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RESEARCH ARTICLE

Simultaneous LC determination of two co-prescribed analgesics with anti-hypercholesterolemic agent in bulk drug, dosage formulations and in human serum with programmed UV detection

Arman Tabassum[1], Muhammad Saeed Arayne[1] and Najma Sultana[2]

1.Department of Chemistry, University of Karachi, Karachi-75270.

2.Research Institute of Pharmaceutical Sciences, University of Karachi, Karachi-75270.

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Corresponding Author

Arman Tabassum

Abstract

A simple, accurate and sensitive High-Performance Liquid Chromatography-UV detection method was developed for simultaneous determination of rosuvastatin with co-administered NSAIDs (piroxicam and diclofenac) in bulk drug, dosage formulations and human serum. Isocratic separation was employed on pre-packed Purospher STAR® C_{18} (5 µm, 25 x 0.46 cm) column at ambient temperature. Mobile phase consisted of methanol, water and acetonitrile (80:17.5:2.5 v/v), pH adjusted to 3.0 with *o*-phosphoric acid at 1 $mLmin^{-1}$. Drugs in eluent were monitored at isosbestic point of drugs 230 nm for their similar absorptivity and under wavelength-program to provide their maximum absorbance.

Linear behavior was observed between 0.33-16.66 μgmL^{-1} for rosuvastatin and piroxicam and 0.25-12.5 μgmL^{-1} for diclofenac with $r^2 \geq 0.998$. %RSD for intra-day and inter-day precision was less than 2 in all bulk, formulations and human serum samples. % recoveries for all drugs were 98.18-101.46% in API, pharmaceutical formulations and in human serum at isosbestic point. Wavelength-programmed analysis made the method more sensitive where 11-38 $ngmL^{-1}$ LOQs and 3-12 $ngmL^{-1}$ LODs for bulk, 11-21 $ngmL^{-1}$ LOQs and 4-7 $ngmL^{-1}$ LODs for formulations; and 8-14 $ngmL^{-1}$ LOQs and 3-4 $ngmL^{-1}$ LODs for samples in human serum shifted to 4-11 $ngmL^{-1}$ LOQs and 1-3 $ngmL^{-1}$ LODs for API, 9-13 $ngmL^{-1}$ LOQs and 3-5 $ngmL^{-1}$ LODs for pharmaceutical formulations; and 3-14 $ngmL^{-1}$ LOQs and 1-5 $ngmL^{-1}$ LODs for samples in human serum recorded at isosbestic point respectively for rosuvastatin, piroxicam and diclofenac. Recovery of drugs was 97.26-102.46% in API, 97.23-102.65% in formulations and 97.68-102.71% in serum samples at %RSD less than 2 for all. Both methods are equally applicable for implementation in industries for QC analyses, forensic, clinical and research work due to the high throughput of methods within 5 minutes with non-toxic and non-corrosive solvents and conventional detector for quantities present in little amounts.

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INTRODUCTION

Cholesterol is biosynthesized in animal hepatocytes for the manufacturing of cell membrane, vitamin D, bile acids and steroid hormones. It circulates throughout body through blood with lipoproteins as carrier. If the concentration of cholesterol in low-density-lipoproteins, very-low-density-lipoproteins and intermediate-density-lipoproteins increases, it increases the risk of atherosclerosis and coronary heart disease. This deposited cholesterol may be due

to intake of excess diet containing fat and cholesterol. It is insoluble in water and so becomes the precursor to hypertension, heart stroke, diabetes, obesity, inflammation and underactive thyroid etc.

Rosuvastatin is one of the fastest acting drugs that inhibit cholesterol synthesis in patients of hypercholesterolemia, hypertriglyceridemia and dyslipidemia. It is inhibitor of HMG-CoA reductase enzyme that is responsible for its synthesis inside body. Piroxicam and diclofenac are non-steroidal anti-inflammatory drugs (NSAIDs) widely recommended for the treatment of acute or chronic pains and inflammations such as rheumatoid arthritis and osteoarthritis [1].

Quantitative determination of rosuvastatin has been carried out using liquid chromatography [2-4]. Less time consumption has been carried out by determining more than a drug simultaneously for which our research group has long been working on simultaneous quantitation of drugs [5-11]. Diclofenac is determined in combination with diflunisal by HPLC [12]. Spectrophotometry is used for determination of piroxicam with diclofenac [13, 14]. Degradation studies of both drugs is done on HPLC [15]. Concerning these three drugs in combination no research report is found for the simultaneous determination of of rosuvastatin, piroxicam and diclofenac however they have been co-prescribed for anti-inflammatory and anti-pyratic responses in ischemia, thrombosis and hypertension.

The aim of present work is to establish less time consuming, fast, economical, sensitive method for determination of these drugs simultaneously on RP-HPLC with little solvent consumption. The method is especially beneficial for the areas where conventional instruments are used. It can determine the drugs at nanogram level. It can be applicable in pharmacokinetic and research studies and pharmaceutical and clinical routine analyses.

1. Experimental

2.1 Materials and reagents

Reference standards of Rosuvastatin, Piroxicam and Diclofenac were obtained from Pharm Evo Pvt. Ltd. Pakistan, Pfizer laboratories Pvt. Ltd. Pakistan and Yung Shin Pharmaceuticals Pvt. Ltd. Pakistan respectively. Their tablet formulations X-plended® 5 mg, Fleden flash® 20 mg and Voren® 50 mg respectively with expiry not less than 365 days (at the time of study) were purchased from local pharmacy. HPLC grade methanol (MeOH), acetonitrile (ACN) and *o*-phosphoric acid were purchased from Merck, Germany. Freshly prepared doubly distilled de-ionized water was used throughout analysis.

2.2 Instrumentation

Isosbestic point and maximum wavelength of the drugs under consideration were determined on Shimadzu1800 UV-visible spectrophotometer. Two liquid chromatographic systems were used for development and validation of methods. Systems were consisted of three main parts: a Shimadzu 10A HPLC System with LC-20-AT HPLC pump (with Rheodyne manual injector of 20 microL loop), SPD-20A Shimadzu UV visible detector and CBM-102 communication Bus Module. Another system was Shimadzu 20A HPLC System provided with pump, detector and CBM of same configuration discussed above. CLASS-GC 10 software (version 2) was used for chromatographic data acquisition. Columns of C_{18} (5 µm, 250 × 0.46 cm) configuration of Purospher® STAR (Merck Millipore) and Sapilco® (Sigma-Aldrich, St Louis, MO) were used for separation of analytes. Micropore microliter filtration syringe was used in the study. Stedec CSW-300 deionizer (Stedec (Pvt) Ltd., Karachi, Pakistan) was used for de-ionization of doubly distilled water from distillation assembly. Elma Ultrasonic LC 30 H sonicator (Elmer, NY) was used to degas the samples for chromatography.

2.3 Preparation of Standard solutions from reference standards

Accurately weighed reference standards were separately taken in 100 mL volumetric flasks to prepare stock solutions of 100 µg mL-1 with diluent of 80:17.5:2.5 MeOH: H_2O: ACN. Further working standards were prepared ranging from 0.0125-16.66 µgmL^{-1} of Rosuvastatin and Piroxicam and 0.03125-12.5 µgmL^{-1} of Diclofenac. Stock solutions were stored in refrigerator at 4^{o}C. These solutions were prepared once and analyzed daily for inter and intra day precision studies.

2.4 Preparation of solutions of pharmaceutical formulations

Content of drug in respective dosage formulations was analyzed by triturating ten tablets of each dosage formulation separately in mortar. Amount equivalent to 10 mg of homogenously crushed tablet was separately dissolved in diluents to make their stock solutions and allowed for 30 minutes, sonicated and filtered. Aliqoutes of respective drugs were prepared from their stock solutions like discussed above.

2.5 Preparation of solutions in human serum

Blood sample from a healthy volunteer of 24 years age was collected at Fatmid Foundation Karachi. Plasma was separated immediately from it by centrifuging blood sample @ 1600 g min^{-1} at 4^{o}C. 9.0 mL ACN was added in 1.0 plasma and vortexed following centrifugation for 10 minute.The blood sample from a healthy person was collected at Fatmid Foundation Karachi and plasma was separated from it by centrifuging at 10,000 rpm. Clear supernatant

called serum was obtained that was collected for *in vitro* analysis of drugs in serum by spiking drugs in desired concentrations. Analysis was carried out at isosbestic point and λ_{max} of each drug.

2.6 Chromatographic condition and experimental parameters

Mobile phase consisted of MeOH: water: ACN in the volumetric ratio of 80:17.5:2.5 with pH adjustment to 3.0 with 85% *o*-phosphoric acid. Mobile phase was filtered with0.45 µm pore size filter millipore vacuum filter system and degassed with an ultrasonic bath prior to chromatographic analysis. Separation of analytes was carried out on Purosper STAR C_{18} (5 µm, 250 × 0.46 cm) column. The analysis was carried out at ambient temperature under isocratic conditions. Flow rate of mobile phase was adjusted at 1.0 mL min^{-1} at 230 nm (isosbestic point of drugs). For detector-pump programmed analysis detector was programmed at 243 nm for 0-4.0 min for Rosuvastatin, 331 nm for 4.1-5.2 min for Piroxicam and 279 nm for 5.3-7.8 min for Diclofenac.

2.7 Optimization of method

2.7.1 Selection of stationary phase (column): Reversed phase C_{18} columns from different manufacturers were attempted for better resolution of peaks and Purospher STAR® and Supalico® were of configuration 5 µm, 25×0.46 cm gave desired results.

2.7.2 Mobile phase composition and pH: mobile phase was adjusted with combination of methanol and water in increasing and decreasing ratio (95:05, 90:10, 85: 15, 80:20, 75:25, 70:30). Shoulders were rectified with addition of ACN. The pH (acidic) was adjusted for better retention of drugs when flow in mobile phase. All these conditions met better resolution and lesser retention times of drugs.

2.7.3 Flow rate: the pump was adjusted with several experiments of low rate adjustment so that drugs could resolve clearly in least time as possible, neither too slow to waste the mobile phase nor too fast to separate out distinguished peaks. Flow rates observed were 0.8-2.0 mL min^{-1}.

2.7.4 Detector wavelength: Maximum wavelength of individual drug was taken on UV-Visible spectrophotometer. Isosbestic point was calculated from their spectra. Analysis was carried out by setting the detector at this wavelength. Further the maximum wavelengths taken on UV-Visible spectrophotometer were also used to program the detector so that maximum sensitivity of the instrument is achieved

2.8 Method validation

Validation of methods developed was carried out according to the recommendations of ICH 2006 guidelines [16]. System suitability, specificity and selectivity, linearity, accuracy, precision, limits of detection and quantitation, robustness and ruggedness were studied and are discussed in detail.

2. Results and Discussion

Nowadays co-prescription has increased the complications of health in patients which needs careful monitoring. Over-the-counter analgesic drugs Piroxicam and Diclofenac can be easily administered by patients. With the fact of still no such method for simultaneous determination of Rosuvastatin with Piroxicam and Diclofenac, the aim is to develop a method for determination of drugs of this combination that is simple, rapid, economical and sensitive, can be applied in routine QC analysis and research studies.

3.1 Method development and optimization

After developing the methods they were optimized for appropriate chromatographic conditions. Isocratic separation of peaks for drug analytes was carried out for reference standards, pharmaceutical formulations and human serum samples with methanol:water:ACN in 80:17.5:2.5 v/v/v ratio as the mobile phase adjusted to pH 3.0 with *o*-phosphoric acid. Since the aim of work was associated to research including *in vitro* and *in vivo* interactions, pharmacokinetics and QC studies, it needed accuracy and sensitivity and so optimization was carried out after studying various parameters.

Looking at the chemical structures of drug compounds (figure 1) reversed-phase chromatographic separation was selected for which C18 column was needed as stationary phase. Purospher STAR® (5 µm, 25×0.46 cm) and Supalico® (5 µm, 25×0.46 cm) were best suited. They were efficient for the reproducible separation of non-polar compounds, minimizing solvent usage with typical peak symmetry. Supalico® (5 µm, 25×0.46 cm) was selected for ruggedness studies.

Peaks could be read unless wavelength is selected. Stronger the absorbed light from analyte, stronger is the peak. Based on wavelength, two methods were developed, one at the isosbestic point (230 nm) and second at the λ_{max} (243 nm, 331 nm, and 279 nm for Rosuvastatin, Piroxicam and Diclofenac respectively). Spectra are seen in figure 2.

Mobile phase was adjusted with the idea of economical and less toxic. Methanol and water was taken as starting test solvents. After various trials of different ratios 80: 20 MeOH: H_2O showed better resolution. Lower ratios gave broader peaks that were near to merge or overlap. Reducing water content and adding ACN removed tailing and produced sharper and even better resolved peaks.

pH is important for stability of compounds in mobile phase. After investigation for pH effect from 2-4.5, well resolved, sharp and reproducible peaks were observed at pH 3.0. Acidic pH was maintained with *o*-phosphoric acid which is inert to column packing. Increase in pH above 3.0 showed reduced heighted peaks. Strong acidic medium is not suitable for column and the waste eluent.

3.2 Method validation

ICH and USP guidelines were followed for validation of developed methods [17-19].

3.2.1 Specificity

Chromatograms of blank solvent, solutions of pacebo, reference standards, pharmaceutical formulations and serum samples were recorded with consecutive injections. There were no interfering peaks for external material, excipient or endogenous plasma component. Sharp peaks were recorded every time at 3.5, 4.4 and 7.2 minutes fro Rosuvastatin, Piroxicam and Diclofenac only respectively. Mobile phase of methanol:water:ACN in 80:17.5:2.5 v/v/v ratio with pH 3.0 was suitable for the system. (Figure 3)

3.2.2 System suitability test

Ten subsequent injections of mixture of reference standards were applied for reproducible peak separation, resolution and column efficiency. Theoretical plates of all drug analytes were found greater than 2000. Tailing and capacity factor less than 2 confirm the system suitability of the method. Calculated parameters for methods at isosbestic point and λ_{max} are presented in table 1.

3.2..3 Linearity

Linearity and range correspond each other. Calibration curves so plotted were linear for concentration range of 0.33-16.66 µg mL^{-1} for Rosuvastatin and Piroxicam and 0.25-12.5 Diclofenac. Correlation coefficients (R^2) were found not less than 0.998. Regression characteristics i.e., slope and intercepts are scheduled in table 2.

3.2..4 Precision

Interday and intraday study of method was carried out by applying replicates of injections of above discussed concentrations of drug analytes for verification by means of percent relative standard deviation. %RSD values so obtained were less than 2. Table 3 shows precision of method from standard.

3.2..5 Accuracy

Percent recovery at three levels of concentration in specified range for pharmaceutical formulations and samples in human serum (80, 100 and 120%) were calculated to determine the accuracy of method. Results exhibited recoveries not less than and greater than 98.03-102.65%. Results suggested good accuracy of method for all samples. Accuracy data are scheduled in table 4. Formula applied to calculate percent recoveries is as under:

Recovery (%) = [(Measured concentration −Original concentration)/Spiked concentration] × 100

3.2.6 Detection and quantitation limits

Limits of detection (LOD) and quantitation (LOQ) are measure of sensitivity levels of a method. They are determined from the slope and standard deviation found from regression statistics. They are representations level of least concentration that an analyte can be detected or quantitated that can be measured by a method. The LOD values are the ratio of three and ten times the chromatographic signal of an analyte to noise in that chromatogram respectively.

In the study under consideration the LOD and LOQ from concentration levels in µgmL^{-1} were found to be 5 and 14 ngmL^{-1} Rosuvaastain, 3 and 11 ngmL^{-1} Piroxicam and 12 and 38 ngmL^{-1} Diclofenac respectively in API. The same for tablet formulations were 5 and 14 ngmL^{-1} Rosuvaastain, 4 and 11 ngmL^{-1} Piroxicam, 3 and 8 ngmL^{-1} Diclofenac. The serum samples exhibited 7 and 21 ngmL^{-1} Rosuvaastain, 4 and 12 ngmL^{-1} Piroxicam, 4 and 11 ngmL^{-1} Diclofenac. (Table 2)

3.2..7 Robustness

Robustness studies are the measure of toughness and stability of method when parameters are changed to little extent. This is done in validation step by making deliberate changes in parameters of method. Mobile phase composition, flow rate showed results leading to approx. 0.1% shift in retention time. pH change did not show distinguished change. Hence the method was found robust. Robustness was also applied on serum samples and pharmaceutical formulations. (Table 5)

3.2..8 Ruggedness

Ruggedness is another form of the measure of toughness and stability of method by changing the environment and instruments of the method. For this purpose the whole method was peroformed in another laboratory Research Institute of Pharmaceutical Sciences, University of Karachi, with new samples of drug analytes. The instrument used was Shimadzu LC-10AT in this laboratory. Different column of another manufacturer, Sapilco C18 (5 µm, 250 × 0.46 cm), was used for the analysis. Results are shown in table 5 with robustness studies. %RSD less than 2 show the ruggedness of method.

3.3 Programmed detector analysis

Individual wavelength maxima of compounds were taken through UV-spectra. Further the chromatographic conditions were modified and validated by programming the detector to λ_{max} with respect to retention time of respective drugs for maximum absorbance. Programming was carried out at 240 nm for 0-4.0 min Rosuvastatin, 331 nm for 4.1-5.2 min Piroxicam and 279 nm for 5.3-7.8 min for Diclofenac. Concentration ranges for calibration curves were 0.0125-16.66, 0.0125-16.66 and 0.03125-12.5 μgmL^{-1} were followed for Rosuvastatin, Piroxicam and Diclofenac respectively. LOD and LOQ values were 3-12 and 11-38 $ngmL^{-1}$ for all drugs. Method is precise and accurate also. ICH guidelines were also followed for this method and results have been presented along with corresponding results obtained from isosbestic point for comparison and validity of method.

Overall results of the method suggest that by programming the detector, the concentration level for analysis can easily be reduced that can reduce the amount of analytes utilized. The LOD and LOQ values also exhibited increased level of sensitivity. Analytes can be detected and quantitaed to three times smaller values of concentration (table 2). The optimaztion and validation is applied on the samples of pharmaceutical formulations and human serum also. The method is found equally reliable, precised (table 3), accurate (table 4), robust and rugged (table 5). Figure 4 distinguishes a difference in peak height of both methods developed with specificity and system suitability.

Conclusion

Simultaneous methods developed for combination of Rosuvastatin and two analgesics Piroxicam and Diclofenac on RP-HPLC at isoscbestic point and individual λ_{max} of compounds was validated according to ICH guidelines and USP. Both methods proved to be facile, reproducible, robust, rugged, economical and less toxic. The sensitivity level of both methods were high and could measure very little amount of analyte in mixture in nanogram per milliliters of concentration. Both methods are applicable in routine QC analysis and further drug-interactions, pharmacokinetic and pharmacodynamic research studies.

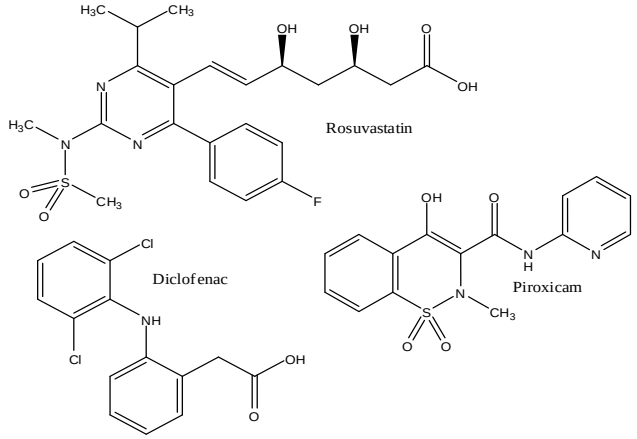

Figure 1: Representative chemical structures of Rosuvastatin (ROS), Piroxicam (PIR) and Diclofenac (DIC)

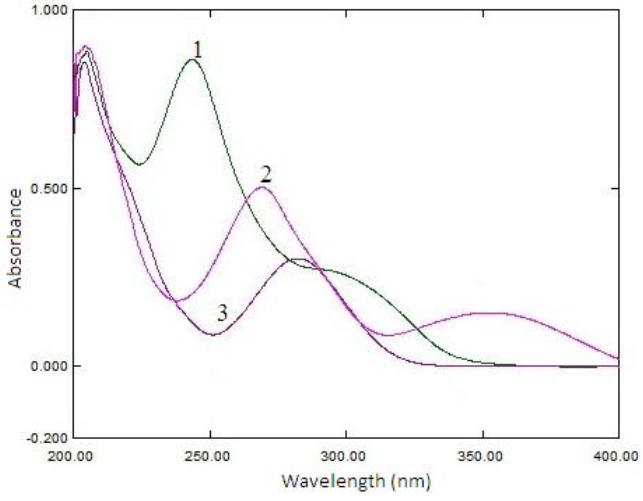

Figure 3.9: Representative UV spectra of rosuvastatin (1), piroxicam (2) and diclofenac (3).

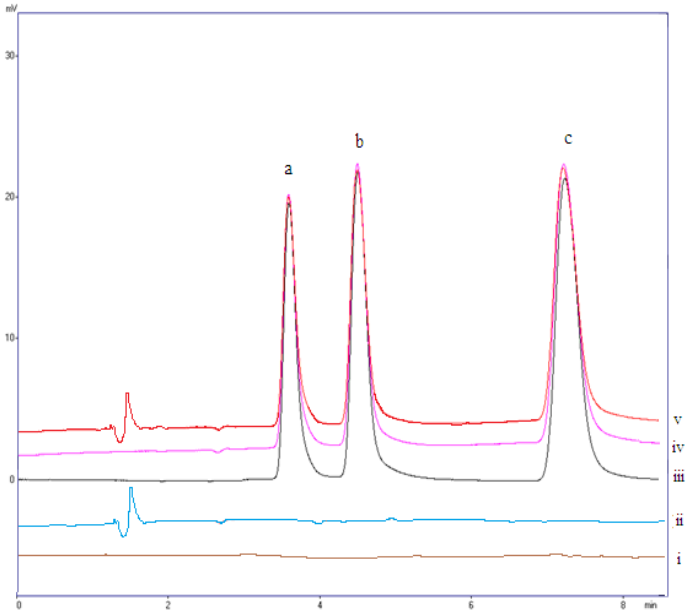

Figure 3: Representative chromatograms of placebo (i), blank human serum (ii), rosuvastatin (a), piroxicam (b) and diclofenac (c) in bulk (iii), pharmaceutical formulation (iv) and human serum sample spiked with standards (v).

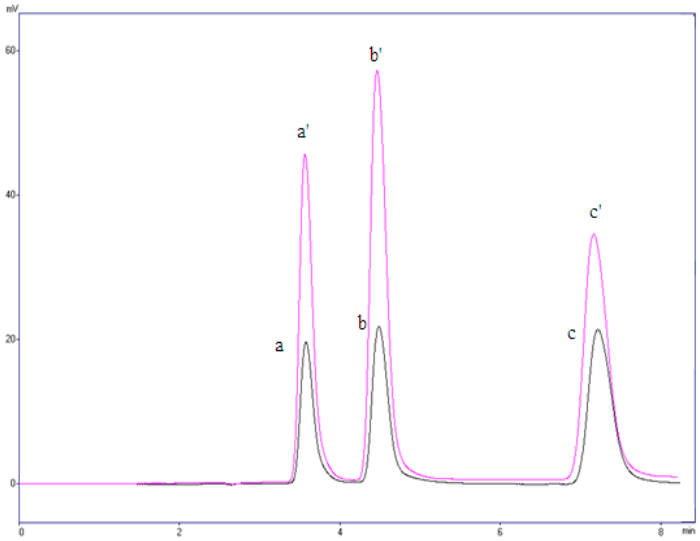

Figure 4: Representative chromatograms of rosuvastatin (a and a'), piroxicam (b and b') and diclofenac (c and c') at isosbestic point and individual λ_{max} respectively.

Table 1: System suitability parameters of Rosuvastatin and NSAIDs in proposed methods

At isosbestic point					
Analytes	t_R	k'	N	T	Rs
ROS	3.577	0.29	2305	1.62	2.02
PIR	4.489	0.62	2425	1.67	2.42
DIC	7.233	1.61	2860	1.45	6.06
At individual λ_{max}					

ROS	3.554	0.00	2270	1.56	1.01
PIR	4.453	0.25	2399	1.45	2.72
DIC	7.157	1.01	2770	1.42	5.96
Retention time (t_R), Capacity factors (k'), Theoretical plates (N), Tailing factor (T), Resolution (Rs)					

Table 2: Regression characteristics of ROS and NSAIDs in proposed methods

Parameter	ROS	PIR	DIC	ROS	PIR	DIC
	At isosbestic point			At individual λ_{max}		
Bulk						
Conc.(µgmL^{-1})	0.33-16.66	0.33-16.66	0.25-12.5	0.0125-16.66	0.0125-16.66	0.03125-12.5
Slope	36086	44850	93577	54767	59653	10308
Intercept	35382	10842	61830	14989	21786	4747
LOD (ngmL^{-1})	5	3	12	1	3	3
LOQ(ngmL^{-1})	14	11	38	4	10	11
R^2	0.998	0.999	0.998	0.999	0.999	0.999
Human serum						
Conc.(µgmL^{-1})	0.33-16.66	0.33-16.66	0.25-12.5	0.0125-16.66	0.0125-16.66	0.03125-12.5
Slope	36983	50118	88719	54779	59527	10274
Intercept	24340	22803	10737	14424	21604	4656
LOD (ngmL^{-1})	7	4	4	4	3	5
LOQ(ngmL^{-1})	21	12	11	12	9	13
R^2	0.998	0.999	0.999	0.999	0.999	0.999
Tablet formulations						
Conc.(µgmL^{-1})	0.33-16.66	0.33-16.66	0.25-12.5	0.0125-16.66	0.0125-16.66	0.03125-12.5
Slope	37009	50128	88746	54774	59583	10294
Intercept	24613	22942	11230	14731	21773	9960
LOD (ngmL^{-1})	5	4	3	1	5	2
LOQ(ngmL^{-1})	14	11	8	3	14	6
R^2	0.998	0.999	0.999	0.999	0.999	0.999

Table 3: Intra and inter day precision of ROS and NSAIDs in proposed methods

Conc. e^{-3} ngmL^{-1}	%RSD											
	At isosbestic point						At individual λ_{max}					
	Bulk		Human serum		Tablet formulations		Bulk		Human serum		Tablet formulations	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
ROS												
0.5	0.03	0.01	0.01	0.26	0.03	0.06	0.01	0.02	0.03	0.04	0.03	0.01
1	0.06	0.04	0.02	0.10	0.04	0.01	0.02	0.02	0.06	0.03	0.03	0.02
3	0.02	0.04	0.01	0.18	0.05	0.10	0.03	0.05	0.14	0.13	0.02	0.02
6	0.05	0.02	0.02	0.04	0.08	0.02	0.01	0.03	0.20	0.17	0.02	0.07
11	0.05	0.05	0.04	0.05	0.05	0.29	0.02	0.07	0.21	0.14	0.12	0.04
PIR												
1	0.01	0.01	0.01	0.05	0.02	0.01	0.017	0.01	0.025	0.06	0.023	0.01
2	0.03	0.01	0.01	0.02	0.05	0.03	0.040	0.02	0.100	0.02	0.007	0.01
6	0.16	0.02	0.03	0.04	0.03	0.02	0.019	0.04	0.046	0.02	0.020	0.07
12	0.01	0.02	0.01	0.04	0.13	0.11	0.026	0.03	0.183	0.11	0.025	0.04
22	0.11	0.03	0.08	0.14	0.72	0.12	0.017	0.02	0.23	0.13	0.018	0.04

DIC												
1	0.01	0.01	0.05	0.04	0.04	0.02	0.01	0.01	0.03	0.03	0.02	0.02
2	0.02	0.02	0.01	0.02	0.02	0.03	0.02	0.01	0.05	0.05	0.32	0.03
6	0.02	0.01	0.02	0.03	0.03	0.03	0.03	0.04	0.04	0.09	0.03	0.02
12	0.02	0.02	0.02	0.04	0.08	0.05	0.04	0.02	0.09	0.06	0.05	0.14
22	0.01	0.01	0.01	0.23	0.31	0.07	0.01	0.02	0.20	0.16	0.25	0.16

Table 4: Accuracy studies of ROS and NSAIDs in proposed methods

	At isosbestic point			At individual λ_{max}		
conc.	% Recovery					
%	Bulk	Human serum	Tablet formulations	Bulk	Human serum	Tablet formulations
ROS						
80	99.97	100.0385	99.98216	98.23	101.78	97.23
100	100.02	100.0828	100.0369	99.45	102.71	99.57
120	100.07	99.77126	99.97477	101.45	98.62	101.12
PIR						
80	100.02	99.95032	99.97477	98.46	101.68	100.33
100	100.01	100.025	100.0643	97.26	99.01	101.69
120	99.98	99.98215	99.86131	100.67	98.03	102.65
DIC						
80	99.97	98.23	99.16	102.46	99.00	101.23
100	99.99	99.46	98.19	100.82	98.23	101.97
120	99.98	97.98	101.46	101.46	97.68	100.99

Table 5: Robustness and ruggedness studies of ROS and NSAIDs (mean values) in proposed methods

	Parameters	t_R	N	T
ROS				
pH (3±0.1)		3.577	2305	0.29
Flow rate (1±0.1) ($mLmin^{-1}$)		3.39	2299	0.21
Mobile phase (80/17.5±2) (v/v/v)		3.41	2298	0.21
Column	Purospher STAR	3.42	2300	0.23
	Sapalico	3.50	2301	0.25
System	LC-20AT	3.42	2300	0.23
	LC-10AT	3.41	2305	0.26
PIR				
pH (3±0.1)		4.489	2425	0.62
Flow rate (1±0.1) ($mLmin^{-1}$)		4.325	2424	0.58
Mobile phase (80/17.5±2) (v/v/v)		4.318	2419	0.55
Column	Purospher STAR	4.321	2423	0.59
	Sapalico	4.491	2453	0.72
System	LC-20AT	4.321	2423	0.59

	LC-10AT	4.340	2428	0.64
DIC				
pH (3±0.1)		7.233	2860	1.61
Flow rate (1±0.1) ($mLmin^{-1}$)		7.110	2859	1.51
Mobile phase (80/17.5±2) (v/v/v)		7.115	2860	1.50
Column	Purospher STAR	7.210	2862	1.52
	Sapalico	7.213	2872	1.57
System	LC-20AT	7.210	2862	1.52
	LC-10AT	7.310	2869	1.58

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