Development and Validation of RP-HPLC Method for the Simultaneous Quantification of Losartan Potassium, Naproxen and Pioglitazone Hydrochloride

Muhammad Anisur Rahman[1], Mohammad Farhadur Rahman[1], Md. Abdus Salam[1], Md. Zakir Sultan[2] and A. K. M. Nur Alam Siddiki[1]

[1]Department of Chemistry, University of Dhaka, Dhaka-1000, Bangladesh
[2]Centre for Advanced Research in Sciences (CARS), University of Dhaka, Dhaka-1000, Bangladesh

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ABSTRACT: A reversed phase high-performance liquid chromatographic (RP-HPLC) method was developed and validated for the simultaneous determination of losartan potassium, naproxen and pioglitazone hydrochloride. Chromatographic separation was performed on a Phenomenex Gemini C18 column (250 x 4.6 mm, 5 µm) with a mobile phase of acetonitrile: 2% acetic acid in water (60:40, v/v) at a flow rate of 0.7 mL/min. Detection was performed at 230 nm using a UV detector. Under the optimized conditions, the retention times for losartan potassium, naproxen and pioglitazone hydrochloride were 6.24, 8.34 and 14.86 minutes, respectively, with well-resolved and symmetrical chromatographic peaks. The method showed good linearity within the concentration range of 40-60 µg/mL for all analytes, with correlation coefficients greater than 0.998. Precision studies showed low %RSD values for both intra-day (0.01-0.79%) and inter-day (0.08-0.85%) analyses. Accuracy was evaluated through recovery studies, with intra-day recoveries ranging from 99.31-101.74% and inter-day recoveries ranging from 99.83-101.56%. Robustness testing confirmed that small variations in chromatographic conditions did not influence the analytical performance. The method was validated according to ICH Q2(R1) guidelines and demonstrated excellent linearity, precision, accuracy and robustness. Therefore, the developed RP-HPLC method is suitable for simultaneous quantitative analysis of these drugs.

Key words: RP-HPLC; losartan potassium; naproxen; pioglitazone hydrochloride; method validation.

INTRODUCTION

The co-existence of hypertension, type 2 diabetes mellitus, and inflammatory conditions has become increasingly prevalent in modern clinical practice.[1] As a result, polypharmacy involving antihypertensive, antidiabetic, and anti-inflammatory agents is often prescribed to manage these comorbidities simultaneously.[2–4] Among these therapeutic agents, losartan potassium, naproxen, and pioglitazone hydrochloride are widely used for managing hypertension, inflammation, and type 2 diabetes, respectively.[5]

Losartan potassium is an angiotensin II receptor antagonist that is primarily indicated for the treatment of hypertension. It provides effective blood pressure control and is often prescribed to patients who are intolerant to angiotensin-converting enzyme inhibitors.[6,7] Naproxen is a non-steroidal anti-inflammatory drug (NSAID) frequently used for managing pain, inflammation, and fever associated with various conditions including osteoarthritis, rheumatoid arthritis and musculoskeletal injuries.[8] Pioglitazone hydrochloride belongs to the thiazolidinedione class of antidiabetic drugs and is used to enhance insulin sensitivity in patients with type 2 diabetes.[4] It has also shown promising results in reducing the risk of progression from prediabetes to overt diabetes. [9,10]

Correspondence to: A. K. M. Nur Alam Siddiki
Email address: nuralam@du.ac.bd
Mobile: +8801716580850

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Given the therapeutic value of these three agents, their co-administration is not uncommon. However, simultaneous administration also necessitates the availability of robust analytical methods for their combined quantification, especially for the purposes of quality control, dosage form analysis, and pharmacokinetic studies.[11,12] Currently, there is a significant lack of validated analytical methods for the simultaneous estimation of losartan, naproxen, and pioglitazone in a single run using high-performance liquid chromatography (HPLC).[13] Existing methods typically focus on single or dual drug combinations, and do not adequately address the analytical challenges posed by this specific three-drug combination.[11,14]

Although several HPLC methods have been reported for individual drugs and their binary combinations, no validated method has been reported for the simultaneous determination of this specific three-drug combination in a single run. Therefore, the objective of the present study was to develop and validate a simple, rapid and cost-effective RP-HPLC method for the simultaneous determination of losartan potassium, naproxen and pioglitazone hydrochloride. The method was validated according to the guidelines of the International Council for Harmonisation (ICH) with respect to linearity, precision, accuracy and robustness. The aim was to establish a reliable analytical procedure for routine pharmaceutical analysis.

MATERIALS AND METHODS

Chemicals and reagents. Reference standards of losartan potassium (99.05%), naproxen (99.9%) and pioglitazone hydrochloride (99.61%) were kindly supplied by Drug International Ltd., Dhaka, Bangladesh. HPLC-grade acetonitrile and glacial acetic acid were obtained from the Drug Research Laboratory at the Centre for Advanced Research in Sciences (CARS), University of Dhaka. Distilled water was used throughout the study. All reagents and solvents used were of analytical or HPLC grade.

Instrumentation and chromatographic conditions. The chromatographic analysis was carried out using a Shimadzu UFLC Prominence HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a binary pump, an autosampler (SIl-20AC HT) and a UV-visible detector (SPD-20A). Data acquisition and processing were performed using LC-Solution software.

Separation of the analytes was achieved on a Phenomenex Gemini C18 column (250 × 4.6 mm, 5 µm). The mobile phase consisted of acetonitrile and 2% acetic acid in water in the ratio of 60:40 (v/v). The mobile phase was pumped at a flow rate of 0.7 ml/min and the injection volume was fixed at 20 µl. Detection was carried out at a wavelength of 230 nm. All analyses were performed at ambient temperature (25 ± 2°C). Before use, the mobile phase was filtered through a 0.45 µm membrane filter and degassed to remove any dissolved gases.

Preparation of standard and working solutions

Stock solution. Stock solutions of losartan potassium, naproxen and pioglitazone hydrochloride were prepared by accurately weighing 10 mg of each drug and transferring them into a 100 ml volumetric flask. The drugs were dissolved in a mixture of acetonitrile and water and the volume was made up to the mark with the same solvent system to obtain stock solutions containing 100 µg/ml of each analyte. The selected concentration levels for linearity, precision, and accuracy studies were chosen to represent the working concentration range and typical assay levels used in routine pharmaceutical analysis.

Linearity solutions. Working solutions for linearity studies were prepared by appropriate dilution of the stock solution with distilled water to obtain concentrations of 40, 45, 50, 55 and 60 µg/ml.

Accuracy solutions. Accuracy studies were carried out using solutions containing 34 and 36 µg/mL, which were prepared by suitable dilution of the stock solution with distilled water.

Precision solutions. For precision studies, working solutions of 38, 42 and 44 µg/ml were prepared by further dilution of the stock solution.

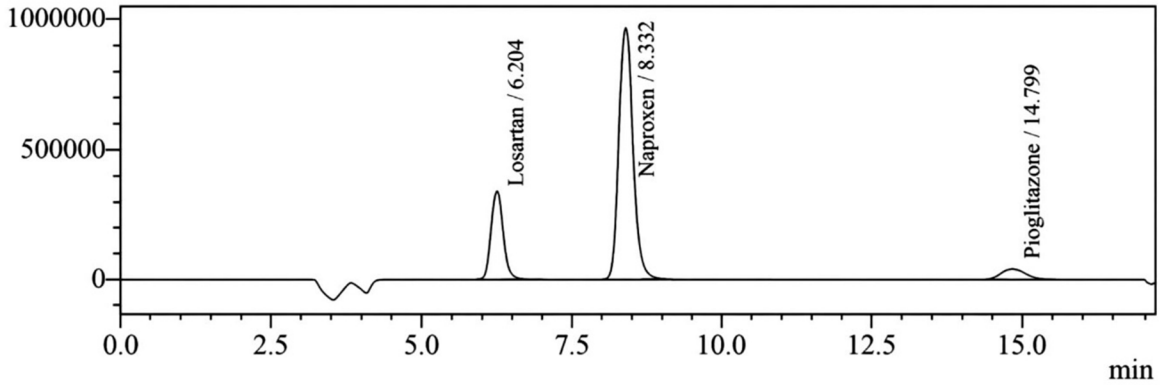
Method validation. The developed RP-HPLC method was validated in accordance with the

international council for harmonisation (ICH) guideline Q2(R1). The validation was carried out by evaluating key performance characteristics including linearity, precision, accuracy and robustness.

System suitability. Prior to analysis, system suitability tests were performed by repeated injection of the standard solution under optimized chromatographic conditions. Parameters such as retention time, theoretical plate count, tailing factor and resolution were evaluated to ensure that the chromatographic system was operating properly.

RESULTS AND DISCUSSION

Method development and optimization. Several mobile phase compositions were tested using mixtures of acetonitrile and aqueous acetic acid (2-5% v/v) at different pH values (2.5-4.5) and ratios. The flow rate was examined from 0.50-0.70 ml/min and the detection wavelengths were tested between 215-254 nm. Initial trials produced broad peaks with poor resolution, indicating the need for fine optimization. The optimal chromatographic conditions were achieved using a mobile phase consisting of acetonitrile : 2% acetic acid in water (60:40, v/v; pH 2.7) at a flow rate of 0.7 mL/min under ambient temperature (25 ± 2°C). This acidic condition was selected to suppress ionization of naproxen and maintain pioglitazone in its protonated form, resulting in improved peak shape, resolution, and symmetry. The injection volume was set at 20 µL. Under these conditions, all three drugs were well-resolved with sharp, symmetric peaks (Figure 1).



Name	Ret. Time (min)	Area	Theoretical Plate (N)	Tailing Factor	Resolution
Losartan	6.204	4,646,386	4,818.167	1.183	0.000
Naproxen	8.332	17,129,992	5,605.472	1.182	5.301
Pioglitazone	14.799	1,079,871	6,814.235	1.168	11.128

Figure 1. Representative RP-HPLC chromatogram showing separation of losartan potassium, naproxen and pioglitazone hydrochloride under optimized conditions.

The retention times of the drugs under optimized conditions were as follows.

Drug	Retention time (min)
Losartan potassium	6.24 ± 0.1
Naproxen	8.34 ± 0.1
Pioglitazone HCl	14.86 ± 0.1

The method provided reproducible, sharp peaks with good resolution, indicating its suitability for simultaneous quantification of the three drugs in mixture. These conditions were subsequently used for validation studies.

Method Validation. The developed RP-HPLC method was validated as per ICH Q2(R1) guidelines with respect to linearity, precision, accuracy and robustness.

Linearity. Linearity was assessed using five different concentrations (40, 45, 50, 55, 60 µg/ml) for each drug. Each solution (20 µl) was injected in triplicate, and the peak area was plotted against concentration. The selected concentration range was chosen to reflect the typical working concentrations obtained after dilution of pharmaceutical dosage forms for routine analysis. The calibration curves (Figure 2) exhibited excellent linearity, with correlation coefficients (r^2) ≥ 0.998 for all three drugs. The regression analysis indicates a strong linear relationship between concentration and peak area over the studied range.

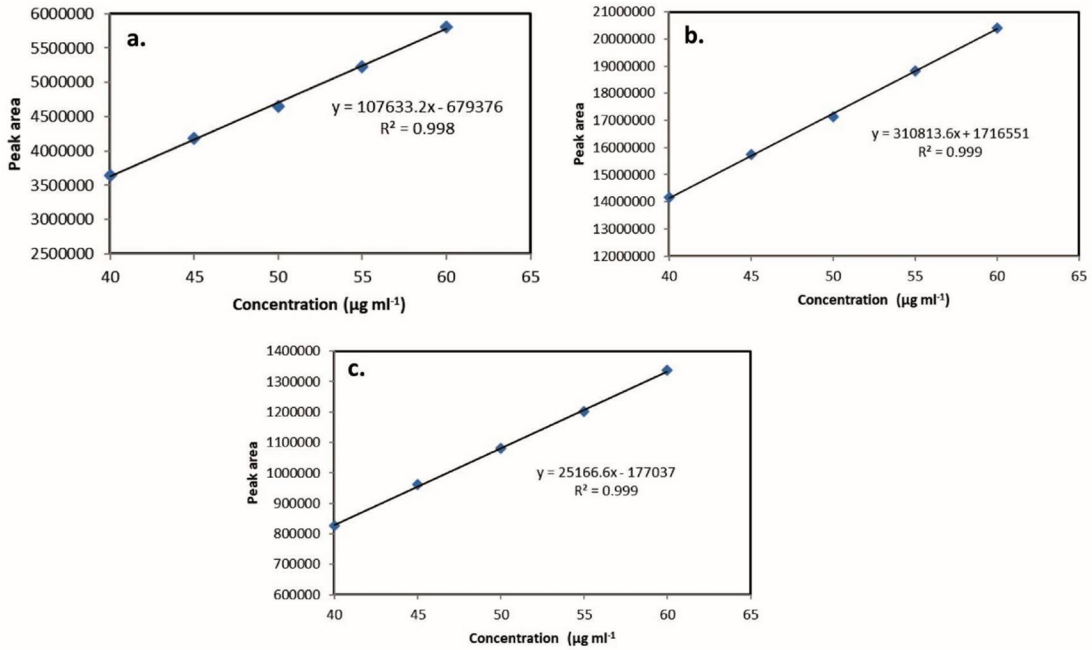


Figure 2. Calibration curves showing the linear relationship between peak area and concentration for (a) losartan potassium, (b) naproxen and (c) pioglitazone hydrochloride in the range of 40-60 µg/ml.

Table 1. Linearity parameters of the developed RP-HPLC method for losartan potassium, naproxen and pioglitazone hydrochloride.

Drug	Concentration (µg/ml)	Regression equation	r^2
Losartan potassium	40-60	y = 107633.2x – 679376	0.998
Naproxen	40-60	y = 310813.6x + 1716551	0.999
Pioglitazone HCl	40-60	y = 25166.6x – 177037	0.999

Limit of detection (LOD) and limit of quantification (LOQ). The limit of detection (LOD) and limit of quantification (LOQ) of the developed RP-HPLC method were determined in accordance with ICH Q2(R1) guidelines using the equations LOD = 3.3σ/S and LOQ = 10σ/S, where S represents the slope of the calibration curve and σ is the standard deviation of the analytical response. The value of σ was calculated from replicate peak area responses (n = 3) at the lowest concentration level of the calibration curve. The calculated LOD values were found to be 0.61 µg/ml for losartan potassium, 0.05 µg/ml for naproxen, and 0.56 µg/mL for

pioglitazone hydrochloride, while the corresponding LOQ values were 1.84 µg/ml, 0.15 µg/ml and 1.71 µg/ml respectively (Table 2). These results indicate that the developed method exhibits adequate sensitivity for the simultaneous determination of all three analytes. The comparatively lower LOD and LOQ values observed for naproxen may be attributed to its higher detector response and lower variability in peak area measurements, resulting in an improved signal-to-noise ratio under the optimized chromatographic conditions.

Table 2. Limit of detection (LOD) and limit of quantification (LOQ) of the developed RP-HPLC method.

Drug	LOD (µg/ml)	LOQ (µg/ml)
Losartan potassium	0.61	1.84
Naproxen	0.05	0.15
Pioglitazone hydrochloride	0.56	1.71

Table 3. Intra-day precision of the developed RP-HPLC method for simultaneous determination of losartan potassium, naproxen and pioglitazone hydrochloride.

Drug	Injected concentration (µg/ml)	Mean peak area ± SD (n=3)	Mean recovered concentration ± SD (µg/ml)	% RSD
Losartan potassium	38	3,435,880 ± 21,044	38.23 ± 0.20	0.52
	42	3,907,578 ± 2,629	42.62 ± 0.02	0.05
	44	4,023,240 ± 1,376	43.69 ± 0.01	0.02
Naproxen	38	13,661,344 ± 504	38.43 ± 0.01	0.01
	42	14,815,282 ± 1,215	42.36 ± 0.01	0.01
	44	15,236,976 ± 13,172	43.50 ± 0.04	0.10
Pioglitazone HCl	38	772,905 ± 4,298	37.74 ± 0.17	0.46
	42	887,172 ± 3,216	42.28 ± 0.12	0.29
	44	939,388 ± 9,073	44.36 ± 0.35	0.79

Precision / Reproducibility

Intra-day precision. The intra-day precision of the developed RP-HPLC method was assessed by analysis of three replicate injections of losartan potassium, naproxen and pioglitazone HCl at three concentration levels (38, 42 and 44 µg/ml) on the same day. Precision was expressed as relative standard deviation (%RSD) of the concentrations recovered. The results showed that the chromatographic method had an excellent repeatability. The values for %RSD of losartan potassium were found to range between 0.02-0.52% and % RSD values for naproxen were between 0.01-0.10%. Similarly, the %RSD values of pioglitazone HCl ranged from 0.29-0.79%. According to ICH Q2(R1) guidelines, % RSD values below 2% are considered to be acceptable analytical precision. The low %RSD values demonstrated excellent repeatability of the method. The mean recovered concentrations were in a very good agreement with the nominal injected concentrations, confirming the reliability and quantitative capability of the developed analytical method.

Inter-day precision. The inter-day precision of the method was assessed by analysis of standard solutions of the three drugs at three concentrations (38, 42 and 44 µg/ml) on two consecutive days. The reproducibility of the method was evaluated by determining the mean concentrations recovered, standard deviation (SD) and % RSD. For losartan potassium, the % RSD values ranged from 0.13 to 0.34%. The minimal variation between Day 1 and Day 2 results demonstrates the stability of the chromatographic system under repeated analytical conditions. Similar behavior was observed for naproxen with values of % RSD ranging between 0.08 and 0.18%. These low variability values are a reflection of a high level of reproducibility and indicated that the method gives reliable quantification in different analytical runs. In the case of pioglitazone HCl, slightly higher values of % RSD (0.38-0.85%) were obtained in comparison to the other two analytes. Since all observed values were well within the acceptable limits (<2%), the results

indicate that the RP-HPLC method developed has adequate reproducibility and can be applied for routine quantitative analysis.

Accuracy/Recovery. A representative chromatogram obtained at the intermediate concentration level (42 µg/ml) is presented in Figure 3, showing well-resolved peaks with stable retention times and symmetrical peak shapes, which supports the precision and consistency of the chromatographic system.

Table 4. Inter-day precision of the developed RP-HPLC method for simultaneous determination of losartan potassium, naproxen and pioglitazone hydrochloride.

Drug	Concentration (µg/ml)	Day 1 recovered (µg/ml)	Day 2 recovered (µg/ml)	Mean ± SD	% RSD
Losartan potassium	38	38.16	38.00	38.08 ± 0.11	0.29
	42	42.68	42.60	42.64 ± 0.06	0.13
	44	43.91	43.70	43.81 ± 0.15	0.34
Naproxen	38	38.48	38.40	38.44 ± 0.06	0.16
	42	42.41	42.36	42.38 ± 0.04	0.08
	44	43.67	43.55	43.61 ± 0.08	0.18
Pioglitazone HCl	38	38.39	37.94	38.17 ± 0.32	0.84
	42	42.65	42.14	42.40 ± 0.36	0.85
	44	44.49	44.25	44.37 ± 0.17	0.38

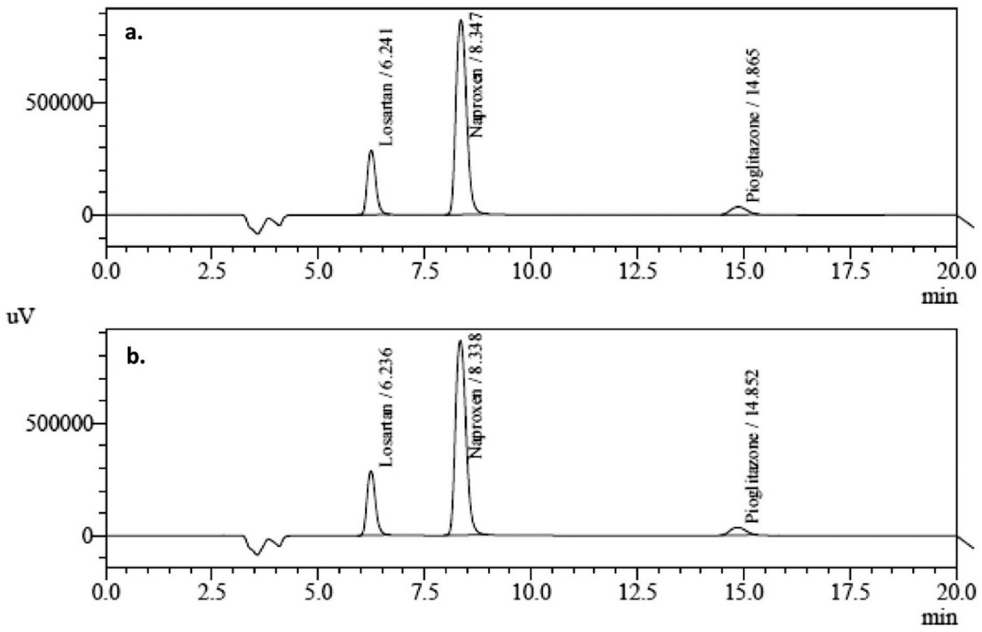


Figure 3. Representative chromatograms obtained at 42 µg/ml showing reproducible peak responses for losartan potassium, naproxen and pioglitazone hydrochloride: (a) intra-day chromatogram and (b) inter-day chromatogram.

Table 5. Intra-day accuracy (recovery) of the developed RP-HPLC method for losartan potassium, naproxen and pioglitazone hydrochloride.

Drug	Injected conc. (µg/mL)	Mean recovered (µg/ml) ± SD	Mean recovery (%) ± SD	%RSD
Losartan potassium	34	34.27 ± 0.02	100.78 ± 0.04	0.04
	36	36.63 ± 0.21	101.74 ± 0.60	0.59
Naproxen	34	34.39 ± 0.02	101.13 ± 0.04	0.04
	36	36.10 ± 0.02	100.29 ± 0.06	0.06
Pioglitazone HCl	34	34.46 ± 0.10	101.36 ± 0.28	0.28
	36	35.75 ± 0.30	99.31 ± 0.84	0.85

Intra-day accuracy. The intra-day accuracy of the developed RP-HPLC method was evaluated by performing recovery studies at two concentration levels (34 and 36 µg/ml) for losartan potassium, naproxen and pioglitazone HCl. The selected concentration levels were used to evaluate method accuracy near the lower concentration region. Each concentration level was analyzed in triplicate within the same day and the accuracy of the method was expressed as percentage recovery of the injected concentration. The recovery values for losartan potassium ranged from 100.78 to 101.74% and naproxen with recovery values between 100.29 and 101.13%. Similarly, pioglitazone HCl also showed recovery values of 99.31 to 101.36%. According to ICH Q2(R1) guidelines, recovery values in the range of 98-102% are acceptable values for pharmaceutical analysis. The values of %RSD for all the analytes were found to be less than 1% which indicated excellent accuracy for an analytical procedure within the same day.

Inter-day accuracy. The inter-day accuracy of the developed RP-HPLC method was evaluated by conducting the recovery studies on two different days at the same concentration levels (34 and 36 µg/ml). The recovered concentrations achieved at Day1 and Day2 were used to calculate the mean recovery values, standard deviation (SD) and % RSD. For losartan potassium, the recovery values were between 100.72-101.12% and for naproxen between 100.59-101.22%. In case of pioglitazone HCl, recovery values were between 99.83- 101.56%. The % RSD values for all analytes were calculated to be less than 2% which demonstrates good reproducibility of analytical method between different days. The fact that the recovery results are close to 100% consistently demonstrate that the method is accurate for normal day-to-day simultaneous analytical measurement of losartan potassium, naproxen and pioglitazone HCl.

Table 6. Inter-day accuracy (recovery) of the developed RP-HPLC method for losartan potassium, naproxen and pioglitazone hydrochloride.

Drug	Injected conc. (µg/ml)	Day 1 recovered (µg/ml)	Day 2 recovered (µg/ml)	Mean ± SD (µg/ml)	%Recovery	%RSD
Losartan potassium	34	34.24	34.25	34.25 ± 0.01	100.72	0.03
	36	36.43	36.38	36.41 ± 0.04	101.12	0.11
Naproxen	34	34.42	34.40	34.41 ± 0.01	101.22	0.03
	36	36.31	36.12	36.22 ± 0.13	100.59	0.36
Pioglitazone HCl	34	34.50	34.56	34.53 ± 0.04	101.56	0.12
	36	36.31	35.56	35.94 ± 0.53	99.83	1.47

Table 7. Robustness study of the developed RP-HPLC method for losartan potassium, naproxen and pioglitazone hydrochloride.

Parameter	Condition	% RSD Losartan	% RSD Naproxen	% RSD Pioglitazone
Flow rate (ml/min)	0.6	0.71	0.63	0.82
	0.7 (optimized)	0.52	0.46	0.63
	0.8	0.69	0.58	0.76
Mobile phase (acetonitrile : 2% acetic acid)	62:38	0.65	0.54	0.73
	60:40 (optimized)	0.52	0.46	0.63
	58:42	0.66	0.57	0.75
Detection wavelength (nm)	228	0.60	0.51	0.70
	230 (optimized)	0.54	0.47	0.63
	232	0.62	0.52	0.69

Robustness. The robustness of the developed RP-HPLC method was investigated by varying the chromatographic parameters slightly; flow rate (± 0.1 ml/min), mobile phase composition (± 2% organic solvent), and detection wavelength (± 2 nm). These variations were applied in order to establish the reliability and stability of the method under slightly changed experimental conditions. When the flow rate

was varied from 0.6 to 0.8 ml/min, the percentage relative standard deviation (RSD) values of all the analytes were kept within acceptable limits which implied that small variations in flow rate did not have significant effects on the chromatographic performance. Similarly, changes to the composition of the mobile phase (62:38 and 58:42 acetonitrile: 2% acetic acid in water) did not yield any major differences in the responses of the peaks, which proved that the separation was stable under these conditions. In addition, slight changes in detection wavelength (228-232 nm) also demonstrated minimal variation in the % RSD values for all of the analytes. In all cases the % RSD values were found less than 2% which complies with the generally accepted criteria for analytical method robustness according to the ICH Q2(R1) guidelines. Furthermore, no significant variation in retention times or loss of resolution was observed under these conditions, confirming the stability of peak separation. The developed RP-HPLC method has been found to be robust and capable of producing consistent and reliable analytical results despite small variations in the chromatographic conditions.

Summary of validation parameters. The comprehensive validation results summarized in Table 8 confirm that the developed RP-HPLC method satisfies all the requirements for analytical method validation recommended by ICH Q2(R1) guidelines**.** The consistent analytical performance observed across different validation parameters demonstrates the reliability and stability of the chromatographic method.

Table 8. Summary of validation parameters of the developed RP-HPLC method for simultaneous determination of losartan potassium, naproxen and pioglitazone hydrochloride.

Validation parameter	Losartan potassium	Naproxen	Pioglitazone HCl
Linearity range (µg/ml)	40–60	40–60	40–60
Regression equation	y = 107633.2x − 679376	y = 310813.6x + 1716551	y = 25166.6x − 177037
Correlation coefficient (r^2)	0.998	0.999	0.999
LOD (µg/ml)	0.61	0.05	0.56
LOQ (µg/ml)	1.84	0.15	1.71
Intra-day precision (%RSD)	0.02–0.52	0.01–0.10	0.29–0.79
Inter-day precision (%RSD)	0.13–0.34	0.08–0.18	0.38–0.85
Intra-day accuracy (%Recovery)	100.78–101.74	100.29–101.13	99.31–101.36
Inter-day accuracy (%Recovery)	100.72–101.12	100.59–101.22	99.83–101.56
Robustness (%RSD)	< 2	< 2	< 2

CONCLUSION

A simple, rapid and reliable RP-HPLC method was successfully developed and validated for the simultaneous determination of losartan potassium, naproxen and pioglitazone hydrochloride. Chromatographic separation was performed using acetonitrile and 2% acetic acid in water (60:40, v/v) as the mobile phase at a flow rate of 0.7 ml/min with UV detection at 230 nm. The method showed excellent linearity ($r^2 > 0.998$) over the concentration range of 40-60 µg/ml, with %RSD values below 2% and recovery within 98-102%, confirming its precision and accuracy. Therefore, the proposed RP-HPLC method is precise, accurate and robust and can be applied for the simultaneous quantitative analysis of these drugs. The method offers advantages such as simplicity, cost-effectiveness, and reliable performance for routine quality control analysis of pharmaceutical formulations.

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