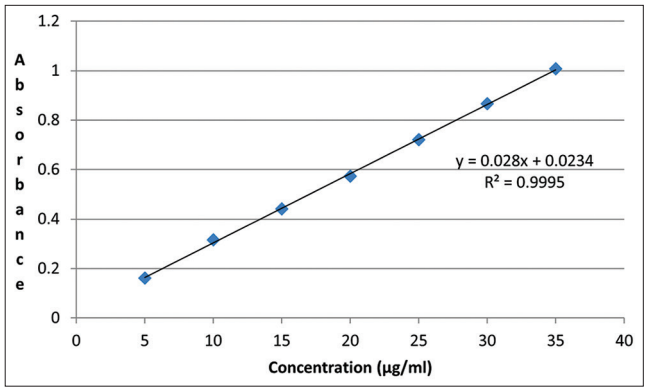

Fig. 9: Standard calibration curve of alogliptin benzoate - UV method

Table 6: Robustness studies of AGP - HPLC method

Parameters	Retention time	% RSD	USP plate count	USP tailing
Flow rate				
0.6	3.15	0.2	9488	1.01
0.8	2.37	0.1	7568	1.00
1.0	1.89	1.1	5986	0.98
Mobile phase				
85:15	2.34	1.2	7560	1.04
80:20	2.37	1.1	7458	1.00
75:25	2.74	1.0	7334	1.02
pH				
4.8	2.39	0.3	7150	1.01
5.0	2.37	0.2	7568	0.99
5.2	2.35	0.4	7382	1.03

HPLC: High performance liquid chromatography, RSD: Relative standard deviation, SD: Standard deviation, AGP: Alogliptin benzoate

Table 7: Results of regression analysis data for the quantitation of AGP by UV method

Parameters	Results
Concentration range (µg/ml)	5-35
Correlation coefficient (r^2)	0.999
Slope (m)	0.028
Intercept (c)	0.023
LOD (µg/ml)	0.872
LOQ (µg/ml)	2.644

AGP: Alogliptin benzoate, LOD: Limit of detection, LOQ: Limit of quantitation

Accuracy

Standard solution of the accuracy of 80%, 100%, and 120% solutions, i.e., 12, 15, and 18 µg/ml were analyzed. Table 10 represents the high percent recovery values indicating that the proposed method is accurate and reproducible.

Robustness

The method remained unaffected by the change in wavelength (± 1 nm). Below tabulated values indicate that the method is robust. The data were presented in Table 11.

LOD and LOQ

The LOD and LOQ of AGP benzoate were found to be 0.872 and 2.644 µg ml, respectively.

CONCLUSION

The present HPLC and UV methods were simple, accurate, efficient, and sensitive and were developed for the determination of AGP and moreover, the developed RP-HPLC method is LC-MS compatible which can be efficiently used for further LC-MS analysis. The UV spectrophotometric method is very simple, rapid, and economical and allows the determination of AGP with sufficient reliability. The methods

Table 8: Linearity data of AGP - UV method

S. No	Concentration (µg/ml)	Absorbance
1	5	0.161
2	10	0.315
3	15	0.442
4	20	0.574
5	25	0.720
6	30	0.867
7	35	1.008

AGP: Alogliptin benzoate

Table 9: Result of precision study - UV method

S. No (n=6)	Absorbance	
	Intraday	Interday
1	0.442	0.441
2	0.440	0.443
3	0.441	0.441
4	0.439	0.452
5	0.443	0.444
6	0.444	0.446
Mean	0.4415	0.4445
SD	0.0018	0.0041
% RSD	0.42	0.93

HPLC: High performance liquid chromatography, RSD: Relative standard deviation, SD: Standard deviation

Table 10: Result for recovery studies - UV method

Sample Id No	Concentration level (%)	Amount added (µg/ml)		Amount found (µg/ml)	% recovery	Statistical parameters
		Standard drug	Sample			
1	80	12.0	15.0	26.90	99.62	Mean=99.783
2		12.0	15.0	26.95	99.81	SD=0.151
3		12.0	15.0	26.98	99.92	% RSD=0.15%
4	100	15.0	15.0	29.99	99.96	Mean=99.95
5		15.0	15.0	29.96	99.86	SD=0.085
6		15.0	15.0	30.01	100.03	% RSD=0.09%
7	120	18.0	15.0	32.95	99.84	Mean=99.76
8		18.0	15.0	32.89	99.66	SD=0.091
9		18.0	15.0	32.93	99.78	% RSD=0.09%

RSD: Relative standard deviation, SD: Standard deviation

Table 11: Robustness studies of AGP by UV method

S. No	Wavelength (nm)		
	275	276	277
1	0.440	0.441	0.446
2	0.438	0.442	0.443
3	0.441	0.440	0.448
4	0.439	0.444	0.442
5	0.442	0.443	0.450
6	0.441	0.439	0.441
Mean	0.440	0.441	0.445
SD	0.0014	0.0018	0.0035
% RSD	0.33%	0.42%	0.80%

RSD: Relative standard deviation, SD: Standard deviation, AGP: Alogliptin benzoate

were validated as per ICH guidelines, and the results of the parameters were found to be within the limits. The methods were free from the interference of blank with the standard. Hence, it can be concluded that these methods may be employed for the drug analysis in routine quality control for the estimation of AGP.

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