Development of new stability indicating UPLC-UV method for the extraction and quantification of perindopril and indapamide from human plasma

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Abstract

Background: The hypertension and cardiovascular ailments are the leading cause of deaths worldwide. The combination therapy was found to be effective on the cardiovascular illness by reducing the blood pressure. The indapamide and perindopril combination therapy showed excellent results on reducing high blood pressure. With this in mind, the stability indicating reverse phase UPLC method was developed for the simultaneous identification and quantification of indapamide and perindopril from human plasma. In this work, we developed a new solid phase extraction method for the extraction of indapamide and perindopril in human plasma. It is a simple, accurate, and selective method for the extraction of these two drugs from human plasma with elution time of 2 min. The extracted drugs were identified and quantified by using stability indicating UPLC method. The method showed high recovery rate as well as low detection and quantification limits of two drugs.

Results: A novel, simple, highly accurate, and precise stability indicating ultra-performance liquid chromatography (UPLC) method was developed for the identification and quantification of perindopril (PP) (brand name Coversyl) and indapamide (IP) (brand name Lorvas) from human plasma. In this UPLC method, HSS C18 column (100 × 2.1 mm, 1.8 μm) and mobile phase acetonitrile (ACN), 10 mM KH2PO4 buffer solution (pH 3.0) mixture was used in the ratio of 65:35. Column temperature of 30 °C, flow rate of 1.0 mL per minute and UV wave length of 254 nm were used. PP and IP were eluted below 2 min runtime with high resolution. Solid phase extraction (SPE) method was used for the extraction of PP and IP from human plasma. Different solvents were used to extract the analyte from SPE such as ACN, methanol, acetone, tertiary butyl diethyl ether (TBDE), chloroform (CHCl3), and ethanol (EtOH). Among these, ACN gave good recovery percentages (94.56 to 101.58%). From the linearity graph, good correlation coefficient values of 0.9996 for PP and 0.9997 for IP were achieved. The coefficient variance values for intra and inter day precision is in between 1.08 and 12.5%. The LOD and LOQ values were determined by the signal to noise ratio method. LOD and LOQ values for IP and PP were found to be 8.6 and 33.5 ng/mL and 28.33 and 110.5 ng/mL respectively. The developed method was statistically validated as per ICH guidelines.

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**Conclusion:** In summary, a novel stability indicating UPLC-UV method was developed and validated for the simultaneous identification and quantification of perindopril and indapamide drugs in human plasma and tested the stability as per ICH guidelines. It is a simple, accurate, and specific method for the extraction of these two drugs from human plasma and eluted within 2 min runtime. The method showed high recovery rate as well as low detection and quantification limits of two drugs. The developed method is suitable for routine analysis as well as in bioanalytical and clinical studies.

**Keywords:** Perindopril, Indapamide, UPLC, Solid-phase extraction, Hypertension

**Background**
Cardiovascular ailments such as hypertension and strokes are the most leading diseases to cause death worldwide. In general, the monotherapy was used to reduce the blood pressure in patients, but it works in few patients only. Therefore, the combination therapy was introduced to effectively reduce the cardiovascular illnesses [1, 2]. Combination therapy has been found to decrease the hypertension, due to their numerous mechanisms of actions and showed reduced side effects due to the lower dosage of medications [3–6].

Perindopril is commonly used to treat coronary artery ailments such as heart failure and hypertensions [7–10]. The chemical name of perindopril is (2S,3aS,7aS)-1-[(2S)-2-[(2S)-1-ethoxy-1-oxopentan-2-yl] aminol]propionic-octahydro-1H-indole-2-carboxylic acid. It is an ethyl ester of non-sulphahydryl derivative and angiotensin converting enzyme (ACE) inhibitor, it selectively reduces the level of angiotensin I by converting it to angiotensin II due to the hydroxylation of perindopril to produce its active metabolite perindoprilat. Consequently, it inhibits the angiotensin II activities like stimulation of aldosterone secretion in adrenal cortex and vasoconstrictions [11, 12].

Indapamide (4-chloro-N-(2-methyl-2,3-dihydroindol-1-yl)-3-sulfamoylbenzamide) is a thiazide or sulphonamide derivative. It is used to treat the mild to moderate hypertensions due to the activities of calcium antagonist and diuretic effects [13–15]. Different studies revealed the antihypertension activity of indapamide due to the peripheral vascular resistance and decreasing the vascular reactivity.

The indapamide and perindopril combination therapy has shown to be effective for reducing high blood pressure [16–19]. The structures of indapamide and perindopril showed in Fig. 1. This combination therapy has proved to show major effects on microcirculatory alterations, systolic blood pressure and arterial stiffness. Thus, many pharmaceutical industries competing to produce the various combinations of indapamide hemihydrate and perindopril arginine to prevent the hypertension and maintain the normal blood pressure in patients. The British and US pharmacopeias officially approved indapamide, and perindopril was approved by British pharmacopeia [13, 20].

The number of methods was reported for the identification and quantification of indapamide and perindopril individually. To the best of our knowledge, only few methods have been reported for the simultaneous estimation of indapamide and perindopril drugs in biological fluids [21–26]. Till now, no one reported the stability indicating reverse phase UPLC method for the simultaneously identification and quantification of indapamide and perindopril from human plasma. Therefore, the current study demonstrated the stability of perindopril and indapamide by doing the forced degradation studies, successfully extracted and quantified these two drug molecules from human plasma.

**Methods**

**Pure samples**
The reference standards perindopril (potency (99.8)), indapamide (potency (99.8)), and active pharmaceutical ingredients (API) were obtained from Mylan laboratories and Glenmark pharmaceutical as gifted samples.

**Chemical and reagents**
For the UPLC analysis and extraction, analytical grade methanol, ACN, TBDE, chloroform (CHCl₃), and ethanol
(EtOH) were procured from Sigma Aldrich, USA, hydrogen peroxide (H2O2), hydrochloric acid (HCl), KH2PO4, orthophosphoric acid, and sodium hydroxide (NaOH) were purchased from Sigma Aldrich, USA, Milli-Q system Millipore, USA, used for the purification of water.

**Instrumentation**

Stability indicating method development and validation was established on UPLC (Acquity, Waters, USA) by using the empower II software. Acquity HSS C18 column (waters) was used for the eluting the peaks with high resolution. Milli-Q Millipore system (USA) was used for the purification of water. Sonicator (sonica, spinotech-Italy), vortex mixture (India), Elico pH meters, and 0.45-micron nylon filters (Merck, Millipore) were used in the sample preparations.

**Analytical conditions**

Isocratic method was used for simultaneous determination and quantification of indapamide and perindopril in human plasma. Mobile phase acetonitrile ACN and 10 mM KH2PO4 buffer solution (pH 3.0) was used in the ratio of 65:35. Column oven temperature was maintained at 30 °C, flow rate of 1 mL per minute and injection volume of 10 μL, UV wavelength (λ max) of 254 nm and run time is 5 min were used. Stock solutions were prepared in ACN, and the mobile phase was used as a diluent for the further dilutions.

**Preparations of linearity and quality control (QC) sample solutions**

Stock solutions of perindopril (1 mg/mL) and indapamide (1 mg/mL) were prepared in ACN and stored at −4 °C. The working standard solutions were prepared by diluting the stock solutions by using mobile phase. Six different concentrations of perindopril (0.75, 1.5, 3.0, 7.0, 15.0, and 25.0 μg/mL) and indapamide (0.15, 0.45, 1.25, 2.5, 7.5, and 12.0 μg/mL) spiked solutions with human blank plasma were prepared for the construction of calibration curve. Three different quality control samples: low (LQC), middle (MQC), and high (HQC) were prepared individually by spiking with 500 μL aliquots of human blank plasma. The solutions of indapamide at the LQC 0.45, MQC 2.5, and HQC 12.0 μg/mL and perindopril at the LQC 1.5, MQC 7.0, and HQC 25.0 μg/mL solutions were prepared. All spiked solutions were stored in refrigerator at −20 °C.

**Extraction procedure**

Human plasma (250 μL) was taken in 2 mL Eppendorf tube containing 500 μL of potassium phosphate buffer (pH 4). The samples were mixed with vortex for few minutes. Then the samples were loaded into the Oasis HLB (1 mL, 30 mg) cartridges. Before loading the sample in to solid phase (cartridges), these were preconditioned with 1 mL of acetonitrile, 1 mL of methanol, and 1 mL of water: acetonitrile (95:05 v/v). The sample loaded cartridges are washed five times with 1 mL of methanol: water (10: 90, v/v) at −30 kPa [27]. Then, the sorbents were dried for 10 min under the air flow. After drying, the analytes were eluted from the sorbent by using 1 mL of acetonitrile under gentle vacuum. The solution was evaporated by using N2 gas at 40 °C in vacuum oven. The sample residues were reconstituted by adding 100 μL of mobile phase solution, vortexed for 2 min, and sonicated for 1 min. Then, the solutions were filtered by using 0.2-micron nylon filters, and 10 μL of sample solution was directly injected in to UPLC for analysis.

**Forced degradation studies of drug substances**

The stability of the developed UPLC method was examined by doing the forced degradation of drug substances. The stability studies of drugs were carried out in presence of acidic, basic, neutral, oxidative, photolytic, and thermal conditions.

**Acidic degradation**

During the acidic degradation of drug substances, 1.0 N hydrochloric acid (HCl) was used. One milliliter of indapamide (1 mg/mL) and perindopril (1 mg/mL) mixed solution was taken in 10 mL volumetric flask stirred with 1 mL of 1.0 N HCl for 1 h at 60 ± 2 °C. The acid treated solution was neutralized with 1 mL of 1.0 N sodium hydroxide for preventing further degradations and make up to the volume with diluent.

**Basic degradation**

Alkaline degradation study of drug molecules was carried out by using 1.0 N sodium hydroxide solution (NaOH). In total, 1.0 mL stock solution was refluxed with 1.0 mL 1.0 N NaOH in 10.0 mL volumetric flask at 60 ± 2 °C for 1 h. The solution was neutralized by adding 1.0 mL of 1.0 N HCl solution and make up to the mark with diluent.

**Thermal degradation**

Thermal degradation studies were carried out at 80 ± 5 °C for 8 h. One milliliter of sample solution was taken in 10.0 mL volumetric flask and kept under the abovementioned conditions and then solution was diluted with diluent.

**Oxidative degradation**

Oxidative stress study of drugs was carried out by using 10% hydrogen peroxide (H2O2). Suspension of 1.0 mL of drugs solution into 1.0 mL of 30% H2O2 and stirred at
30 ± 2 °C for 1 h then the solution was diluted up to 10.0 mL with diluent.

**Photolytic degradation**
Photolytic degradation was carried out by using UV light. One milliliter of sample solution was exposed to UV light for 24 h in UV chamber, and the solution was diluted up to 10.0 mL by using diluent.

**Neutral degradation**
HPLC grade water was used in the neutral degradation. One milliliter HPLC grade water was added into 1.0 mL sample solution and refluxed at 60 ± 2 °C for 5 h and then solution was diluted up to 10 mL with diluent.

All degradation samples are filtered with 0.45-micron nylon filters and each degradation sample (10.0 μL) was injected individually in to UPLC and recorded the chromatogram.

**Results**

**Method development and optimization**
The main objectives of this work are the isolation, quantification of IP, and PP drug molecules form human plasma by developing a novel stability indicating UPLC method. In the process of method development, different compositions of mobile phases with different pH were used. In general, the pH of the buffer will retain the ionization of compounds and provide low tailing factor values. The pKa values of indapamide and perindopril are 8.8 (nearly neutral) and 5.7 (weak acidic) respectively. The selected mobile phase acetonitrile and KH2PO4 buffer (pH=3) (65:35) would give good retention and tailing factor peaks. The optimized chromatograms were shown in Fig. 2. Among all the columns used in the current method, HSS C18 column (100×2.1 mm, 1.8 μm) was found to be suitable for getting adequate resolution, plate count, and tailing factors (indapamide (1.7) and perindopril (1.6)) for the drug molecules simultaneously. This column gave high theoretical plate count values (indapamide (8254) and perindopril (6411)), which indicates the efficiency of column for the separation with high resolution, narrow, and sharp peaks. The system parameters such as flow rate (1.0 mL/min) and injection volume (10.0 μL) were optimized based on the minimal consumption of mobile phase and peak resolution.

In the forced degradation studies, the drug degradation between 5.0 to 20% is acceptable and that was considered

![Fig. 2 Optimized resulting UPLC chromatograms with different columns](image)
as stability indicating method and is reasonable for the validation of chromatographic method. In the present method, stability studies were carried out in different conditions such as acidic, basic, neutral, thermal, oxidation, and photolysis. In those, the drugs were degraded only in acidic and basic conditions and were stable in remaining conditions. In acidic condition, 1.56% of indapamide and 8.65% of perindopril and in basic condition, 6.42% of indapamide, and 3.59% of perindopril were degraded (Fig. S2 and Table 1). The assay % of drugs were calculated by using the following formula:

$$\% \text{Assay} = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Dilution of standard}}{\text{Dilution of sample}} \times 100$$

Solid phase extraction (SPE) method was used for the extraction of drug molecules from human plasma. Various organic solvents (MeOH, ACN, CHCl₃, EtOH, acetone, and TBDE) were used for the extraction of drug molecules from SPE, comparing with all of those ACN was given good recovery percentages of indapamide (96.64-98.64%) and perindopril (98.51-101.25%).

Method validation
The developed stability indicating UPLC-UV method was validated for the following parameters statistically as per the ICH guidelines [28].

| Stress condition | Time   | Assay% of PP after degradation | Assay% of IP after degradation | Remarks                                      |
|------------------|--------|--------------------------------|-------------------------------|---------------------------------------------|
| Unstressed sample | –      | 99.63                          | 100.2                         | –                                           |
| Acid hydrolysis (1.0N HCl) | 60 min | 91.15                          | 98.64                         | The significant degradation was observed. |
| Base hydrolysis (1.0N NaOH) | 60 min | 96.01                          | 93.78                         | The slight degradation of analytes was observed. |
| Oxidation (10% H₂O₂) | 60 min | 98.65                          | 99.12                         | Significant degradation was not observed.  |
| Thermal (80 °C)   | 8 h    | 99.32                          | 99.81                         | Significant degradation was not observed.  |
| Photolytic degradation | 24 h   | 99.53                          | 99.98                         | Significant degradation was not observed.  |

Selectivity
Simultaneous extraction of perindopril and indapamide was carried out from human plasma by using SPE method. Perindopril and indapamide peaks are arrived (0.87 and 1.16) within 2 min of retention time (Fig. 3). The chromatograms revealed that the peaks are pure, symmetric, well separated, and no other endogenous peaks are eluted. The results indicated that the SPE method was sufficient to isolate the perindopril and indapamide from human plasma.

Specificity
The specificity and selectivity was determined by running six different blank plasma sample solutions using the above optimized UPLC method. No interference peaks were observed at respective retention times of perindopril and indapamide. Moreover, the specified drugs also did not give any interference peaks during the analysis.

Calibration curves
The linearity of the developed method was evaluated by using standard solutions of six different concentrations of indapamide (0.15, 0.45, 1.25, 2.5, 7.5, and 12.0 μg/mL) and perindopril (0.75, 1.5, 3.0, 7.0, 15.0, and 25.0 μg/mL) plasma sample solutions. The linearity graphs were constructed by plotting the peak area against the concentrations of plasma sample solutions (Fig. 4), and the results are shown in Table 2. The linearity plots
gave acceptable correlation coefficient \( (R^2) \) values for indapamide (0.9997) and perindopril (0.9996).

**Precision and accuracy**
The precision was determined by using the different concentrations (LQC, MQC, and HQC) of QC samples. For determining the intra-day precision, the experiments were carried out five times within same day. The analysis was also done in different days for the inter-day precision measurements and both precision values are expressed in percentage (1.08-12.5\%) of cumulative variance (CV) of the peak area of three different QC sample solutions. The accuracy was determined in terms of mean percentage of analytes (94.56-101.2\%) recovered from the plasma (Table 3).

**LOD and LOQ**
Limit of detection (LOD) and limit of quantification (LOQ) of the developed method were assessed by using signal to noise (S/N) method. The LOD and LOQ are useful for the assessment of sensitivity of developed method. In this method, the LOD value of perindopril (33.5 ng/mL) and indapamide (8.6 ng/mL) was determined by using S/N ratio of baseline over 3 times signal level of sample. LOQ values of perindopril (110.5 ng/mL) and indapamide (28.33 ng/mL) was assessed by using S/N ratio of baseline over 10 times signal level of sample.

**Stability of solutions**
The study of solution stability also plays a key role for determining the effects on long time storage of solutions. For knowing stability of sample solutions, three different concentrations QC (LQC, MQC, and

### Table 2 Linearity of IP and PP

| IP (μg/mL) \( [X] \) | Mean peak area \( [Y] \) | PP (μg/mL) \( [X] \) | Mean peak area \( [Y] \) |
|----------------------|------------------------|----------------------|------------------------|
| 0.15                 | 15954                  | 0.75                 | 8139                   |
| 0.45                 | 21585                  | 1.50                 | 11751                  |
| 1.25                 | 38384                  | 3.00                 | 19584                  |
| 2.50                 | 62645                  | 7.00                 | 39258                  |
| 7.50                 | 159785                 | 15.0                 | 75581                  |
| 12.0                 | 242445                 | 25.0                 | 125842                 |

Linear regression equation: 
- IP: \( Y = 19163x + 13957 \)
- PP: \( Y = 4820x + 4710 \)

Slope: 19163, 4820
Intercept: 13957, 4710
Correlation coefficient \( (R^2) \): 0.9997, 0.9996

Average \( (n) =3 \)
HQC) samples are kept under the RT for 4 h, 4 °C for 48 h, and 7 days at −20 °C. No significant changes were observed during the stability studies and hence the sample solutions are stable under these conditions and the results are summarized in Table 4.

**Robustness**
The robustness of the developed method was evaluated by changing the conditions such as flow rate, column temperature, mobile phase ratio, and UV wavelength. The flow rate ±0.2 mL per minute, organic solvent of mobile phase ±5%, column oven temperature ±5 °C and UV wavelength of detector ±4 nm was changed for the evaluation of robustness of the developed method. The analyte solution containing 10 μg/mL concentration of each drug indapamide and perindopril were prepared for these studies. There is no substantial difference observed with the change in the abovementioned conditions and RSD values vary in between 1.04 and 5.77 (Table 5). These results indicate that the developed method have good performance and reliability even with small variation in method conditions.

**Discussion**
A new stability indicating UPLC method was developed to detect at low concentration (nanogram) level of perindopril and indapamide in human plasma. The established UPLC method was optimized adequately by using various parameters, different UPLC columns, mobile phases for satisfactory peak shape, high sensitivity, and good resolutions. The results showed that the UPLC-UV method is suitable for simultaneous quantification and identification of perindopril and indapamide drugs in human plasma. Very few LLE and SPE methods were reported for the quantification of perindopril and indapamide in human plasma [29–33] which are time consuming and cost effective for the separation of drugs from biological samples. However, there is no SPE stability indicating UPLC method was reported for the identification of these drugs. The method reported in the present paper is very simple, easy to operate, quick and less cumbersome, and showed adequate recovery percentage (93.00-101.58) of drugs. The established method was able to give low LOD and LOQ values and able to give high sensitivity, accuracy, and precision to determine indapamide and perindopril in human plasma.

**Conclusion**
In summary, a novel stability indicating UPLC-UV method was developed and validated for the simultaneous

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**Table 3** Validation parameters results for determination of perindopril and indapamide

| Analyte     | Nominal value (μg/mL) | Intra day | Inter day |
|-------------|-----------------------|-----------|-----------|
|             | Found concentration (μg/mL) | Precision (RSD %) | Accuracy (%) | Found concentration (ng/mL) | Precision (RSD %) | Accuracy (%) |
| Perindopril | 1.5                   | 1.46 ± 0.28 | 8.34       | 97.45       | 1.48 ± 0.65 | 6.48       | 97.12       |
|             | 7                     | 7.16 ± 0.83 | 4.66       | 101.58      | 7.24 ± 2.51 | 3.51       | 101.2       |
|             | 25                    | 25.12 ± 2.73 | 2.4        | 98.6        | 24.68 ± 1.54 | 1.08       | 98.05       |
| Indapamide  | 0.45                  | 0.39 ± 0.16 | 12.5       | 95.16       | 0.41 ± 0.62 | 11.12      | 94.56       |
|             | 2.5                   | 2.48 ± 0.79 | 5.36       | 100.4       | 2.49 ± 0.64 | 3.05       | 99.2        |
|             | 12                    | 11.22 ± 1.68 | 4.8        | 98.7        | 11.56 ± 3.5  | 3.61       | 97.92       |

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**Table 4** Stability studies of analytes in human plasma and mobile phase

| Analyte      | Nominal value (μg/mL) | Stability conditions Human plasma (analyte concentration (%)) | Mobile phase 4 °C (24 h) |
|--------------|-----------------------|-------------------------------------------------------------|--------------------------|
|              |                       | RT (4 h) | 4 °C (48 h) | −20 °C (15 days) | 4 °C (24 h) |
| Perindopril  | 1.5                   | 97.25    | 102.4       | 96.15           | 98.45       |
|              | 7                     | 100.06   | 98.2        | 95.29           | 99.1        |
|              | 25                    | 98.33    | 99.65       | 96.72           | 99.58       |
| Indapamide   | 0.45                  | 101.6    | 103.08      | 97.26           | 102.5       |
|              | 2.5                   | 97.05    | 98.15       | 93              | 100.25      |
|              | 12                    | 98.3     | 100.2       | 95.52           | 97          |
identification and quantification of perindopril and indapamide drugs in human plasma and tested the stability studies as per ICH guidelines. It is a simple, accurate, and specific method for the extraction of these two drugs from human plasma with quick elution time of 2 min. The method showed high recovery rate as well as low detection and quantification limits of two drugs. The developed method could be used for routine analysis as well as in bioanalytical and clinical studies.

Abbreviations
PP: Perindopril; IP: Indapamide; SPE: Solid-phase extraction; ACN: Acetonitrile; MeOH: Methanol; CHCl3: Chloroform; TBDE: Tertiary butyl diethyl ether; EtOH: Ethanol; LOD: Limit of determination; LOQ: Limit of quantification; RP: Reverse phase; UPLC: Ultra performance liquid chromatography; RSD: Relative standard deviation

Supplementary Information
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Additional file 1: Figure S2. Acidic and basic degradation HPLC chromatograms.

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Authors’ contributions
All the authors have read and approved the manuscript. BP has framed the methodology of the work, and investigated, validated by performing the formal analysis. BP and JC have drafted the original paper. PN and JC have helped in conceptualization of the work.

Availability of data and materials
All data and materials are available upon request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References
1. McNamara K, Alzubaidi H, Jackson JK (2019) Cardiovascular disease as a leading cause of death: how are pharmacists getting involved? Integr Pharm Res Pract 8:1–11. https://doi.org/10.2147/IPRP.S133088
2. Antonakoudis G, Poulimenos I, Kifnidis K, Zouras C, Antonakoudis H (2007) Blood pressure control and cardiovascular risk reduction. Hippokratia 11(3): 114–119
3. Mogensen CE, Viberti G, Halimi S, Ritz E, Rulope L, Jermendy G, Widimsky J, Sareli P, Taton J, Rull J, Erdogan G (2003) Effect of low-dose perindopril/indapamide on albuminuria in diabetes: preterax in albuminuria regression.

Table 5 Robustness study

| Chromatographic changes | Indapamide | Perindopril |
|-------------------------|------------|-------------|
|                         | RT       | Recovery (%) | TF | N          | RT       | Recovery (%) | TF | N          |
| Mobile phase ratio      |          |              |    |            |          |              |    |            |
| 70:30                   | 0.74     | 97.89        | 1.64 | 8254       | 1.05     | 98.38        | 1.65 | 5957       |
| 65:35                   | 0.87     | 98.06        | 1.74 | 8102       | 1.16     | 99.35        | 1.65 | 6264       |
| 60:40                   | 1.02     | 96.54        | 1.72 | 7650       | 1.82     | 97.64        | 1.61 | 6441       |
| Mean ± SD               | 97.49±0.83 | 98.45±0.85   |    |            |          |              |    |            |
| RSD (%)                 | 0.85     |              |    |            | 0.87     |              |    |            |
| Flow rate (mL/min)      |          |              |    |            |          |              |    |            |
| 0.8                     | 1.14     | 97.49        | 1.65 | 6845       | 1.71     | 98.14        | 1.62 | 6038       |
| 1.0                     | 0.87     | 98.26        | 1.65 | 8125       | 1.16     | 99.52        | 1.71 | 6506       |
| 1.2                     | 0.72     | 96.07        | 1.61 | 7845       | 1.03     | 97.84        | 1.65 | 6382       |
| Mean ± SD               | 97.27±1.11 | 98.5±0.89    |    |            |          |              |    |            |
| RSD (%)                 | 1.14     |              |    |            | 0.9      |              |    |            |
| Column temperature (°C) |          |              |    |            |          |              |    |            |
| 35 °C                   | 0.85     | 99.04        | 1.70 | 8201       | 1.12     | 98.47        | 1.64 | 6218       |
| 30 °C                   | 0.87     | 98.63        | 1.71 | 8054       | 1.16     | 99.04        | 1.64 | 6151       |
| 25 °C                   | 0.89     | 97.82        | 1.71 | 7654       | 1.19     | 97.22        | 1.62 | 6424       |
| Mean ± SD               | 98.49±0.62 | 98.24±0.93   |    |            |          |              |    |            |
| RSD (%)                 | 0.63     |              |    |            | 0.94     |              |    |            |
| UV wavelength           |          |              |    |            |          |              |    |            |
| 258                     | 0.87     | 96.14        | 1.71 | 7951       | 1.14     | 99.81        | 1.63 | 5984       |
| 254                     | 0.87     | 98.36        | 1.72 | 8156       | 1.16     | 98.74        | 1.61 | 6068       |
| 250                     | 0.85     | 97.03        | 1.71 | 7899       | 1.17     | 96.28        | 1.61 | 6248       |
| Mean ± SD               | 97.17±1.11 | 98.27±1.81   |    |            |          |              |    |            |
| RSD (%)                 | 1.14     |              |    |            | 1.84     |              |    |            |
