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Simultaneous Determination of Indapamide, Amlodipine Besylate and Perindopril Arginine Combined in Tablet Dosage Form Using High Performance Liquid Chromatography

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ABSTRACT

We developed and validated a high performance liquid chromatographic method for the simultaneous determination of a single-pill triple therapy containing: Indapamide, Amlodipine Besylate, and Perindopril. The validation parameters are tested by following the international conference of harmonization (ICH) guidelines. The three components were successfully separated in just 14 minutes. The aforementioned components were well-separated on an Inertsil C8- column (250mm x 4.6mm, 5µm) using a mobile phase prepared by mixing of Triethylamine counter ion solution (pH 3.0) with Acetonitrile at a fixed ratio of 2:1. Analysis was performed at a wavelength of 205 nm, eluent flow rate and column oven temperature was 1.5 ml/min, and 40°C, respectively. Linearity was observed in the concentration ranges of (20-30) µg/mL for Indapamide, and (80-120) µg/mL for both Amlodipine Besylate and Perindopril Arginine. We found the recovery percentages to be 99.98%, 101.04%, and 100.58% for Perindopril Arginine, Amlodipine Besylate, and Indapamide, respectively. Further, we found the detection limits to be 0.38 µg/mL, 0.99 µg/mL and 3.65 µg/mL, and the obtained quantitation limits were 1.16 µg/mL 3.01 µg/mL, and 11.06 µg/mL for Indapamide, Amlodipine Besylate, and Perindopril Arginine, respectively.

Keywords: Simultaneous Determination; Indapamide; Amlodipine Besylate; Perindopril Arginine; HPLC; Validation.

1. INTRODUCTION

Hypertension, a prevalent disease among 35-40 % of the adult population, is a major health concern in this century[1]. Hypertension negatively impacts the cardiovascular risk and is implicated to be the culprit in a plethora of severe and life-threatening ramifications which includes heart failure, myocardial infarction, and kidney damage[2]. Current guidelines advocate adopting evidence-based lifestyle modifications as a mean to manage elevated blood pressure. This includes engaging in regular aerobic type of exercise[3], reducing sodium intake[4, 5], stress and alcohol consumption[6]. However, the majority of clinical cases do require antihypertensive medications to lower blood pressure readings to safe levels. Therefore, a huge number of therapeutics has been introduced to the clinical practice to serve such a purpose. An accumulating body of research is now leaning toward promoting the usage of two or more anti-hypertensive

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agents formulated in one dosage form to perform their putative pharmacologic effect via different mechanisms of action[7]. Currently, it is widely accepted within the clinical practice settings to use a combination of two or more anti-hypertensive agents that are formulated in a single tablet given the enhanced efficacy and improved adverse reaction profile compared to the classical single agent dosage form. Even when the dose is doubled[7,8]. Diuretics, beta-blockers[9], calcium channel blockers[10], angiotensin-converting enzyme inhibitors[11] and angiotensin receptor blockers[12] are considered to be the five primary anti-hypertensive classes that are recommended by the American Heart Association and the European Society of Hypertension as an exclusive treatment. The use of combinations of multiple agents in a single pill formulation offers many benefits, among which, better patient compliance, improved adherence to treatment because patients prefer single treatment schedule more than multiple treatment schedules[12,13], faster response to treatment, minimized risk of side effect[13], and reduced cost of production[12].Recently, a tremendous attention was paid to the usage of a triple combination of three active components namely: indapamide (IN), perindopril (PEP)(perindopril arginine), and amlodipine(AM) (amlodipine besylate) to control high blood pressure[14]. Triplixam tablet is an example of a commercial single tablet formulation with triple combination of IN, PEP, AM and acts as an anti-hypertensive medicine.

Indapamide (4-chloro-N-(2-methyl-1 -indoline-) 3-sulfamoylbenzamide) (figure 1a) is a known thiazide-like diuretic agent[15]. In addition to its classical effect of reducing intravascular space by promoting natriuresis and diuresis, Indapamide possesses an intrinsic ability to reduce vascular responsiveness to pressor amines (tyramine, tryptamine, and phenylethylamine)[16]. These Amines constrict the vascular system, and cause an increase in the heart rate and contractile force[15, 16].

Amlodipine (2-[(2-Aminoethoxy) methyl]-4-(2-chlorophenyl)-1, 4-dihydro-6-methyl-3,5-pyridinedicarboxylic acid-3-ethyl-5-methyl ester)(figure 1b) is an agent known to antagonize calcium channels within the heart and the vessels. This feature is employed to control hypertension and to alleviate symptoms of ischemic heart disease such as chronic stable angina pectoris. Amlodipine is available in the market as a besylate salt[17].

Perindopril ((2S, 3aS, 7aS)-1-[(S)-N-[(S)-1-carboxybutyl]alnyl]hexahydro-2-indolinecarboxylic acid-1-ethyl ester)(figure 1c) is recognized as an angiotensin converting enzyme inhibitor and is widely used to treat hypertension and heart failure[18, 19,20, 21]. Perindopril is a prodrug that requires hydrolysis to its active metabolite, perindoprilat[19, 20]. Perindopril is available in the market as perindopril arginine or perindopril erbumine[19].

Several studies are directed toward determining of AM, IN and PEP individually or in-combination with other drugs. Most studies involved simultaneous analysis of only PEP and IN or AM and IN in a combined dosage form by HPLC [21, 22, 23,24].The present study sought to develop a HPLC analysis method for simultaneous determination of AM, IN and PEP in a single tablet formulation. We do acknowledge that El-Bagary et al[25] have conducted a similar experimentation, however, we created and validated a new methodology that aimed to enhance the analytical performance and improve the chromatographic separation of AM, IN and PEP when compared with El-Bagary et al study.

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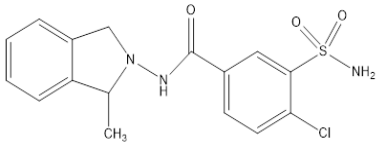

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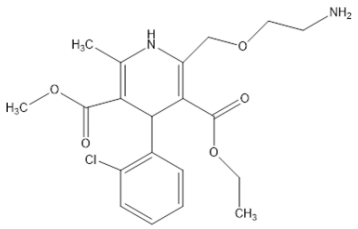

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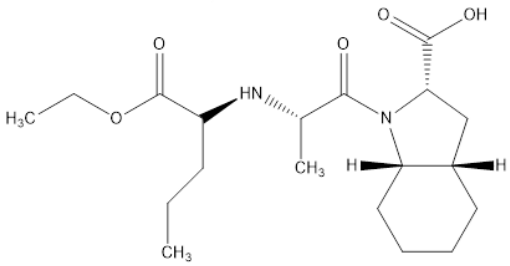


Fig.1: Chemical structures of (a) Indapamide, (b) Amlodipine and (c) Perindopril

2. EXPERIMENTAL

Reagent and materials

Indapamide, Amlodipine Besylate, and Perindopril Arginine raw materials were certified to have a purity of 99.90%, 99.85%, and 99.95%, supplied by Dishman (Ahmedabad, India), Aurobindo pharma (Hyderabad, India), and Aatri (Mumbai, India) respectively. Acetonitrile (HPLC grade) and Nylon Filter membranes (diameter = 47 mm, pore size = 0.45 µm) were obtained from Merck (Germany), trimethylamine (TEA) was purchased from Fisher (U.K). Phosphoric acid 85 % was supplied from Panreac (Spain). Combined tablets containing 3.7 mg of Perindopril Arginine (equivalent to 2.5 mg of PEP), 13.1 mg of Amlodipine Besylate (equivalent to 10 mg AM), and IN (10 mg) were formulated in the department of research and development, Dar Al Dawa, Jordan; as per requested. The approximate weight of the formulated tablet was 300 mg, and the mass percent composition was 7.4 % of active ingredients and 92.6% of expedients.

Instrumentation

The analysis method was developed and validated on HPLC Thermo- Scientific Dionex- Ulti- Mate 3000 system containing a gradient pump, UV-VISIBLE detector, column oven and a manual injector (Rheodyne with a 20 µL sample loop). The computational analysis was performed by Chromeleon 7 software. Specificity test was performed on water HPLC system consisting of Waters Alliance 2695 Pump, 2998 photodiode array detector (PDA) (Waters, Milford, MA, USA), Waters column heater and a Rheodyne injector with a 20 µL loop. Empower. ®. 3 software (Database Version 7.21.00.00) was used to process the data.

Chromatographic conditions

An Inertsil C8 column ((25cm x 4.6 mm, 5µm) GL Science, Japan) was used as the stationary phase. Isocratic elution was performed with a mobile phase composed of 0.7% triethylamine (TEA)(that was

dissolved in water and adjusted to pH 3 with orthophosphoric acid 85%) and mixed with acetonitrile in a ratio of 2:1(v/v). The mobile phase was filtered through a 0.45 µm membrane filter and degassed for at least 10 minutes before use. The flow rate was 1.5 mL/min. Detection was conducted at 205 nm with a UV–visible detector and at 200 to 260 nm with PDA detector.

Preparation of standard solutions

Three stock solutions with a concentration of 1000 µg/mL were prepared by separately weighing a 73.7 mg of Perindopril Arginine (equivalent to 50.0 mg PEP) , 69.5 mg of Amlodipine Besylate (equivalent to 50.0 mg of AM), and 50.0 mg of IN, and then transferring them into three separate 50 mL volumetric flasks that were finally dissolved in Acetonitrile. All stock solutions were kept at 4 °C and allowed to reach room temperature before use.

Preparation of sample solution

A quantity of powdered tablets of 480, 600, 720 mg that was equivalent to (4, 5,6) mg of IN, (16, 20,24) mg of AM and(16, 20,24) mg of PEP, were weighed and placed into 200 mL volumetric flasks. Around thirty milliliters of the mobile phase were added to the flasks and the solutions were placed in ultrasonic bath for 30 minutes to be dissolved. After that, the sample solutions were allowed to reach room temperature and the mobile phase was added to complete volumes. The solutions were filtered through Whatman filter papers No. 41 and 0.45 µm nylon filtration membranes. The obtained final concentrations were 20, 25, 30 µg/mL of IN, 80, 100, 120 µg/mL of AM, and 80, 100, 120 µg/mL of PEP.

Method validation

The developed method was subjected to validation test obtained from the International Conference of Harmonization (ICH) Guidelines for Validation of Analytical Methods[27]. Many parameters were determined, among which, system suitability test, linearity, specificity, sensitivity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision and robustness.

A system suitability test was performed by repeating injection of a standard solution (25 µg/mL of IN, 100 µg/mL of AM, and 100µg/mL of PEP) for ten times. A number of measurements including: peak shape, peak resolution, capacity factor (K'), and theoretical plate number (N) were carried out.

Stock solutions (1000 µg/mL) of IN, AM and PEP were mixed, diluted to various concentrations by adding different volumes of the mobile phase. Concentrations of the prepared solutions were: 20-30 µg/mL for IN, 80 – 120µg/mL for AM, and 80 –120µg/mL for PEP. Concentration ranges of the prepared solutions were equivalent to IN, AM, and PEP in the formulated drug samples (within range or at the extremes). These prepared solutions were used to study linearity and create calibration curves. An aliquot (20 µL) of each ternary mixture solutions was injected three times under the optimized chromatographic conditions and responses were collected by calculating the average peak area of the repeated injections. Calibration curves were made by plotting peak areas versus the concentrations and the correlation coefficients (R^2) were determined.

The percentage recoveries were calculated for AM, IN and PEP by three different concentration levels in order to evaluate the accuracy of the method. Standard solutions of IN (20, 25, and30 µg/mL), AM (80, 100 and 120µg/mL) and PEP (80, 100 and 120 µg/mL) were deliberately added to many placebo samples. Then those components were extracted and the main recovery was calculated using the following equation:

$$\text{Recovery \%} = \frac{\text{amount found in the samples}}{\text{Spiked amount}} X\ 100\%$$

The intra-day (same day) and inter-day (different days) precision of the suggested analytical method were evaluated by recording three responses for each concentration level on the same day and three responses for each concentration level on three different days, and the concentration levels used were 25 µg/mL for IN, 100 µg/mL for PEP and AM. The precision, which is usually

expressed as relative standard deviation (RSD), was required to be less than 2%.

Repeatability was evaluated by recording response ten times at one concentration level. It was performed on 25 µg/mL for IN and concentrations of 100 µg/mL for AM and PEP.

The LOD and LOQ were calculated for this method to evaluate its sensitivity. The LOD and LOQ were detected by using the following mathematical equations[27]:

LOD = 3.3x σ_{n-1}/m,

LOQ =10 x σ_{n-1}/ m

where σ_{n-1} is standard deviation of the blank and m is the slope of regression equation.

Specificity test was performed by HPLC equipped with PDA detector to determine the purity of the peaks obtained for IN, AM, and PEP. A standard of PEP (100 µg/mL), AM (100 µg/mL), IN (25 µg/mL) and placebo sample (3000 µg/mL) were analyzed. The obtained chromatographic peaks for all components were investigated for peak purity by comparing the spectra at three specific regions: peak start, peak apex and peak end. The used scanning range was 200 –260 nm.

Robustness of the methods was tested. The same standard mixture (containing 25 µg/mL IN, 100 µg/mL AM and 100 µg/ml PEP) was reanalysed by HPLC for several times after intended alteration in the method parameters. Changes in the responses of AM, PEP and IN were recorded. Method parameters were altered including: the mobile phase pH (∓0.1), column oven temperature (± 2° C), mobile phase composition (± 3% of acetonitrile), the flow rate (∓0.2 mL), and the detection wavelength (∓ 2 nm). The precision (RSD) was calculated and investigated if it was less than 2%.

3. RESULTS AND DISCUSSION

A system suitability test for HPLC was assessed before measuring other validation parameters and running quantitative analysis. The responses were recorded for ten replicate injections of the prepared standard with a concentration of 25 µg/mL IN, 100 µg/mL AM and 100 µg/mL PEP. Several parameters were evaluated for the optimized HPLC method, among which the capacity factor, the peak resolution (K'), asymmetry of the peak, theoretical Plate (N), selectivity factor, and RSD of the peak area (Table 1).

Table 1. System Suitability Parameters for the HPLC Method

Parameter	PEP	AM	IN
Capacity Factor	2.69	4.88	8.72
Resolution Factor	3.47	10.5	-
Asymmetry Factor	1.0	1.3	1.2
Theoretical Plate	380	4776	3265
Selectivity Factor	1.81	1.79	-
RSD of the peak area (%)	0.28	0.03	0.14

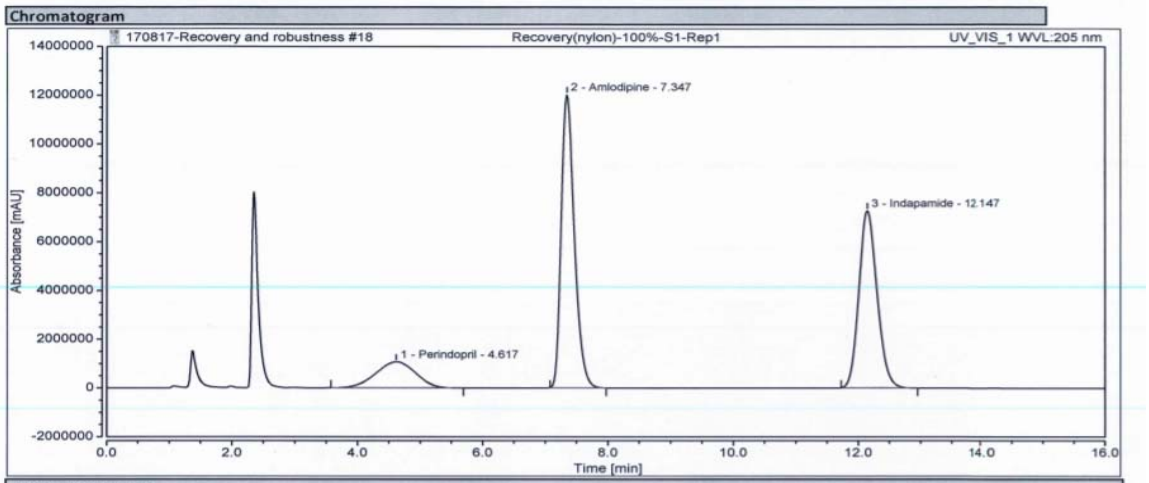
The capacity factor values were estimated at 2.69, 4.88 and 8.72 for PEP, AM, IN, respectively. Compared to El-Bagary et al study, the values of capacity factors were estimated at 0.77, 2.05 and 4.38 for PEP, AM, IN, respectively. This method showed a good improvement in the capacity factor of PEP. The resultant small capacity factor for PEP (0.77) highlights a short interaction time between PEP and the stationary phase[26].

The estimated resolution factors were 3.47 and 10.5 which indicated that all components were totally separated and not eluted together. One of the estimated resolution factors in our study is higher than that reported by El-Bagary et al (3.37 and 4.59)[25]. The reported values of asymmetry factors (1.0, 1.1 and 1.3) confirmed that all peaks were symmetrical[28]. These reported values of asymmetry factors are in line with those reported in the El-Bagary et al study. Theoretical plates were estimated at 380, 4776, and 3265 for PEP, AM, IN, respectively. The theoretical plate values revealed an excellent chromatographic performance for both AM and IN and not for PEP which exhibited a reduced efficiency of

separation. Comparing theoretical plate values with that reported from El-Bagary et al study. We found the efficiency of separation for PEP to be lower by 0.27 times compared to that reported by El-Bagary et al whereas theoretical plate's values for AM and IN were higher by 1.5 and 1.3 times respectively compared to that reported in the El-Bagary et al study. Moreover, PEP owned the broadest peak due to its high band diffusion[26]. The estimated selectivity coefficients in this method were 1.81 and 1.79. Selectivity factors were reported by El-Bagay et al as 2.66 and 2.14. Both of the two methods showed high selectivity and absence of coeluting[28]. The percent relative standard deviation (%RSD) of the peak area responses were 0.28% for PEP, 0.03% for AM, and 0.14% for IN in this study. El-Bagary et al reported the percent relative standard deviation (%RSD) of the peak area responses as 0.77% for PEP, 0.58% for AM, and 0.29% for IN.

The mobile phase was selected after testing several mobile phases to achieve optimized HPLC parameters, such as reasonable run time, absence of noisy lines, sharp peaks with high resolution and acceptable symmetry. Perindopril arginine was hydrolysed to arginine and PEP. As shown in Figure 2, four symmetrical peaks with good separation were obtained on a C 8 column through use of a mobile phase composed of 0.7% TEA solution (its pH was adjusted to 3.0 with 85 % H_3PO_4) and acetonitrile in a ratio of 2:1(v/v). The obtained retention times were 2.4 minutes for Arginine, 4.6 minutes for PEP, 7.3 minutes for AM, and 12.1 minutes for IN when analysis was performed at a flow rate of 1.5 mL/min and detection wavelength of 205 nm as shown in Figure 2.

The suggested analytical method was validated in obedience with the International Conference on Harmonization guidelines (ICH). Results of the various parameters are presented in Table 2.



Integration Results

No.	Peak Name	Retention Time	Area	Asymmetry (EP)	Resolution (USP)	K'	Peak Type	Rel Area
		min	mAU*sec					%
1	Perindopril	4.617	786900	1.0	3.47	2.69	Autodetect	12.77
2	Amlodipine	7.347	2924017	1.3	10.50	4.88	Autodetect	47.46
3	Indapamide	12.147	2450432	1.2	n.a.	8.72	Autodetect	39.77
Total:			**6161349.374**	**3.46**	**13.97**			

Fig 2: HPLC chromatogram of a standard mixture of PEP (100 µg/mL), AM (97 µg/mL), and IN (25 µg/mL).

Three linear calibration curves were obtained after plotting peak area versus concentration for IN, AM, and PEP in the ranges of 20–30, 80–120, and 80–120 µg/mL, respectively (Table 2). The obtained linear equations were: Y = 97468 x+ 28462, Y = 30756 x -53413, and Y = 6927 x + 95761 for IN, AM and PEP, respectively. El-

Bagary et al reported concentration ranges: 5-20 µg/mL for IN, 2.5-80 µg/mL for AM, and 5-80µg/mL for PEP. The obtained linear equations from El-Bagary et al study were Y = 1.3587x− 0.1295 for IN, Y = 0.4718 x -0.0081 for AM, and Y = 0.1623x + 0.0288 for PEP. Slopes values are higher in our study than those obtained from El-Bagary et al study and this confirms that the chromatographic conditions and concentration ranges in our study participate in having better instrumental response toward IN, AM and PEP. The correlation coefficient (R^2) values were 0.998 for IN, 1.00 for AM and 0.995 for PEP. The LOD detected values were 0.99, 0.38 and 3.56 µg/mL for AM, IN and PEP, respectively. The LOQ detected values were 3.01, 1.16 and 11.06 µg/mL for AM, IN and PEP, respectively. IN owned the lowest level of LOD and LOQ as shown in the table 2.

The percentage recovery values of IN, AM, and PEP were extended from 99.9 to 101.4%. These values agreed with those reported by El-Bagary et al. High values of recovery with RSD values less than 1% for all drugs at different concentration confirms the accuracy of this analysis method. The values of the accuracy studies are summarized in Tables 2.

The obtained results from Inter-day and intra-day precision test showed that the RSD values were less than 2% for all injected samples. Low obtained values of RSD can be considered a confirmation to that the analysis method is precise and repeatable. The precision values are summarized in table 2.

Table 2. Summary of Validation Parameters for the Proposed Method

Parameter	PEP	AM	IN
Range of linearity (µg/mL) n =3	80–120	80–120	20–30
Regression equation	Y = 6927 x+ 95761	Y=30756 x -53413	Y = 97468 x + 28462
Correlation Coefficient R^2	0.995	1.000	0.998
Inter-day precision (RSD %) n=6	0.56	0.43	0.12
Intra-day precision (RSD %) n=6	0.45-1.0	0.25-0.99	0.49-0.98
Specificity	Specific	Specific	Specific
Repeatability (RSD %) n=10	0.14	0.04	0.24
LOD (µg/mL)	3.65	0.99	0.38
LOQ (µg/mL)	11.06	3.01	1.16
Recovery (%)±SD (n= 3)	99.92±0.39-100.02± 0.64	100.5±0.04-100.7±0.13	100.9±0.1- 101.4±0.08

The peak purity of AM, IN and PEP was investigated through comparing their respective spectra at three regions: peak start, apex and peak end. The peak purity value for all three drugs exceed 990 (ideal value, 1,000), which means that the peaks were pure and free from interfering peaks. Therefore, the proposed analysis method was specific for the three drugs and the excipients had no effect on the separation process, retention times, shape and width of the peaks.

The robustness of the method was studied by achieving assays of a standard mixture containing 20 µg/mL of IN, 100 µg/mL of AM and 100 of µg/mL PEP.

The parameters of the method were purposely changed, and alterations in the responses of AM, IN and PEP were recorded. The assay values of the three drugs were calculated in the changed parameters. Changes of the different parameters did not highly affect the retention times and peak areas. The proposed method proved to be robust, because these minor changes in flow rate, pH, and wavelength, mobile phase composition and column oven temperature do not significantly affect the obtained results. The RSD was less than 2% under different experimental conditions as shown in Table 3.

Table 3. Robustness test of IN, AM, and PEP by the HPLC Method

Altered Parameter	IN(assay ± SD)	AM(assay ± SD)	PEP(assay ± SD)
Acetonitrile composition (±3%)	100.8 ± 0.3	100.4 ± 0.2	99.6 ± 0.4
pH(±0.10)	100.3 ± 0.1	100.2 ± 0.2	99.8 ± 0.3
Wavelength (±2 nm)	100.4 ± 0.2	100.6 ± 0.4	99.0 ±1.0
Column oven temperature (± 2°C)	100.5 ± 0.5	100.6 ± 0.4	98.3 ±1.0
Flow Rate (±0.2 mL/min)	100.3 ±0.2	100.6 ± 0.3	99.2 ±0.6

4. Conclusion

This study shows that the developed high performance liquid chromatographic method was applied successfully to simultaneously determine Indapamide, Amlodipine Besylate and Perindopril Arginine in a combined single formulated tablet. Validation data studies showed that the method was precise, sensitive, selective, robust, and linear over the concentration range of 20-30 µg/mL for Indapamide, and 80-120 µg/mL for Amlodipine Besylate and Perindopril Arginine. This method was also free from interference from the excipients used in the formulations. This method showed improvement in capacity factor of Perindopril Arginine and theoretical plates for Amlodipine Besylate and Indapamide when compared to the previously reported study. The main advantages of this method were rapidness and easiness. These results allowed us to conclude that the developed method can be used in routine analysis.

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التحديد المتزامن للانداباماید والأملودیبین والبیرندوبریل في الأقراص باستخدام الفصل الكروماتوغرافي عالي الكفاءة

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ملخص

أجريت هذه الدراسة لتطوير طريقة تحليل دقيقة للتحديد المتزامن للانداباماید والأملودیبین والبیرندوبریل في الأقراص و التثبت منها باستخدام الفصل الكروماتوغرافي عالي الكفاءة. تم تنفيذ الفصل الكروماتوغرافي باستخدام عامود فصل من نوع (Inertsil C8) أبعاده (25 سنتيمتر*4.6 ملمتر*5 ميكرومتر) محفوظ في درجة حرارة 40°س, و طور متحرك يتكون من خليط محلول ثلاثي ايثيل الامين المنظم (رقمه الهيدروجيني 3.0) و الاسيتونايتريل بنسبة (2:1) بمعدل تدفق 1.5 مللتر/دقيقة و على طول موجي 205 نانومتر للكشف, بحيث ينتهي فصل المواد الثلاثة في أقل من 20 قيقة. تم التثبت من طريقة التحليل استناداً إلى المبادئ التوجيهية للمؤتمر الدولي للتنسيق. من خلال هذه الدراسة يمكننا التوصية باستخدام طريقة التحليل هذه في كافة مختبرات تحليل الأدوية لتحليل الانداباماید والأملودیبین والبیرندوبریل.

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