STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF PIOGLITAZONE, AMLODIPINE, ATORVASTATIN AND CLOPIDOGREL

Monika[a], Pandey S.[b] and Chawla P.[a,c*]

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ABSTRACT

A new simple, rapid, isocratic and stability indicating reverse phase high performance liquid chromatography (HPLC) method has been developed for simultaneous estimation of pioglitazone (P), amlodipine (AM), atorvastatin (AT) and clopidogrel (CP). Isocratic separation was achieved on Millennium M Sil C18 (125 mm ´4.6 mm) column, with mobile phase comprising of methanol: 0.02 M KH_2PO_4 (55:45 V/V), pH adjusted 4.0 with 0.01% ortho phosphoric acid. The flow rate was maintained 1mL/min and analytes were screened with U.V. detector. The method was validated according to ICH guidelines with respect to linearity, accuracy, precision and specificity. The drugs were exposed to various stress conditions like acidic and basic hydrolysis, oxidation, exposure to UV light and temperature (dry heat) and the stressed sample were analysed by the proposed method. No co-eluting, interfering peaks from excipients, impurities were observed during stress condition and all the degraded peaks are well resolved from parent peaks.

Keywords: Pioglitazone, Amlodipine, Atorvastatin, Clopidogrel, Validation, Stability -indicating study.

INTRODUCTION

Hypertension, diabetes and hyperlipidemia often coexist in patients suffering from cardiovascular diseases (CVD) and their combined effects make this disease common.[1] A pill containing different active ingredients (polypill) to overcome these conditions is always beneficial vis a vis the prevalent pills containing only one ingredient, in terms of cost and patient compliance.

Pioglitazone (P) is an oral antidiabetic drug. Pioglitazone is useful in the treatment of type-2 diabetes mellitus (also known as non-insulin dependent diabetes mellitus. Chemically, it is 5-[[4-[2-(methyl-2-pyridinylamino) ethoxyphenyl] methyl]-2, 4-thiazolidinedion (Fig.1). It stimulates the peroxisome proliferator-activated receptor gamma (PPAR-γ) and to a lesser extent PPAR-α. Pharmacological studies indicate that pioglitazone improves sensitivity to insulin in muscle and adipose tissue and inhibits hepatic gluconeogenesis. A literature survey regarding quantitative analysis of pioglitazone alone or its combination with other drugs revealed that attempts have been made to develop analytical method including HPLC in biological fluid and pharmaceutical formulations,[2-4] LCMS/MS,[5] spectrophotometry,[6] and high performance thin layer chromatography (HPTLC).[7] Amlodipine (AM) is chemically described as 2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-3-ethoxycarbonyl-5-methoxycarbonyl-6-methyl-1,4dihydropyridine. It is a long- acting calcium channel blocker. It inhibits the influx of calcium ions

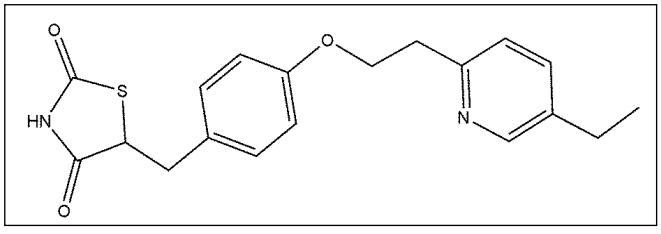

Fig. 1: Chemical structure of Pioglitazone (P)

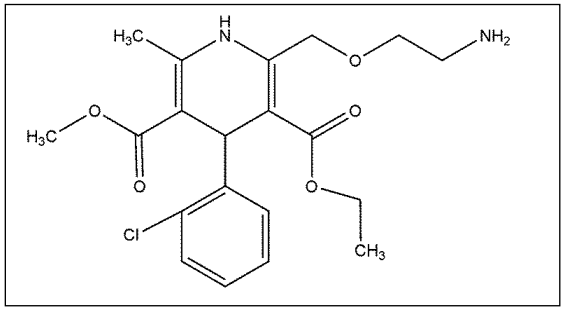

Fig. 2: Chemical structure of Amlodipine (AM)

[a] Department of Pharmaceutical Chemistry, Babu Banarasi Das University, Faizabad Road, Lucknow - 226 028, Uttar Pradesh, India
[b] Department of Pharmacy, Sarojini Naidu Medical College, Agra - 282 003, Uttar Pradesh, India
[c] Department of Pharmaceutical Chemistry, ISF College of Pharmacy, Moga - 142001, Punjab, India
*For Correspondence: E-mail: pvchawla@gmail.com

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into vascular smooth muscle and cardiac muscles. (Fig. 2). Several methods including HPLC,[8-9] LC-ESI-MS/MS[10], spectrophotometry[11] and high performance thin layer chromatography (HPTLC)[12] have been reported for the estimation of amlodipine. Atorvastatin (AT), chemically (3*R*,5*R*)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-propan-2-ylpyrrol-1-yl]-3,5-dihydroxyheptanoic acid, is an inhibitor of 3- hydroxyl-3-methylglutaryl coenzyme A (HMG-Co A) reductase enzyme. This enzyme participates in the conversion of HMG-CoA to mevalonate, an early and rate-limiting step in cholesterol biosynthesis. It is mainly use in the treatment of hypercholesterolemia (Fig. 3). Various methods including HPLC in pharmaceutical dosage form,[13-15] LC-MS,[16] HPLC in human serum[17] and HPTLC[18] have been reported for determination of atorvastatin. Clopidogrel hydrogen sulphate (CP) is an oral, thienopyridine-class antiplatelet agent. Chemically it is methyl (2*S*)-(2-chlorophenyl)[6,7-dihydrothieno[3,2-c]pyridin-5(4*H*)-yl]acetate sulphate (Fig. 4). Clopidogrel specifically and irreversibly inhibits the P2Y12 subtype of ADP receptor, which is important in aggregation of platelets and cross-linking by the protein fibrin. Several analytical methods involving HPLC,[19-20] LC-MS/MS[21] and LC-MS[22] have been reported for the analysis of clopidogrel.

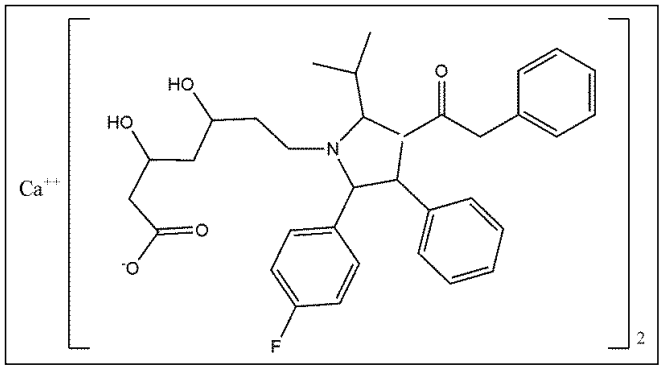

Fig. 3: Chemical structure of Atorvastatin (AT)

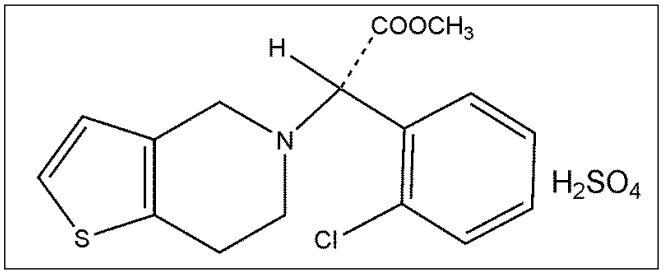

Fig. 4: Chemical structure of Clopidogrel (CP)

We have developed and validated a method for simultaneous estimation of these four drugs[23]. In Continuation of to our research work, stability indicating HPLC methods have been developed for simultaneous determination of the P, AM, AT and CP in the presence of their degradation products. All the selected drugs have different physicochemical properties and polarities, so the main challenge of the present work was to elute all the selected drugs with their degraded products with isocratic mode. To the best of our knowledge, this is the first time that all four selected drugs were estimated simultaneously with isocratic mode. Although stability indicating methods have been reported for assay of various drugs in drug products, most of them describe assay procedures for drug products containing only one active drug substance. The objective of the present work was to develop a simple, accurate, precise, reliable, sensitive and fast high-performance liquid chromatographic analytical method for simultaneous determination of P, AM, AT and CP and its degradation products formed under various stress condition and validate the method as per ICH guidelines[24-25].

MATERIALS AND METHODS

Instrument and Reagents

The HPLC system (Shimadzu, Tokyo, Japan) equipped with LC-10 AT pump and variable length UV visible detector SPD-10A was used to carried out the entire analysis. Best chromatographic separation and quantification were achieved on a Millenium M Sil C18 of 125 × 4.6 mm id, 5µm particle analytical column (ambient temperature). Spinchrom Chromatographic Station ®CRF Version 1.7 (Spinchrom Pvt. Ltd., Chennai, India) was used for chromatographic data recording. Pure analytical drugs of P, AM, AT and CP were provided by Alkem Laboratories, Gurgoan, India as gift samples. HPLC grade chemicals (water and methanol) were purchased from S.D. Fine Chem. Ltd. (India), while analytical grade potassium dihydrogen phosphate and orthophosphoric acid (85 %) were purchase from Merck Limited, Mumbai, India for the preparation of mobile phase. Pioglitazone tablets (P-GLIT-15, Cipla, India.) Amlodipine tablets (Amlip, 5mg, Cipla, India), Atorvastatin tablets (Atrolip, 5mg, Cipla, India) and Clopidogrel tablets (Ceruvin, 75 mg, Ranbaxy India) were purchased from local pharmacy.

Preparation of standards

Individual stock solutions of P (1mg/mL), AM (1mg/mL), AT (1mg/mL) and CP (1mg/mL) were prepared in methanol. Working standards of individual drug and mixtures of selected drugs were prepared in mobile phase over a concentration range of 0.625-10µg /mL. All working standards were stored at 2-8 ^{0}C in refrigerator and were found stable during the period of study, which were brought to room temperature before use.

Table I: Optimized chromatographic condition of proposed HPLC method

Parameter	Optimized condition
Mobile phase	Methanol: 0.02 M KH_2PO_4 55:45% V/V and pH were adjusted to 4.0 with 0.01% ortho phosphoric acid
Column	$C_{18,}$ Millenium M Sil Limited, Length-12.5 cm, i.d.-4.6 mm.
Detection	U.V., 269 nm (0-3.5 min.) 240nm (3.51-12min.) 230nm (12.1 -25min)
Flow-rate	1mL/min
Retention time of P	2.99 min.
Retention time of AM	4.0 min.
Retention time of AT	6.3 min.
Retention time of CP	14.4 min.

Table II: Results of system suitability parameters

Parameter	P	AM	AT	CP
Theroretical plates	1527	2580	7640	4864
Retention Time	2.98	4.02	6.3	14.4
Resolution	-	3.65	3.65	9.41
Asymmetric	1.5	1.4	1.2	1.4

All the results are average of six determinations

Sample solutions preparation

For this purpose, twenty tablets of each drug were weighed individually and mean weight of each category of drugs was determined. The tablets were crushed individually in a glass mortar to a fine homogenous powder. Powered mass equivalent to 15 mg of P, 5 mg of AM, 5 mg of AT and 5 mg of CP was transferred into separate 10 mL volumetric flasks and the volume was made up to the mark with mobile phase. The solutions of drugs were sonicated for 20 minutes for complete extraction followed by centrifugation for 30 min at 1000 rpm. A clear supernatant (1 mL) containing suitable amount of drug was transferred in a volumetric flask (10 mL) and made up the volume (10mL) with mobile phase (100 µg/mL). Mixture was prepared by mixing solution of P, AM, AT and CP in a ratio of 1:1:1:1. The results are shown in Table V.

Stress Studies:

Forced degradation studies on P, AM, AT and CP were carried out under acidic, basic, oxidative, thermal and photolytic stress conditions.[26]

Preparation of Stock Solution for stress studies:

Accurately weighed samples of 10 mg of each drug were carefully transferred to separate volumetric flasks of 10 mL capacity. The volume was made up to the mark with mobile phase (1000 µg/mL). Stress studies were performed in solitary (control) runs and mixture (combined) runs. Chromatograms obtained were subsequently compared.

Acidic degradation

Acidic degradation was performed for all the drugs individually as well as in combination in 0.1N hydrochloric acid at room temperature and in 1 N hydrochloric acid at 70 ^{0}C.

Table III: Linearity Study

Parameters	P	AM	AT	CP
Linearity range (µg/mL)	0.625-10 (µg/mL)	0.625-10 (µg/mL)	0.625-10 (µg/mL)	0.625-10 (µg/mL)
Correlation coefficients (r2)	0.9999	0.9999	0.9998	0.9999
Regression equation	y=34.305 x+0.0038,	44.996x-0.0934,	y= 15.52x-11.586	y=46.12 x-1.5014
Slope (m)	34.305	44.996	15.52	46.12
Intercept (c)	0.0038	0.0934	11.586	1.5014

** y= mx+c, y = peak response, m =slope, x= concentration (mg/mL), c = intercept.*

Table IV: Intra and inter-day precision and accuracy data of P, AM, AT and CP

Parameter	P	AM	AT	CP
Inter-day precision 2.5 (µg/mL) % RSD	0.84	0.35	1.34	0.87
Intra-day precision 2.5 (µg/mL) % RSD	0.79	0.41	1.02	0.75
% Recovery	95-105%	95-105%	95-105%	95-105%

All the results are average of six determinations

Table V: Analysis of formulation

Drug	Quantity claimed mg/tablet	Quantity found mg/ tablet	% Recovery*
Pioglitazone	15	14.99	99.93
Amlodipine	5	5.10	102.0
Atorvastatin	5	5.02	100.4
Clopidogrel	75	75.15	100.2

**% Recovery = 95-105%*

Acid degradation with 0.1N hydrochloric acid at room temperature

To perform acidic degradation 1 mL of stock solution (1000 µg/mL) of P, AM, AT and CP were transferred individually in separate in volumetric flasks (10 mL) followed by addition of 5 mL of 0.1 N hydrochloric acid to all the flasks. Similarly, 1 mL from combined stock solution (1000 µg/mL) containing all four drugs that is P, AM, AT and CP were transferred in one volumetric flask (10 mL) and then 5 mL of 0.1 N hydrochloric acid was added. All these volumetric flasks were kept at room temperature for 24 hr. After 24 h solutions were neutralized with 1 N NaOH and diluted up to mark with mobile phase. Appropriate

Table VI: Result of forced degradation study of proposed RP-HPLC method

Degradation Condition	Area				% degradation			
	P	AM	AT	CP	P	AM	AT	CP
Standard sample	**346.8813**	**508.0397**	**1302.2949**	**457.5707**	**None**	**None**	**None**	**None**
Acidic N HCl, 24 h, R.T.	302.0563	466.3317	583.5	426.47	12.92	8.20	55.19	6.79
1 N HCl, 1 h, 70^{0}C	302.2776	455.6558	596.6381	409.3606	12.85	10.31	54.18	10.53
Basic 0.1N NaOH, 5th days, R.T	243.7887	332.2179	1075.0986	36.2337	29.72	34.61	17.44	92.08
1 N NaOH, 1 h, 70^{0}C	235.8530	300.6096	1056.2613	48.7814	32.00	40.82	18.89	89.33
Oxidation 5 % H_2O_2, 2 h, 70^{0}C	309.7827	469.6903	973.0189	440.4425	10.69	7.54	25.28	3.75
Dry heat 105 ^{0}C, 48 h	341.3022	506.0408	1298.9068	451.7415	1.61	0.39	0.26	1.27
Photolysis U.V. light at 254 nm, 48 h	321.4712	495.2822	1234.2822	422.8208	7.32	2.51	5.18	7.5

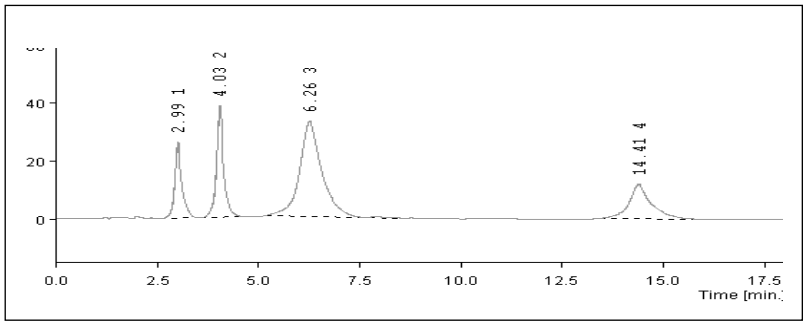

Fig. 5: Typical chromatogram of standard solution of P, AM, AT and CP

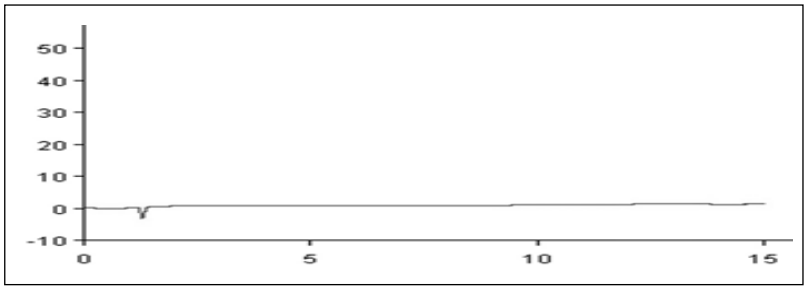

Fig. 6: Chromatogram of blank sample (mobile phase)

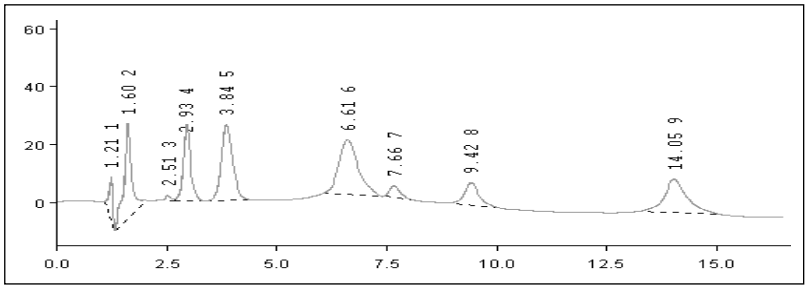

Fig 7a: Chromatogram of mixture in 0.1 N HCl 24 h (RT)

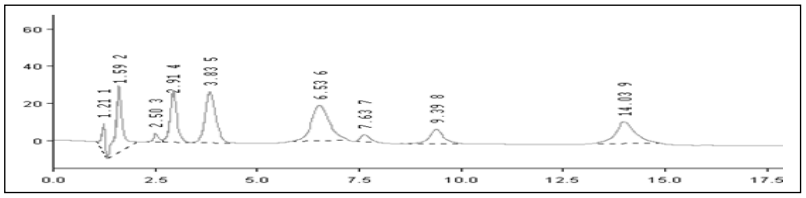

Fig 7b: Chromatogram of mixture in 1 N HCl after 3 h., (70 °C)

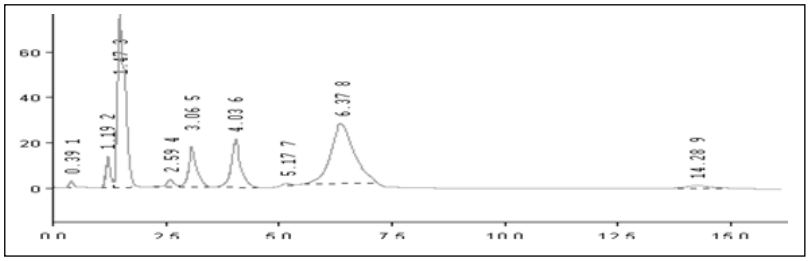

Fig 8a: Chromatogram of mixture after 5th day in 0.1 N NaOH (RT)

aliquots were taken from the above volumetric flasks and finally the concentration of 10 µg/mL of P, AM, AT and CP separately and in mixture was prepared.

Acid degradation with 1N hydrochloric acid at 70 °C:

The single drugs and combination of all the four drugs were also exposed to 1 N hydrochloric acid l under reflux for 1 h (70 °C). After 1 h solutions were neutralized with 1 N sodium hydroxide and diluted up to the mark with mobile phase. Appropriate aliquots were taken from the above volumetric flasks and finally prepare the concentration of 10 µg/mL of P, AM, AT and CP separately and in mixture was prepared.

ALKALI DEGRADATION:

Alkali degradation with 0.1N sodium hydroxide at room temperature for 5 days:

To perform alkali degradation, 1 mL of stock solution (1000 µg/mL) of P, AM, AT and CP were transferred individually in separate in volumetric flasks (10 mL) followed by addition of 5 ml of 0.1 N sodium hydroxide to all the flasks. Similarly, 1 mL from combined stock solution (1000 µg/mL) containing all four drugs that is P, AM, AT and CP were transferred in one volumetric flask (10 mL) and then 5 mL of 0.1 N sodium hydroxide was added. All these volumetric flasks were kept at room temperature for 5 days. After 5th day solutions were neutralized with 1 N hydrochloric acid and diluted up to the mark with mobile phase. Appropriate aliquots were taken from the above volumetric flasks and finally the concentration of 10 µg/mL of P, AM, AT and CP separately and in mixture was prepared.

Alkali degradation with 1N NaOH at 70 °C:

The single drugs and combination of all the four drugs were also exposed to 1 N sodium hydroxide under reflux for 1 h (70 °C). After 1 h solutions were neutralized with 1 N hydrochloric acid and diluted up to the mark with mobile phase. Appropriate aliquots were taken from the above volumetric flasks and finally prepare the concentration of

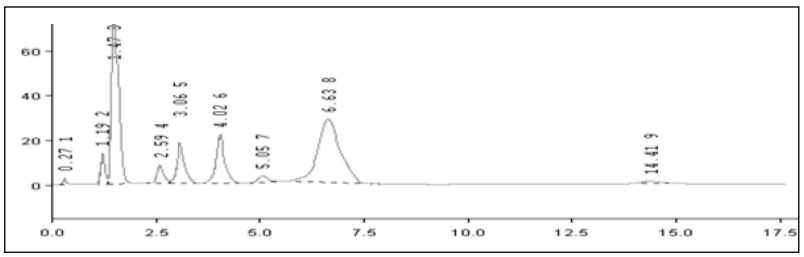

Fig 8b: Chromatogram of mixture in 1 N NaOH, (70 °C)

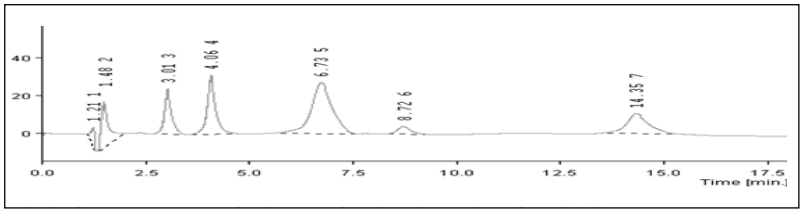

Fig 9: Chromatogram of mixture after 2h, in 5 % H_2O_2 (70 °C)

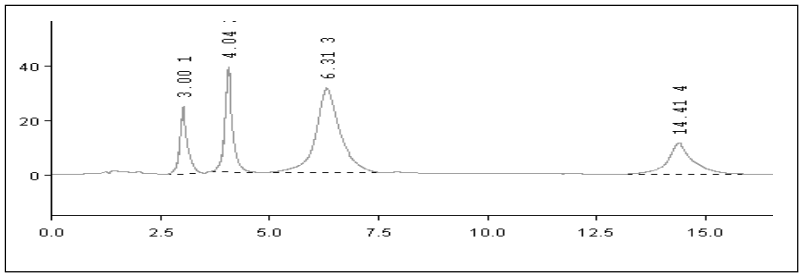

Fig 10: Chromatogram of mixture after 48 h. (105 °C)

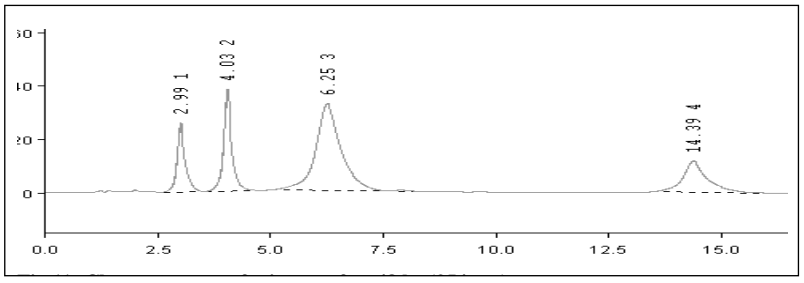

Fig 11: Chromatogram of mixture after 48 h (254 nm)

10 µg/mL of P, AM, AT and CP separately and in mixture was prepared.

Oxidative degradation:

For oxidative degradation study, 1 mL of stock solution (1000 µg/mL) of P, AM, AT and CP was transferred individually in separate volumetric flasks (10 mL) followed by addition of 5 mL of 5% hydrogen peroxide (H_2O_2) to all the flasks. Similarly, 1 mL from combined stock solution (1000 µg/mL) containing all four drugs that is P, AM, AT and CP were transferred in one volumetric flask (10 mL) and then 5 mL of 5% H_2O_2 was added.

All these volumetric flasks were kept on water bath and heated at 70 ^{0}C for 2 h. After 2 h solutions were diluted up to mark with mobile phase. Appropriate aliquots were taken from the above volumetric flasks and finally the concentration of 10 µg/mL of P, AM, AT and CP separately and in mixture was prepared.

Thermal degradation

For thermal degradation study, drugs were exposed in an oven at 105 °C for 48 h. After 48 hrs, accurately weighted 10 mg of each drug and transferred separately in 10 mL volumetric flasks and stock solution of 1000 µg/mL prepare with mobile phase. Appropriate aliquots were taken from the above volumetric flasks and finally prepare the concentration of 10 µg/mL of P, AM, AT and CP separately and in mixture was prepared.

Photolytic degradation

For photolytic degradation drugs were exposed in UV radiation at 254 nm for 48 h. After 48 h. accurately weighted 10 mg of each drug and transferred separately in 10 ml volumetric flasks and stock solution of 1000 µg/mL prepared with mobile phase. Appropriate aliquots were taken from the above volumetric flasks and finally prepare the concentration of 10 µg/mL of P, AM, AT and CP separately and in mixture was prepared.

RESULTS

Method development and optimization

The HPLC separation and quantification was achieved on a Millenium M Sil C_{18} (125 × 4.6 mm id, 5µm) reverse phase column. Mobile phase having composition of methanol: 0.02M KH2PO4 (55:45 % V/V) with pH 4.0 showed good peak resolution in the presence of degradation products and impurities. The mobile phase pumped at flow rate of 1mL/min, with an injection volume of 20 µL. Retention time of selected drugs under optimized chromatographic conditions were 2.99 min, 4.04 min 6.3 min and 14.4 min for P, AM, AT and CP, respectively. The typical chromatogram of the selected drugs is shown in Fig. 5 and the results are presented in Table I. System suitability test was carried out as per USP guidelines and the results are summarized in Table II.

Method validation parameter

Method validation was carried out as per ICH (Q2) guidelines in terms of linearity, specificity, precision, accuracy, limit of quantification (LOQ) and limit of detection (LOD).

Linearity

Linearity of the method was evaluated at five different concentration levels over the range of 0.625 to 10 µg/mL for all drugs. The linear regression equation of pioglitazone, amlodipine, atorvastatin and clopidogrel were found to be y= 34.305x+0.0038, 44.996x-0.0934, y= 15.52x-11.586 and y=46.12 x-1.5014 respectively, and the regression coefficient values were found to be 0.9999, 0.9999, 0.9998 and 0.9999 respectively, which show a high degree of linearity for all drugs. The results are presented in Table III.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. A representative chromatogram (Fig.6) indicates that commonly used tablet excipients did not interfere with the method and no significant changes in retention times of the drugs were observed in the presence and absence of excipients.

Precision and accuracy

For determination of intra-and inter-day precision, three concentrations i.e. 0.625 µg/mL, 2.5 µg/mL and 10 µg/mL were prepared for P, AM, AT and CP. The intra-day and inter-day precision study of drugs were carried out by analysing responses three times on the same day and on three different days. Standard solutions were analysed within one day (morning, afternoon and evening) and for inter-day variability, the same were analysed for fourteen days. The low level of %RSD for intra-and inter-day reveal that method is precise. The accuracy was expressed as the percentage of analytes recovered by the assay. Results of accuracy and precision are shown in Table IV.

Sensitivity

Limits of detection and quantification were based on the standard deviation and the slope of the calibration plots. Limits of detection (LOD) and quantification (LOQ) were calculated using formula LOD =3.3 (σ/ s); LOQ =10 (σ/ s); where s = slope of the calibration curve and σ = standard deviation of the response. LOD of P, AM, AT and CP were 0.03, 0.026, 0.028 and 0.026 µg/mL respectively, and limit of quantitation were 0.10, 0.07, 0.86 and 0.79 µg/mL respectively.

Accelerated degradation

The results of forced degradation studies are given in Table VI. Pioglitazone show maximum degradation in alkaline condition (1 N sodium hydroxide) and it is less sensitive to acidic condition (1 N hydrochloric acid). Similarly, amlodipine is more sensitive in 1N NaOH and showed 40% degradation in this condition. Atorvastatin showed less than 20% degradation in basic condition, in acidic condition (1N hydrochloric acid) atorvastatin showed 54.18% degradation. Under acidic condition, atorvastatin showed two additional peaks at 7.6 min. and 9.3 min. Clopidogrel showed 89.335 % degradation in alkali condition and is less sensitive in acidic condition. The chromatogram of acid hydrolysis is represented in Fig. 7a and 7b and the chromatograms of alkali degradation are represented in Fig. 8a and 8b. Under oxidation condition (5 % H_2O_2) only atorvastatin showed 25.28% degradation. However, pioglitazone, amlodipine and clopidogrel did not show any strong degradation (Fig. 9). During thermal (Fig. 10) and photolytic conditions (Fig. 11) drugs did not show any signiticant degradation.

DISCUSSION

In the present work, the selected drugs belonged to different categories and having different polarity and physiochemical properties, so the main task of this work was to developed such chromatographic conditions which are suitable for separation of selected drugs along with their degraded products. So, during study, different compositions of mobile phase having acetonitrile: methanol: water in different ratios and in different pH were tried for the simultaneous elution of selected drugs. When acetonitrile and water were used as mobile phase, then peaks were merged and showed poor resolution. In mixture of methanol and water peaks showed broadening. So, buffer was tried. Mobile phase with composition methanol: 0.02M KH_2PO_4 (65:35 % V/V) with pH 4.5 show good peak resolution, but during stability study it was found that the degraded peak of pioglitazone in basic condition interfered with the principal peak of atorvastatin. A methanol: 0.02 M KH_2PO_4 mixture in (55:45%V/V) ratio, pH adjusted 4.0 with 0.01% ortho phosphoric showed appropriate and rapid separation of selected drugs and their degraded products produced during stress condition. The proposed work was carried out on Millenium M Sil C_{18} (125 × 4.6 mm id, 5µm) column which show better reproducibility, good resolution, low tailing and low back pressure. The proposed method was completely validated according to ICH guidelines and showed satisfactory results for all validation parameters. During stability study, it was found that all degraded products which were produced in

stress conditions showed good resolution from the parent peaks. As the proposed method is capable of separating the selected drugs and their degraded products under stress condition with isocratic mode, this method could be successfully applied in routine analysis of these bulk drugs and pharmaceutical dosage forms.

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REFERENCES

1. Wei M., Mitchell B.D., Haffner S.M., Stem, M.P.: Effects of cigarette smoking, diabetes, high cholesterol, and hypertension on all-cause mortality and cardiovascular disease mortality in Mexican Americans: the San Antonio Heart Study. Amer. **J. Epidemiol.,** 1996, 144 (11) 1058-1065.
2. Sane R.T., Menon S.N., Inamdar S., Mote M., Gundi G.: Simultaneous determination of pioglitazone and glimepiride by high performance liquid chromatography, **Chromatographia,** 2004, 59 (7) 451-53.
3. Sripalakit P., Neamhom P., Saraphanchotiwitthaya A.: High-performance liquid chromatographic method for the determination of pioglitazone in human plasma using ultraviolet detection and its application to a pharmacokinetic study, **J. Chromatogr. B, 2009,** 843 (2) 164-169.
4. Peraman R., Mallikarjuna S., Ammineni P., Kondreddy V.K.: RP-HPLC method development and validation for simultaneous estimation of atorvastatin calcium and pioglitazone hydrochloride in pharmaceutical dosage form, **J. Chromatogr. Sci.**, 2014, 52 (9)1038-42.
5. Lin Z. J., Ji W., Desai-Krieger D., Shum L.: Simultaneous determination of pioglitazone and its two active metabolites in human plasma by LC-MS/MS, **J. Pharm. Biomed. Anal.,** 2003, 33 (1) 101–108.
6. Chaturvedi P. K., Sharma R.: Simultaneous spectrophotometric estimation and validation of three component tablet formulation containing pioglitazone hydrochloride, metformin hydrochloride and glibenclamide, **Anal. Letters,** 2008, 41 (12) 2133-2142.
7. Singh D., Dwivedi S.C., Kushnoor A.: Development and validation of a HPTLC method for simultaneous estimation of pioglitazone and glimepiride in bulk and tablet dosage form, **Int. J. Biomed. Adv. Res.,** 2011, 2 (9) 368-377.
8. Jain P.S., Patel M.K., Gorle A.P., Chaudhari A.J., Surana S.J: Stability-indicating method for simultaneous estimation of olmesartan medoxomile, amlodipine besylate and hydrochlorothiazide by RP-HPLC in tablet dosage form, **J. Chromatogr. Sci.** 2012, 50 (8) 680-7.
9. Dai S.Y., Qiu S.T., Wu W., Fu C.M.: Development and validation of an RP-HPLC method for simultaneous determination of ramipril and amlodipine in tablets, **J. Pharm. Anal.,** 2013, 3 (6) 440-446.
10. Gadepalli S.G., Deme P., Kuncha M., Sistla R.: Simultaneous determination of amlodipine, valsartan and hydrochlorothiazide by LC-ESI-MS/MS and its application to pharmacokinetics in rats, **J. Pharm. Anal.,** 4(6) 2014, 399-406.
11. Mishra P., Gupta A., Shah K.: Simutaneous spectrophotometric estimation of atorvastatin and amlodipine besylate from tablets. **Indian J. Pharm. Sci.,** 2007, 69 (6) 831-833.
12. Rao N.M., GowriSankar D.G.: Development and validation of HPTLC method for the simultaneous estimation of amlodipine besylate and atorvastatin calcium in combined dosage form, **Eurasian J. Anal. Chem.,** 2016, 11 (3)155–168
13. Shah D.A., Bhatt K.K., Mehta R.S.: Development and validation of a RP-HPLC method for determination of atorvastatin calcium and aspirin in a capsule dosage form, **Indian J. Pharm. Sci.,** 2007, (69) 546-49.
14. Jain N., Raghuwansh R., Jain D.: Development and validation of RP-HPLC method for simultaneous estimation of atorvastatin calcium and fenofibrate in tablet dosage forms, **Indian J. Pharm. Sci.,** 2008, 70 (2) 263-5.
15. Qutab S.S., Razzaq S.N., Khan, I.U.: Simultaneous determination of atorvastatin calcium and ezetimibe in pharmaceutical formulations by liquid chromatography, **J. Food Drug Anal.** 2007, 15 (2) 139-144.
16. Crevar-sakac M., Vujic Z., Vujcic Z., Markovic B., Vasiljevic D.: LC–MS/MS method for quantification of atorvastatin, o-hydroxyatorvastatin, p-hydroxyatorvastatin, and atorvastatin lactone in rat plasma, **Acta Chromatogr.**, 2016, 28 (3) 281–298.
17. Shah Y., Iqbal Z., Ahmad L., Khan A., Khan M.I., Nazir S., Nasir F.: Simultaneous determination of rosuvastatin and atorvastatin in human serum using RP-HPLC/UV detection: method development, validation and optimization of various experimental parameters, **J. Chromatogr B Analyt Technol Biomed Life Sci.,** 2011, 879 (9) 557-63.
18. Panchal H.J., Suhagia B.N.: Simultaneous analysis of atorvastatin calcium and losartan potassium in tablet dosage forms by RP-HPLC and HPTLC, **Acta Chromatogr.,** 2010, 22 (2) 173–87.
19. Silvestro L., Gheorghe M.C., Tarcomnicu I.: Development and validation of an HPLC-MS/MS method to determine clopidogrel in human plasma. Use of incurred samples to test back-conversion, **J. Chromatogr B Analyt Technol Biomed Life Sci.,** 2010, 878 (30) 3134-42.
20. Robinson A., Hillis J., Neal C., Leary A.C.: The validation of a bioanalytical method for the determination of clopidogrel in human plasma, **J. Chromatogr. B,** 2007, 848 (2) 344–354.
21. Takahashi M., Pang H., Kawabata K., Farid N.A., Kurihara A.: Quantitative determination of clopidogrel active metabolite in human plasma by LC–MS/MS, **J. Pharm. Biomed. Anal.,** 2008, 48 (4) 1219-1224.
22. Ksycinska H., Rudzki P., Bukowska-Kiliszek M.: Determination of clopidogrel metabolite (SR26334) in

human plasma by LC–MS, **J. Pharm. Biomed. Anal.,** 2006, 41 (2) 533-539.

23. Monika, Chawla P., Pandey S.: Synchronised separation of antihypertensive, antidiabetic and antihyperlipidemic drugs by RP-HPLC, **Asian J. Chem.,** 2017, 29 (7) 1573-1577.
24. ICH Guidelines: Validation of Analytical Procedures: Q2B, 1996.
25. ICH Guidelines: Validation of Analytical Procedures: Q2A, 1994.
26. Blessy M., Patel R.D., Prajapati P.N., Agrawal Y.K.: Development of forced degradation and stability indicating studies of drug- A review. **J. Pharm. Anal.,** 2014, 4 (3) 159-165.