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Article

Simultaneous determination of anti-diabetic drugs

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A novel reverse phase, isocratic HPLC method is described to separate five anti-diabetic drugs *i.e.,* glimepiride, metformin, sitagliptin, rosiglitazone and pioglitazone. Nucleosil C_{18} analytical column was used as stationary phase, while mobile phase consisted of acetonitrile:phosphate buffer: methanol (40/40/20, v/v) pH 2.0. Effluent was monitored at a flow rate 1 mL/min and detected at wavelength of 240 nm. This research produced excellent chromatography over a wide concentration range of 25-10000 ng/mL. Sepprated and well resolved quantifiable peaks were obtained and test results were linear in this range. Correlation coefficient of more than 0.9990 was witnessed as well as Low %RSD values *i.e.,* maximum 2.0% documented excellent precision of the method. Good recoveries from pharmaecutical (99-101%), urine and plasma samples (>96%) in a range of concentrtion granted very good linearity, accuracy and precision. The projected method has satisfactory applications in quality control of these molecules as well as quantification of these molecules in urine and plasma samples.

Keywords: Metformin/pharmacokinetics. High Pressure Liquid Chromatography, Urine/Plasma methods. Determination/prevention. Diabetes Mellitus/drug therapy. Drug Liberation/drug effects.

INTRODUCTION

Diabetes is a complicated disorder of endocrine system, wherein body do not produce insulin also known as (diabetes type1) or become resistance to the effect of insulin also known as (diabetes type II). As a result body blood sugar level remains consistently above normal. If it remains untreated, major complication may arise which affect various organs including eyes, kidneys, brain and heart (Kim, Newton, Knopp, 2002). A great deal of scientific activities is focused on the treatment of diabetes these days. Research in this area has produced new classes of medicines which are effective in countdown diabetic with less associated risks and harmful repercussions. These includes the biguanides such as metformin (MF), sulfonylureas such as glimepiride (GP), thiazolidinediones also known as PPARγ agonist such as pioglitazone (PGH), and rosiglitazone (RGM), meglitinides (repaglinide) and dipeptidyl peptidase IV (DPP-IV) inhibitor such as sitagliptin (SGP) etc. Although, type 1 and pre-diabetics can be controlled through monotherapy, however, its failure is non deniable fact in case of type II diabetes. To curb this menace generally combination therapy has been in practice (Valsamakis, Kumar, 2000). Co-administration of MF, GP, SGP, insulin and PPARγ agonist in whole or in part have resulted clinically meaningful glycemic improvements and is an ideal combination, considering contraindications, risk of hypoglycaemia, obesity and short and long term effectiveness (Fonseca *et al.*, 2000; Goodarzi, Bryer-Ash, 2005). Structures of MF,GP, SGP, PGH and RGM are shown in Figure 1.

MF is chemically defined as *N*,*N*-dimethyl-imidodicarbonimidic diamide hydrochloride. It is an important hypoglycemic agent, designed as a first line therapy for type II diabetese. GP is chemically defined as 1-{(*p*-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamide)ethyl]phenyl)sulfonyl}-3-(*trans*-4-methylcyclohexyl)urea. It stimulates pancreatic β-cells to secret insulin and is a useful combination for type II diabetic mellitus. SGP is named chemically as 7-[(3*R*)-3-Amino-1-oxo-4-(2,4,5-trifluorophenyl)butyl]-5,6,7,8-tetrahydo-3-(trifluoromethyl)-1,2,4-triazolo[4,3-a] pyrazine phosphate. It is active, well-tolerated, and selective inhibitor of DPP-4, which enhances GLP-1 concentration and provides glycemic improvement. PGH

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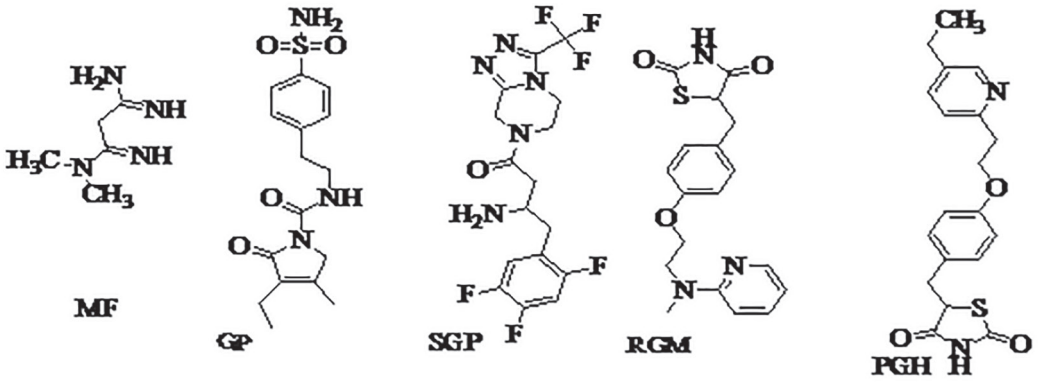

FIGURE 1 - Structure of the studied drugs.

is a thiazolidinediones derivative, chemically defined as 5-(4-[2-(5-ethylpyridin-2-yl)ethoxy]benzyl) thiazolidine-2,4-dione), widely recommended in type II diabetes. RGM is another thiazolidinedione, chemically defined as (±)-5-[[4-{2-methyl-2-(pyridinylamino) ethoxy} phenyl]methyl]methyl]-2,4-thiazolidinedione (*Z*)-2-butenedioate (1:1)]. Both are established PPARs-γ agonist and exert their glucose lowering effect by making the cells more responsive to insulin (Katzung, Masters, Trevor, 2004).

Drug industries and clinical laboratories are significantly interested in developing simultaneous analytical method. Anti-diabetic drugs are generally working together in form of bi or tri-therapy to combat the disease. Therefore, it is important to develop a validated analytical method which can quantitate these drugs individually as well as simultaneously. Literature survey revealed a number of reports for analysis of these molecules in pharmaceutical as well as biological samples. A protocol (Kolte *et al.,* 2004) was developed for analysis of MF and PGH in pharmaceutical dosage form. Another report describes simultaneous quantification of MF, PGH and GP, in pharmaceutical samples (Jain *et al.*, 2008). Liquid chromatographic method for plasma level determination of anti-diabetic drugs (Aburuz, Millership, McElnay, 2005; Venkatesh *et al.*, 2006) as well as anti-diabetics in presence of some ACE inhibitors (Siddiqui *et al*, 2017) are well reported. GP and related impurities have been analyzed through liquid chromatography (Khan *et al.*, 2005). Arayne *et al.* (2010, 2013) established a simaltaneous method for analysis of metformin in presence fo H-2 receptor antagonist as well as in presence of captopril and lisinopril. Sultana *et al.* (2011) developed HPLC-UV method for analysis of diltiazem in association with anti-diabetics drugs, capable in both pharmaceuticals and human serum. LC-MS/MS method has been comprehended by Ding *et al.* (2007), Ho *et al.* (2004), Lin *et al.* (2003), Mistri, Jangid, Shrivastav (2007) and Wang, Miksa (2007) to analyze anti-diabetic drugs. However, despite of all these reports, literature is short on the simultaneous quantification of MF, GP, PGH, RG, and SGP. The objective of this endeavor was to produce a rapid, reliable and accurate RP-HPLC method for the quantification of the aforesaid five antidiabetic drugs, simultaneously in pharmaceutical preparations and in physiological samples, covering clinical level concentration. Substantial method validation study was performed and the proposed method holds well for quantification of these drugs in studied samples (ICH, 2005).

EXPERIMENTAL

Material and method

MF (99.4%), GP (98.3%), PGH (99.2%), RGM (99.3%) and SG (99.5%) were gifted by local pharmaceutical company. Commercial product containing these drugs were procured from local market. HPLC grade chemicals and solvents were used for mobile phase preparation. Phosphoric acid, diethyl amine and triethyl amine were from Merck (Germany), methanol, acetonitrile were from Fisher scientific (UK). Dosage formulations of MF, GP, SGP, PGH and RGM were procured from the local market. Blank blood was procured from national institute of cardiovascular diseases Karachi.

HPLC system of Schimadzu Corporation consisting of SIL 20A auto injector, LC-20 AT pump, SPD 20A prominence UV/VIS detector controlled by LC solution software was used in this study. Other equipments include Sartorius analytical balance (TE2145), UV-visible Shimadzu spectrophotometer (1650 PC) with UV Probe software, and pH meter (Jenway 3240).

Chromatographic resolutions of the peaks were achieved with a Nucleosil® C_{18} HPLC column (4.6 x 250 mm; 5 µm). Mobile phase consist of acetonitrile:

phosphate buffer: methanol (40/40/20) pH 2.0, drawn at a flow rate of 1 mL/min, with a detecting wavelength of 240 nm, at ambient temperature.

Preparation of standard solutions

Standard primary stock solution of 100 µg/mL were arranged individually in amber volumetric flask, for each analyte in diluent (mobile phase). Stock solutions were further diluted and mixed together appropriately to obtain a composite solution of required concentrations for all the analytes. A volume of 20-µL was injected in to the chromatographic system and was analyzed under the given conditions.

Preparation of pharmaceutical sample

Stock sample solution of 100 µg/mL of each drug were prepared by taking a finally grounded homogenous bulk sample of each drug product into an amber glass volumetric flask, 30 mL diluent was added, followed by sonication and shaking for 15 minutes each. After volume correction, further dilutions were made to get the required concentration for each drug.

Preparation of in-vitro urine and plasma sample

Urine was collected from healthy volentears. Plasma was obtained by mixing blood with heparin and centrifugation at 15,000 rpm for few minutes and was stocked at -20 °C for pending analysis. In a 5 mL volumetric flask 1 mL of urine or plasma sample was added individually, followed by the addition of 1 ml of stock composite solution and 2 mL of diluent. After sonication and stirring for few minutes the sample was diluted up to the mark level. Further dilutions were made according to the requirements and before HPLC analysis samples were passed through 0.45µm filter (Karlsen, Blomhoff, Gundersen, 2005; Wang, Miksa, 2007; Siddiqui *et al.*, 2013; Siddiqui *et al.*, 2014; Siddiqui *et al.*, 2017).

Method validation

Method validation studies were performed as guided by international conference on hormonization. It includes linearity and range, specifity, accuracy, precision as well as robustness (ICH, 2005). Before method validation, justification of system suitability was established by injecting five replicates of the same composite standard solution. Degree of reproducibility, tailing factor and column efficiency were calculated.

Specificity and robustness

Specifity is the determination of an analyte unequivocally in presence of coaddetives or other ingredients that are supposed to be present in a sample. Plasma, urine as well as pharmaceutical excipients were fortified with these drugs and invistigated through the proposed method. Similary method was tested for small but deliberate variation expected during day today analysis. Solution stability was also checked by placing composite standard solution in refregerator for 48 hours (Sher *et al.*, 2014).

Linearity and range

Linearity is the verification through a mathamtical procedure that a given method produced results proportionate to concentration of the analyte. A range of concentraton 25-10000 ng mL^{-1} of standard composite solutions were prepared by diluting stock standard composite solution. Linearity was determined by plotting peak area vs concentrtion which produced least-square regression calibration curves (Sher *et al.*, 2015).

Quantification and detection limit

Limits of detection (LOD) and quantitation (LOQ) were evaluated by applying empirical formula i.e, 3.3(σ/s) for LOD and 10(σ/s) for LOQ, where σ is the standard deviation of the response and S is the slope of the calibration curve.

Accuracy and precision

The closeness of resutls to the individual reading when a bulk homogenous sample is subjected to multiple analysis is known as precision while, the closeness of experimental results to the true value is called as method accuracy. Multiple analysis were conducted on urine, plasma and pharmaceuticals and percent relative standard deviation were determined to present precision. Percent recovery was used to describe accuracy of the method. Sample were studied in a range of concentration covering low, middle and high concentration (Sher *et al.*, 2016).

RESULTS

Acetonitrile: Phsphate buffer: methanol (40/40/20 v/v) pH 2.0, was found to be suitable mobile phase with established flow rate of 1 mL/min and detecting wavelength of 240 nm. Typical chromatogram are shown in Figures 2-6, which shows retention time for MF at 5.9,

SG at 9.1, RGM at 10.9, PGH at 12.6 and GP at 13.8. Before application, projected method was validated in line with ICH guidelines (ICH, 2005).

Stock composite standard solution of 100 µg/mL was serially diluted to get a range of concentration covering low and high dosage. Before method validation system suitability was ensured by calculating reproducibility, tailing factor and column efficiency as well as peak resolution. Reporduciblity of less than 0.5% was witnessed. Tailing factor ranged from 1.03-1.34, column efficiency ranged from 9480-26832, while resolution ranged from 4.4-9.4 as shown in Table I, which shows excellent chromatographic setup at the outset. After chromatography the calibration curve was produced by constructing plot of peak area vs. concentrations. Linearity

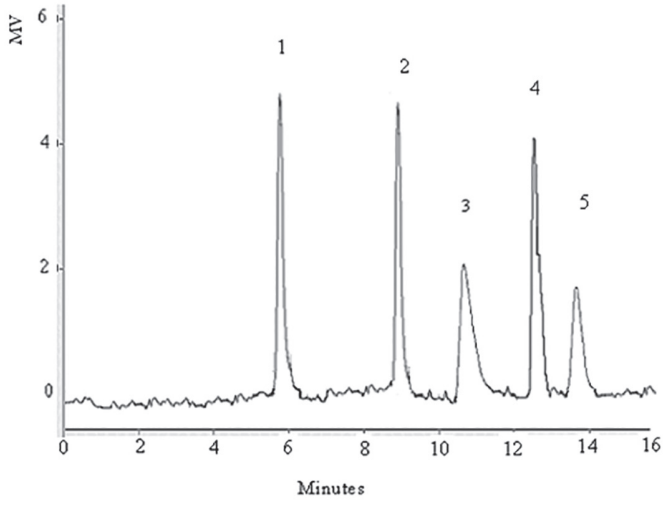

FIGURE 2 - Chromatogram in API .1=Metformin, 2=Sitagliptin, 3= Rosiglitazone, 4=Pioglitazone, 5=Glimepiride.

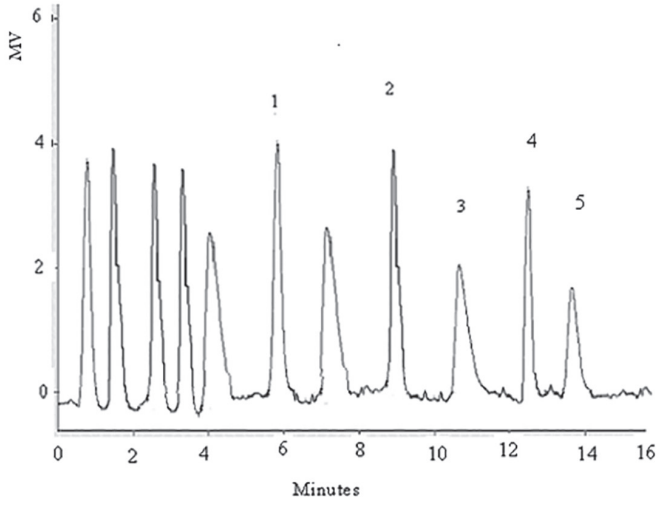

FIGURE 3 - Chromatogram in Urine samples.1=Metformin, 2=Sitagliptin, 3= Rosiglitazone, 4=Pioglitazone, 5=Glimepiride.

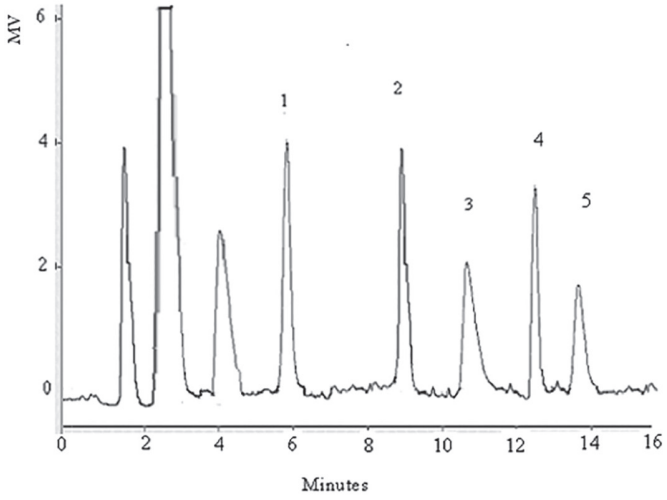

FIGURE 4 - Chromatogram in plasma samples.1=Metformin, 2=Sitagliptin, 3= Rosiglitazone, 4=Pioglitazone, 5=Glimepiride.

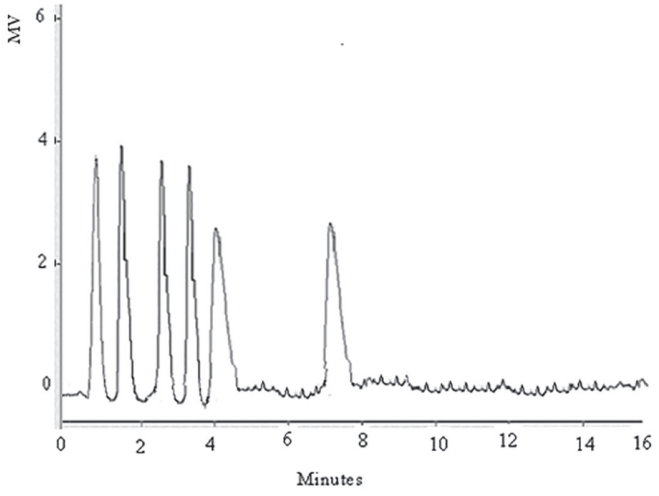

FIGURE 5 - Blank urine.

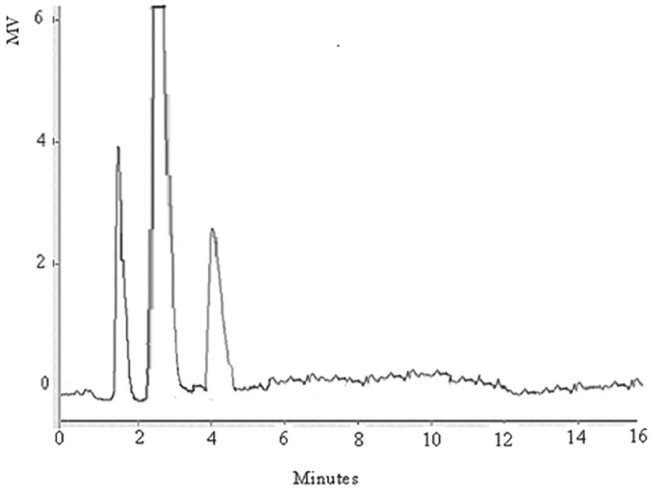

FIGURE 6 - Blank plasma.

correlation coefficient value as well as sensitivity test such as limit of detection and limit of quantification were calculated statistically. Limit of detection was defined as LOD = $3.3\sigma/S$, while quantification LOQ = $10\sigma/S$. The values were experimentally evaluated through chromatography while gradually decreasing analytes concentration. A signal-to-noise ratio of 3:1 was established for LOD, while 10:1 for LOQ, respectively.

TABLE 1 - System suitability

Sr. No.	Parameter	Result found (range)
1	%RSD of five replicates (NMT2%)	0.05- 0.52
2	Tailing factor (T) (NMT 2)	1.03-1.34
3	Theoretical plates (N) (NLT:4000)	9480-26832
4	Resolution (R)	4.4-9.4

Table II shows study range of 25-10000 ng/mL, correlation coefficient of more than 0.9990 as obtained with detection limit of 0.52-0.90 ng/mL and quantification limit of 1.6-4.5 ng/mL.These results indicates excellent linearity of the porposed method in the given range. As shown in Table III, plasma samples and urine samples recovery ranged from 96-101.8% with %RSD values from 1.5-2.4%. Pharmaceutical sample recovery ranged from 98-101% with %RSD values from 0.8-1.4%. More than 96% accuracy has been established and over all precision of less than 2% is shown in pharmaceutical while maximum 2.0% precision was exhibited in urine and plasma samples.

DISCUSSION

Plasma level RP-HPLC UV quantification of the five anti-diabetic drugs i.e., MF, PGH, RGM, GP and SGP, was the main objective of this study. These molecules are shown in Figure 1. MF and SGP were soluble in water, whereas PGH, RG and GP were soluble in organic solvent. Early experiments suggested phosphate buffer and acetonitrile combination a better option of mobile phase. However, this combination (60:40 v/v) resulted in late elution of SGP. Moreover, peak of RGM and PGH merged together. Acetonitrile addition negatively impacted the separation. However, by increasing aqueous part of mobile phase (40:60 v/v), PPARs were separated but it resulted in late elution of SGP with distracted peak symmetry, owing to strong interaction with stationary phase at high pH. Decreasing pH gradually resolved the two PPARs, however, owing to probably basic nature of SGP, its peak was broad due to strong interaction with stationary phase. Methanol was added to mobile phase to increase polarity and at last, acetonitrile: phosphate buffer: methanol (40/40/20 v/v) pH 2.0 was found to be a suitable mobile phase. Decreasing pH below 2.0 resulted in very early elution showing little retention, especially in case of MF and SGP owing to their hydrophilic basic nature.

Earlier simple mobile phases such as aqueous methanol and aqueous acetonitrile were tried, but unsatisfactory and poorly resolved peaks were obtained. Best separation was achieved with Nucleosil C_{18} analytical column, although, other analytical columns such as C_{18} and C_8 of different brands were also evaluated. Excellent chromatographic resolution as shown in Table I and Figure 2-6 is granted with sound sensitivity, when mobile phase was drawn at flow rate of 1 mL/min and detected with 240 nm wavelength. This resulted in a fast, accurate and reliable analysis of the five anti-diabetic drugs in simultaneous mood in pharmaceuticals and physiological fluids. The method was found to be free of interference from endogenous plasma and other excipients and sensitive enough to determine these drugs in concentration as low as 25 ng/mL as shown in Table II and III. Substantial method validation studies were conducted to establish validity of the method. Figure 2-6, shows selective and

TABLE II - Linearity and sensitivity of the method

Parameter	Inference				
	MF	GP	PGH	RGM	SG
Linearity range (ng /mL)	25-10000	25-10000	25-10000	25-10000	25-10000
Correlation coefficient	0.9998	0.9992	0.9995	0.9997	0.9993
Slope	81005.1	32025.5	66684.1	64327.6	28430.2
Intercept	-2531	-1113	-2098	-2101	-1007
(LOD) (ng /mL)	0.52	0.88	0.66	0.73	0.90
(LOQ) (ng /mL)	1.62	3.96	2.56	2.33	4.55

TABLE III - Applications of the method

Nominal conc. ($ng.mL^{-1}$)	% Recovery in Plasma				
	MF	**GP**	**PGH**	**RGM**	**SGP**
25	97.60	96.33	97.82	97.32	96.31
50	101.91	97.60	98.42	96.61	96.91
100	100.22	98.82	98.93	98.72	98.32
1000	99.81	96.91	96.41	100.81	99.81
5000	101.40	98.90	101.40	101.22	97.43
10000	99.71	101.41	101.81	99.82	101.81
Mean % recovery	100.12	98.22	99.12	99.07	98.42
%RSD	1.51	1.82	2.11	1.82	2.01
	% Recovery in Urine				
25	98.62	96.61	97.82	96.31	96.12
50	97.43	97.50	98.40	96.42	97.43
100	99.80	101.82	100.42	97.83	100.41
1000	101.41	96.83	99.81	100.81	99.82
5000	97.34	100.41	101.43	98.42	97.32
10000	100.74	101.52	98.42	99.80	100.72
Mean % recovery	99.73	99.12	99.32	98.21	98.61
%RSD	1.72	2.43	1.43	1.82	1.90
	% Recovery in API				
25	101.11	101.03	100.71	100.33	101.13
50	98.13	100.12	100.43	101.12	99.22
100	101.02	100.21	98.32	100.11	100.41
1000	98.33	101.01	100.10	99.60	99.41
5000	101.12	99.71	101.11	98.82	100.90
10000	99.11	98.42	99.62	99.32	101.12
Mean % recovery	99.97	100.11	100.08	99.69	100.13
%RSD	1.41	0.89	0.91	0.88	0.97

specific determination of the ingredients, while, Table II and III shows excellent linearity, accuracy and precision in a wide range.

CONCLUSION

The method facilitates simultaneous determination of MF, GP, SGP, PGH and RGM. The newly developed LC-method is cost-effective, rapid, and sensitive enough to assess plasma level qunatification of analytes under the study. Short chromatographic run time, less sample volume and low limit of quantification are highly appreciated from routine quality control perspective. The developed method is more than simple and robust, and statistical results suggest validity of the method. Reliability, sensitivity, rapidness, good recovery and precision as well as wide applications of the projected method present superiority over the other reported HPLC methods.

CONFLICT OF INTEREST

All the authors declare that they do not have any conflict of interests regarding the publication of this paper.

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