

Original Article

VALIDATED HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF SITAGLIPTIN, METFORMINE AND ATORVASTATIN IN PURE FORM AND IN PHARMACEUTICAL FORMULATIONS

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ABSTRACT

**Objective:** The main objective of current study is to develop and validate a selective HPLC method for determination of the studied drugs either in single or in mixture form. The method is rapid, precise, accurate and specific for the separation and determination of sitagliptin phosphate, metformin hydrochloride, and atorvastatin calcium in pure form and in pharmaceutical formulations in presence of quetiapine as an internal standard.

**Methods:** This method is based on HPLC separation of the three drugs on HyperSil GOL column (150x4.6mm, 5 $\mu$ ). The mobile phase-A was prepared by mixing buffer (containing 1% of concentrated nitric acid 65% and 2% of concentrated ammonia solution 28-32%, pH  $\approx$ 8.5) and methanol in the ratio 30:70. The flow rate is 1 ml/min, with isocratic elution and UV detection at 254nm. The retention time of each of sitagliptin phosphate, metformin hydrochloride, atorvastatin, quetiapine internal standard was found to be at 3.384, 2.640, 4.837 or 6.000 min. respectively.

**Results:** the proposed method was successfully applied for the quantitative determination of each of sitagliptin, metformin and atorvastatin as single component or as combined mixture. The linear regression analysis data for calibration plots showed a good linear relationship over a concentration range of 3.125-100  $\mu$ g/ml for sitagliptin, 0.625-25  $\mu$ g/ml for metformin, and 0.3125-10  $\mu$ g/ml for atorvastatin. The mean values of the correlation coefficient, slope and intercept were 0.9976, 96.92 and +274.21 for sitagliptin, 0.9995, 218.82 and +14.97 for metformin, 0.9994, 828.87 and +81.95 for atorvastatin. The method was validated as per the ICH guidelines. The limit of detection (LOD) and limit of quantification (LOQ) was 0.82 and 2.46  $\mu$ g/ml for sitagliptin, 0.4 and 1.2  $\mu$ g/ml for metformin, and 0.09 and 0.27  $\mu$ g/ml for atorvastatin.

**Conclusion:** The developed and validated HPLC method and the statistical analysis showed that the method is repeatable and selective for the estimation of the three studied drugs in presence of quetiapine as an internal standard.

**Keywords:** Metformin, Sitagliptin, Atorvastatin and Chromatography.

INTRODUCTION

Sitagliptin phosphate monohydrate (3R)-3-amino-1-[3-(trifluoromethyl)-6,8-dihydro-5H-[1,2,4]triazolo[4,3-a]pyrazin-7-yl]-4-(2,4,5-trifluorophenyl)butan-1-one } (fig.1) [1] is an oral hypoglycemic drug of the dipeptidyl peptidase-4 (DPP-4) inhibitor classes, which represent a new therapeutic approach in treatment of type 2 diabetes. (DPP-4) enzyme breaks down the incretin hormones including glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). GLP-1 and GIP are gastrointestinal hormones released in response to meals. By preventing GLP-1 and GIP inactivation, they are able to increase the secretion of insulin and suppress the release of glucagon by pancreas this drive blood glucose level toward normal [2].

As a number of individuals affected by diabetes are continuing to increase worldwide, the need for effective management assumes ever greater urgency. Newer classes of medications, particularly those works via the incretin pathway, achieve glucose lowering and minimize risks associated with traditional therapies. Ideally, combination therapies should be well tolerated, convenient to take, have few contraindications, have a low risk of hypoglycemia and weight gain, and reasonably effective over both the short and long term such as the combination of sitagliptin and metformin [3].

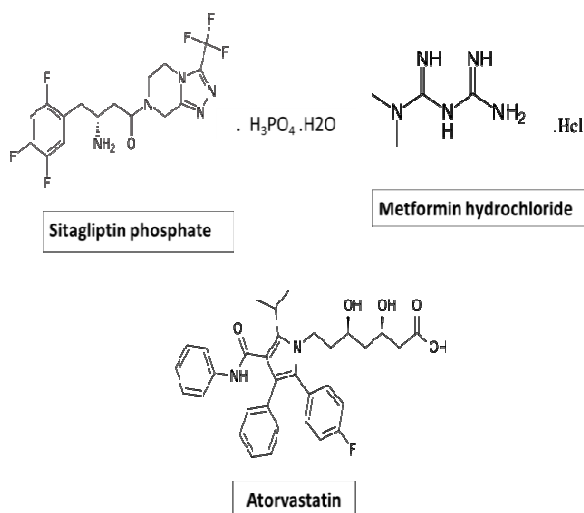
Metformin (1-carbamimidamido-N, N-dimethylmethanimidamide) (fig.1) [1] is an anti-diabetic drug from the biguanide class of oral hypoglycemic agents. It exerts its glucose lowering effect primarily through increased hepatic insulin sensitivity and the resultant suppression of hepatic glucose output. Metformin may also modestly enhance glucose uptake in peripheral tissues and increase glucose metabolism in the splanchnic bed [4].

Current American Diabetes Association guidelines suggest that all adult with diabetes should be managed to achieve low density lipoprotein (LDL) cholesterol less than 100 mg/dl employing statins as first-line therapy [5].

Atorvastatin ([R-(R\*, R\*)]-2-(4-fluorophenyl)- $\beta$ ,  $\delta$ -dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino)carbonyl]-1H-pyrrole-1-heptanoic acid) (fig.1) [1] is a lipid-lowering agent that specially, competitively, and reversibly inhibits 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, which catalyzes the conversion of HMG-CoA to mevalonic acid, the rate limiting step in cholesterol biosynthesis [6,7]. From the previous information it is reasonable to use sitagliptin, metformin and atorvastatin combination in the management of type-2 diabetes mellitus. Literature survey reveals several methods for the determination of sitagliptin in pharmaceutical preparation or biological fluids including spectrophotometry [8-10], HPLC [11, 12], UPLC [13], liquid chromatography-tandem mass spectroscopy [14-16], gas chromatography [17], and capillary electrophoresis [18].

Several methods have also been described for the determination of metformin either alone or in combination with various drugs, such as spectrophotometry [19-21], LC [22-24], LC-MS-MS [25-27] and capillary electrophoresis [28-30]. As per the literature, several methods have been reported for the determination of atorvastatin alone or with some other drugs [31-42].

The main objective of this work is to develop a validated, selective and inexpensive HPLC method for separation and quantitative determination of sitagliptin, metformin, and atorvastatin, which was not done before. Using an internal standard in the method makes it suitable for plasma or urine application.



**Fig. 1: structures of metformin hydrochloride, Sitagliptin phosphate and Atorvastatin calcium.**

## MATERIALS AND METHODS

### Reagents and Chemicals

All the used solvents were of HPLC grade, concentrated nitric acid 65% and concentrated ammonium hydroxide solution 28-30% supplied from Sigma-Aldrich (St. Louis, Missouri, USA). Chromatographic-grade water was produced by a Milli-Q system (Millipore, Billerica, MA). The reference standards of metformin hydrochloride, sitagliptin phosphate and atorvastatin calcium were obtained as gift samples from Saudi Food & Drug Authority. Sitagliptin phosphate monohydrate (equivalent to 100mg sitagliptin) in Januvia tablets manufactured by Merck Sharp and Dohme Ltd. Atorvastatin calcium (equivalent to 20mg atorvastatin) in lorvast tablets manufactured by Tabuk pharmaceutical Mfg. Co., Tabuk- Saudi Arabia. Metformin hydrochloride, (equivalent to 500mg metformine) in Dialon tablets manufactured by Julphar, Gulf pharmaceutical industries.

### Preparation of buffer

The buffer solution consist of water containing 1% of concentrated nitric acid 65%, 2% concentrated ammonium hydroxide solution 28-30% and adjust pH to 8.

### Preparation of mobile phase

The mobile phase was prepared as a mixture of buffer: methanol in the ratio 30:70 v/v. The mobile phase was filtered on 0.45µm nylon filter.

### Preparation of standard stock solutions

Standard stock methanolic solutions of the three studied drugs and the internal standard were prepared in concentrations of, (1mg/ml) of each of sitagliptin, metformin or quetiapine and (100 µg/ml) of atorvastatin. Solution were prepared by transferring an accurately weighed 100 mg of each of sitagliptin, metformin or quetiapine, and 10 mg of atorvastatin into separate 100 ml volumetric flasks containing 40 ml of methanol, sonication for 5 min.; and cooled to room temperature. The volume was made up to the mark by methanol. The stock solutions were stored at 2-8°C.

### Preparation of sample solution

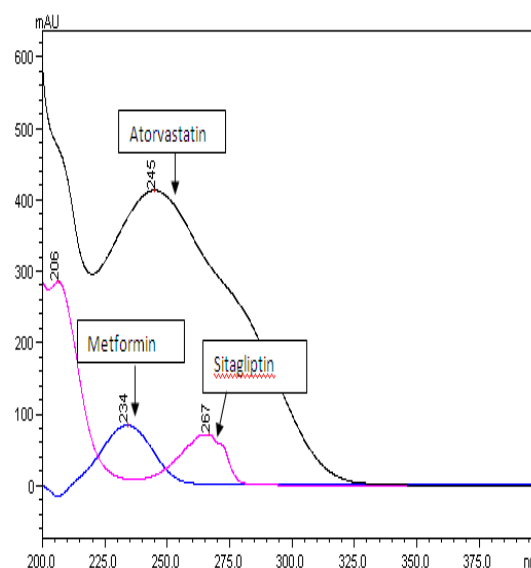
Ten tablets of each of studied drug were accurately weighed, transferred to a clean, dry mortar and ground to fine powder. A powder equivalent to 100mg sitagliptin, 100mg metformin and 20mg atorvastatin were transferred into a separate 100 ml volumetric flask, 40 ml methanol was added, sonication for 10 min; and diluted to the volume with methanol. This solution was filtered through 0.45µm pore size nylon filter membrane.

## Instrument and Chromatographic conditions

HPLC system consists of Agilent HP 1100 system equipped with an auto sampler (Waldbronn Germany), quaternary pump, auto injector, column compartment and photodiode array (PDA) detector. The used analytical column was HyperSil GOLD (150mmx4.6mm-4µm). Mobile phase was (Buffer: Methanol in ratio 30:70 v/v). The mobile phase was filtered through 0.45µm pore size nylon filter membrane. The method applied isocratic elution with flow rate 1 ml/min, and injection volume 10µl. The column was at room temperature. The method used UV- detection at 254 nm and the run time was 7 min.

## Optimization of HPLC method

All drugs were subjected to chromatographic analysis using mobile phases of different pH values, flow rates and different stationary phases. The changes in the retention time, sensitivity and selectivity of all drugs were noted as a function of changing mobile phase, pH and stationary phase. Initially methanol: water in different ratios was tried but incomplete separation of peaks was observed, then acetonitrile: water and acetonitrile: buffer in different ratio and different pH values was tried but splitting of peaks and low sensitivity were observed. Later methanol with buffers in different pH values were tried, the best results were given on using pH 8, then different ratios of buffer: methanol were tried until the best separation, resolution, peak symmetry and selectivity were obtained at ratio 30:70 v/v of mobile phase with flow rate 1ml/min on HyperSil GOLD (150x4.6mm-4µm). Fig.2 shows the overlaid spectra of the three studied drugs, several wavelengths for UV-detection were tried (Fig.3) and best sensitivity was obtained at 254nm as shown in (Fig. 4).



**Fig. 2: Overlay of UV spectra of metformin, sitagliptin and atorvastatin by PDA detector of HPLC.**

## RESULTS AND DISCUSSIONS

### Optimization of the chromatographic conditions

The objective of this chromatographic method development was reducing analysis time, maintaining good efficacy, retention time below 7 minutes, along with resolution between the studied drugs and the used internal standard. The chromatographic separation was achieved using HyperSil GOL column (150x4.6mm, 5µ), mobile phase methanol: buffer pH ≈ 8 in a ratio 30:70. The flow rate was 1 ml/min, with isocratic elution and UV detection at 254nm.

### Validation of the method

Validation of the optimized HPLC method was carried out with respect to the following parameters.

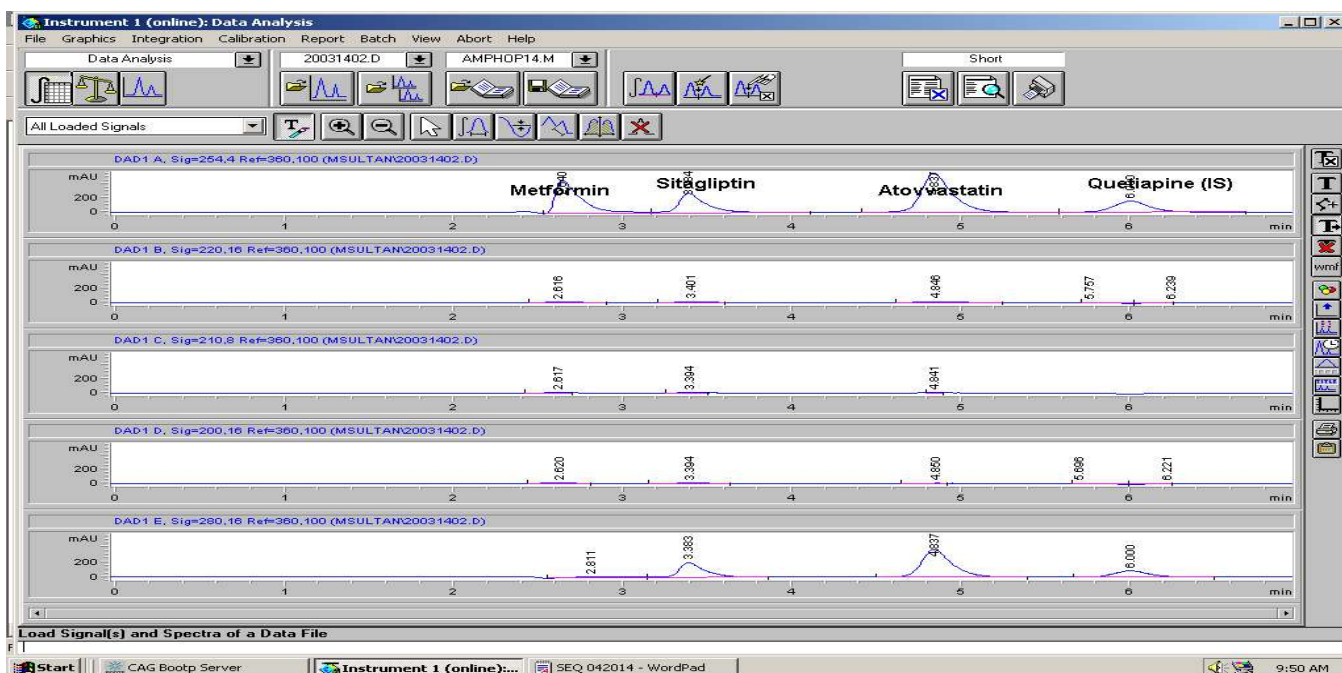


Fig. 3: Optimization of the best wavelength for UV detection of HPLC.

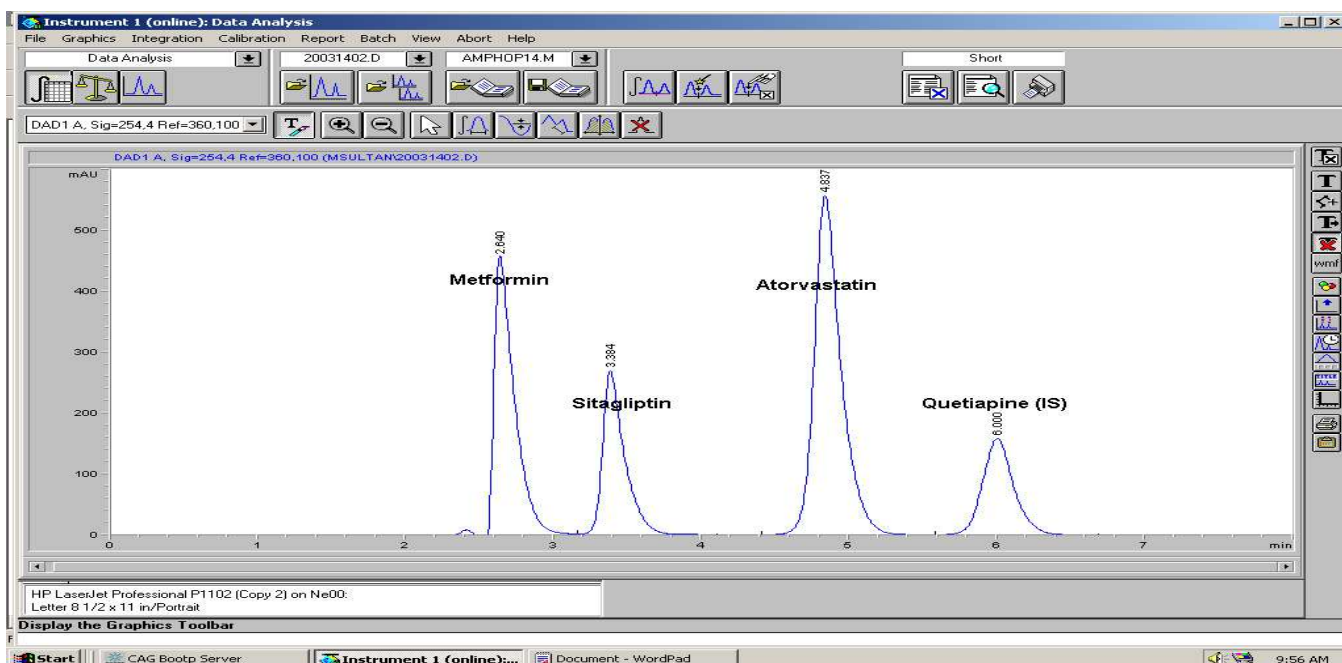


Fig. 4: HPLC chromatograms of Metformin, Sitagliptin, Atorvastatin and internal standard Quetiapine.

#### Accuracy

Accuracy of the method was verified by studying recovery at three different concentrations of sitagliptin, metformin, and atorvastatin by replicate analysis ( $n = 3$ ). The results obtained from the determination of accuracy, expressed as percentage recovery, were recorded in (Table 2)

#### Linearity and rang

Linearity of the method was studied by injecting six concentrations of methanolic solution of the studied drugs in concentration range of 3.125-100  $\mu\text{g/ml}$  for sitagliptin phosphate, 0.625-25  $\mu\text{g/ml}$  for metformin hydrochloride, and 0.3125-10  $\mu\text{g/ml}$  for atorvastatin

triplicate into the HPLC system keeping the injection volume constant. The peak areas were plotted against the corresponding concentrations to obtain the calibration curves Fig. 4, 5, 6. The average correlation coefficient ( $R^2$ ) was observed to be  $\geq 0.997$  during the course of validation.

#### Precision

The precision of the method was verified by repeatability and intermediate precision studies. Repeatability was performed by analysis of three different concentrations six times on the same day. The intermediate precision of the method was checked by analysis of three different concentrations on three different days [43]. Results were recorded in (Table 1).

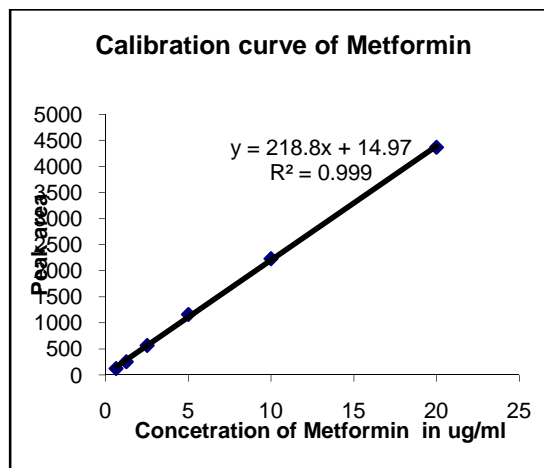


Fig. 4: Calibration curve of metformin

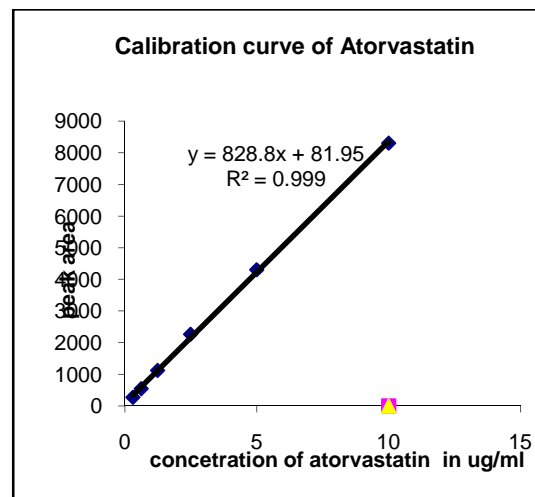


Fig. 5: calibration curve of atorvastatin

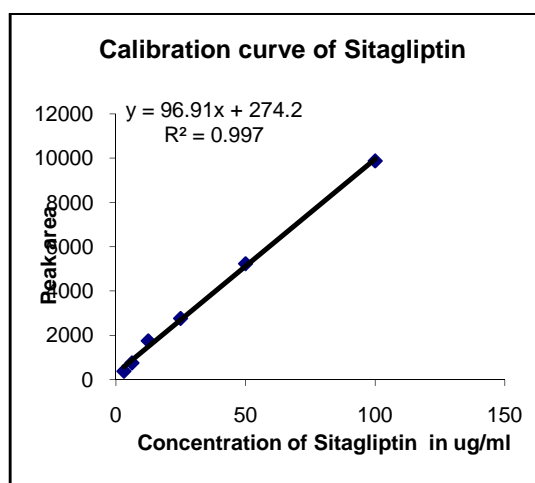


Fig. 5: calibration curve of sitagliptin phosphate monohydrate

**Limit of detection (LOD) and limit of quantification (LOQ)**

The LOD and LOQ were separately determined on the basis of standard calibration curve. The residual standard deviation of the regression line or the standard deviation of y-intercepts of regression lines was used to calculate LOD and LOQ. Sensitivity of the proposed method was estimated in terms of Limit of Detection (LOD) and Limit of Quantitation (LOQ).  $LOD = 3.3 SD/S$  and  $LOQ = 10 S.D/S$ , where S.D is the residual standard deviation of regression line and S is the slope of the line. The results of LOD and LOQ obtained for studied drugs were recorded in (Table 3).

**Robustness of the method**

The robustness of the proposed HPLC method was assessed by the ability to remain unaffected by small changes in experimental conditions. Change in flow rate by  $\pm 0.2\%$  and small changes in mobile phase organic strength and in pH by  $\pm 2\%$  has no significant effect on chromatographic resolution.

Table 1: Results of precision study.

Drugs	Concentration (µg/ml)	Repeatability (n = 6)		Intermediate Precision (n = 6)	
		R.S.D.%	R.S.D.%	R.S.D.%	R.S.D.%
Metformin hydrochloride	5	1.413	1.696		
	10	0.567	1.249		
	20	0.456	1.025		
Sitagliptin phosphate	25	0.298	1.790		
	50	0.289	1.156		
	75	1.008	1.011		
Atorvastatin	2.5	0.490	1.465		
	5	1.435	1.520		
	10	0.379	1.285		

Table 2: Results of accuracy study

Metformin (5µg/ml)			Sitagliptin(25 µg/ml)			Atorvastatin(2.5µg/ml)		
%Recovery	Statistical Analysis		%Recovery	Statistical Analysis		%Recovery	Statistical Analysis	
98.00	Mean	99.33	97.80	Mean	97.60	99.98	Mean	99.80
101.00	SD	1.247	99.00	SD	1.2328	99.65	SD	0.1352
99.00	%RSD	1.255	96.00	%RSD	1.2622	99.79	%RSD	0.1354
Metformin (10µg/ml)			Sitagliptin (50µg/ml)			Atorvastatin(5 µg/ml)		
96.00	Mean	97.88	99.00	Mean	99.08	99.00	Mean	99.06
99.00	SD	1.339	99.76	SD	0.5180	98.67	SD	0.3412
98.65	%RSD	1.386	98.50	%RSD	0.5227	99.50	%RSD	0.3444
Metformin (20 µg/ml)			Sitagliptin (75µg/ml)			Atorvastatin(10µg/ml)		
96.00	Mean	97.33	98.00	Mean	98.08	97.90	Mean	97.30
97.00	SD	1.247	97.75	SD	0.3118	96.00	SD	0.9202
99.00	%RSD	1.281	98.50	%RSD	0.3180	98.00	%RSD	0.9457

Table 3: System Suitability Parameters

Parameters	Metformin hydrochloride	Sitagliptin phosphate	Atorvastatin
Linearity	0.625-20 µg/ml	3.125-100 µg/ml	0.3125-10 µg/ml
Retention time	2.640 min.	3.384 min.	4.837 min.
Resolution	4.40 7.63		
More than 1.5 Theoretical plates*			
More than 2000	4767	5446	9605
Peak width	0.1529	0.1834	0.1974
Symmetry	0.349	0.467	0.674
LOD	0.40	0.82	0.09
LOQ	1.20µg	2.47µg	0.27µg

\*Tangent Line Method

Table 4: Results for application of developed HPLC method on pharmaceutical formulations

Dosage form of drug	Labeled amount in mg/tab.	Recovered of amount in mg Mean ± SD	%CV	% of Assay
Dialon tablet 500mg/tab. Of Metformin hydrochloride	100	99.67 ± 0.624	0.626	99.67
Januvia tablets 100mg/tab. Of Sitagliptinphosphat	20	19.95 ± 0.1225	0.613	99.75
Lorvast tablet 20mg/tab. Of Atorvastatin	500	501.667 ± 6.944	1.390	100.33

### Specificity

The specificity of the method was assessed from the chromatogram where complete separation of sitagliptin, metformin, atorvastatin, and internal standard were achieved. The peaks obtained were sharp, well separated at the baseline as shown in (Fig.4) and the resolution for all peaks more than 1.5 as recorded in (Table 3).

### Application

Analysis of each of the pharmaceutical formulation was done by injecting different concentrations of sample solution into HPLC in triplicates under the mentioned optimum conditions. Concentration of each drug in tablet was calculated using the calibration curve, and the results were recorded in (Table 4). Validity of the method was assessed applying the standard addition technique.

### CONCLUSION

RP-HPLC method of 7 min runtime was successfully developed for the simultaneous determination of three drugs with good resolution (more than 2) between all drugs. The method shows high degree of accuracy and precision with less than 2% RSD. The method is simple, accurate, rapid, and precise and can easily be used for routine analysis of the three drugs in pure and formulated forms (tablets, capsules and powder). The method can also be applied in plasma and urine samples.

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