RESEARCH ARTICLE

Concurrent Analysis of Amlodipine, Rosuvastatin, and Hydrochlorothiazide in Solution and Biological Fluid Using LC-MS/MS

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ABSTRACT

A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was created and verified in this investigation. This methodology offers a fast, easy, affordable, economical, and reliable approach to simultaneously determining amlodipine, rosuvastatin, and hydrochlorothiazide in solution and human plasma. Through the extraction process, acetonitrile was utilized as a precipitating agent for the proteins in plasma. The C18 column was injected with a volume of 10 µL, including ondansetron as an internal standard. The analytes were eluted using an isocratic mobile phase (0.1% acetic acid and 1:4 (v/v) methanol). The method was sensitive and selective for the analytes over a dynamic concentration range of 5 to 600 µg/mL in solution and 0.5 to 60.0 µg/mL in plasma. At a minimum of 0.9988 coefficient of determination, linearity was attained. Within and between runs, the precision and accuracy were 2.87 to 11.41% and 86.2 to 113%, respectively. A comparatively quick run time of less than two minutes (high throughput) was achieved. The current method can be used in many applications, such as bioequivalence investigations and therapeutic medication monitoring, to quantify the relevant analytes.

Keywords: Amlodipine, Rosuvastatin, Hydrochlorothiazide, LC-MS/MS, Plasma.

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INTRODUCTION

Cardiovascular diseases cover a wide range of ailments affecting the heart and blood vessels and currently stand as the most common death cause worldwide as they have claimed the lives of almost 14 million individuals in 2000 and nearly 18 million in 2019. The expanding and aging of the global population is a major factor contributing to the rising death toll[1]. Hypertension, angina, strokes, and cardiomyopathy are a few examples of cardiovascular diseases. They progressively appear as people age, particularly in those who have one or more risk factors, such as high blood pressure, dyslipidemia, alcoholism, and an unhealthy lifestyle.[2]

Elevated blood pressure, or hypertension, is characterized by a systolic blood pressure of at least 140 mmHg and a diastolic blood pressure of at least 90 mmHg and is one of the most common chronic disorders in humans, impacting over 1 billion individuals globally. It is arguably the most powerful factor that leads to heart failure and other peripheral vascular diseases.[3]

Dyslipidemia leads to hypertension and a root cause of atherosclerosis and conditions associated with it, including ischemic cerebrovascular disease and coronary heart disease. It is characterized by abnormally high blood levels of lipids and lipoproteins due to faulty metabolism. Nowadays, the majority of industrialized and developing nations attribute heart disease and death to dyslipidemia and atherosclerosis.[4,5] The prevalence of lipitension, the co-existence of hypertension and dyslipidemia, is estimated by various epidemiological studies to be in the range of 15 to 31%.[6]

While it is common to begin hypertension treatment with a single medicine, good blood pressure control often necessitates using a combination of drugs. In this regard, thiazide diuretics and calcium channel blockers are among the effective combinations, along with lipid-lowering medication, if necessary, and some lifestyle modifications.[7,8]

Thiazide diuretics are considered the cornerstone of the current antihypertensive therapy, constituting a well-tolerated and effective first-line treatment for individuals with mild

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to moderate, uncomplicated hypertension that considerably decreases cardiovascular morbidity and mortality.[9] The benzothiadiazine derivative, hydrochlorothiazide (HC), has been used as a diuretic and antihypertensive medication since 1957.[10] It works primarily in the distal nephron by blocking the luminal transmembrane-coupled Na-Cl transport pathway.[11] Almost two hours following a single oral dose, HC attains a maximum concentration (C_{max}) of 0.075 mg/L, and the drug's half-life is reported to be about 6.5 – 9 hours.[11,12]

Calcium channel blockers (CCBs) are drugs that differ in their pharmacological and chemical makeup. These drugs obstruct the transmembrane passage of calcium, which moves via certain channels from the extracellular space to the cytoplasm. The pharmacologic efficacy and cellular binding locations of CCBs vary greatly, where they can selectively interact with either cardiac or vascular (or both) L-type voltage-dependent transmembrane calcium channels.[13,14] Amlodipine (AML) is a basic dihydropyridine derivative that inhibits the calcium influx by blocking the channels in peripheral and coronary smooth muscle cells, leading to significant vasodilation in peripheral and coronary vascular beds.[15] After administering a 10 mg oral dose of AML, a peak plasma concentration of 5.9 ± 1.2 ng/mL was reached in 6 to 12 hours.[16]

Statins, or the 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase inhibitors, are the primary drugs for treating dyslipidemia. They lower LDL and TG levels and increase HDL levels in familial and severe hypercholesterolemia with atorvastatin and rosuvastatin (ROS) regarded as powerful LDL reducers.[17,18] The latter is a dihydroxyheptanoic acid derivative with fluorinated phenyl and polar sulfone groups, which confer many sites of interaction with the HMG-CoA reductase enzyme, granting ROS the highest affinity for the enzyme among the other genres. After oral administration of the single dose of ROS 40mg, the mean C_{max} was 19–25 µg/L after 3 to 5 hours.[19]

Several analytical methods for measuring plasma levels of AML in biological fluids were reported, which include gas chromatography (GC), liquid chromatography/mass spectrometry (LC/MS), and high-performance liquid chromatography (HPLC) with varied detection modes such as fluorimetric, ultraviolet, and electrochemical detection. High-performance thin layer chromatography (HPTLC), liquid chromatography coupled with tandem mass spectrometric (LC-MS/MS) methods, and ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) have also been performed. AML was estimated in pharmaceuticals using liquid chromatography (LC), spectrophotometry, voltammetry, and micellar electro-kinetic chromatography.[20-22] Compared to LC-MS/MS, the sensitivity of several HPLC methods is insufficient for pharmacokinetic studies and therapeutic drug monitoring. However, a few LC-MS/MS methods are useful for research and have not been validated for pharmacokinetics studies.[21]

Various HPLC, HPTLC, and spectrophotometric methods were reported for determining HC in plasma and pharmaceuticals. However, in these methods, the plasma volume needed was high, the chromatographic run time was longer, and the sensitivity for pharmacokinetic studies was insufficient.[23,24] As an alternative, solid-phase extraction-based LC-MS/MS techniques have been developed to measure ROS levels in plasma. Furthermore, different spectrophotometric, HPLC, and HPTLC methods were used to examine ROS calcium in medicinal formulations.[25,26]

Over the years, it has been recommended to treat hypertension patients with calcium channel blockers, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins), and thiazide diuretics in combination to manage hypertension safely and improve the patient's status.[27,28] The current study aimed to develop a simple, suitable, and accurate LC-MS/MS method to determine AML, ROS, and HC simultaneously in solutions and human plasma and investigate the validity of the developed method based on the European Medicines Agency (2011) guidelines. Moreover, we investigated the possibility of using the validated method in bioequivalence studies.

MATERIAL AND METHODS

Materials, acetonitrile and Acetic Acid were obtained from Fisher Chemical (UK), ondansetron (ONS) the internal standard (IS) powder and triethylamine were purchased from Sigma-Aldrich, amlodpine raw material from JPM (Jordan) as a donation, ROS raw material from Hikma (Jordan) as a donation, HC raw material from JPM (Jordan) as a donation and HPLC grade water from Lichrosolv ® (Germany).

Instruments, the employed liquid chromatography was Dionex Ultimate 3000. Thermo LCQ Fleet mass spectrometer (an ion-trap spectrometer) was the detector. Chromatographic conditions, for the concurrent determination of AML, ROS, and HC in human plasma and solution, an isocratic solution of 0.1% acetic acid and methanol 1:4 (v/v) was used as the mobile phase. The pH of the mixture was adjusted by adding 0.02% triethylamine. The C18 ACE column of the dimensions (4.6 mm x 5 cm), and (2.1 mm x 5 cm) were used to determine the analytes in plasma and solution, respectively. The sample injection volume was 10 µL with a flow rate of 0.5 mL/min and 0.2 mL/min for measuring the analytes in plasma and solution, respectively. The concentration of the internal standard was 1 µg/mL, and 5 µg/mL to determine them in solution.

For the mass spectrometry analysis, mass spectrometric parameters were as follows: 300 °C ion source with an ionization voltage of 5 kV. The gas flow rate was 42 L/min (sheath gas), 14 L/min (auxiliary gas), and 0 L/min (sweep gas). The MS detection modes utilized were AML (+MS1=409, MS2 =237.9 CE=23), ROS (+MS1=482, MS2 =464 CE=37), HCT (- MS1=295.95, MS2 =268.85 CE=25), and ONS (IS) (+MS1=294, MS2=170, CE=27).

Standard calibration concentrations and quality control samples preparation, Initially, a 10000 µg/mL stock solution of AML, ROS, and HC was prepared by dissolving 100 mg of each drug in 10 mL of methanol. Next, the subsequent working solutions (5, 10, 15, 30, 80, 200, 300, 400, 500, and 600 µg/mL) were prepared as shown in Table 1.

Table 1: Quality control sample preparation

Stock solution concentration	*10000 µg/mL*	
Working solution final volume	*10000 µL*	
Calibrator and QC[a]	*Stock solution volume (µL) taken*	*Working solution concentration (µg/mL)*
1 (LLOQ[b])	5	5
2	10	10
3	30	30
4	80	80
5	200	200
6	400	400
7	600	600
QC Low	15	15
QC Mid	300	300
QC High	500	500

[a] QC is quality control, and [b] LLOQ is the lower limit of quantitation

To prepare the internal standard and precipitating agent, 10 mg of ondansetron, the internal standard (IS), was accurately weighed and dissolved in 10 mL acetonitrile using a vortex to yield a 1 mg/mL stock solution. Then, a volume of 0.1 mL from the stock solution (1 mg/mL) was taken and diluted up to 100 mL with acetonitrile to create the IS working solution. This solution (1 µg/mL) was used later in the extraction procedure.

For plasma sample preparation, the calibration concentrations (0.5, 1, 1.5, 3, 8, 20, 30, 40, 50, and 60 µg/mL) were prepared by adding 100 µL of each previously prepared working solution to 900 µL plasma (plasma spiking). The next step is plasma extraction, in which the precipitating agent (acetonitrile) having 1 µg/mL of ondansetron (internal standard) was added in a 2:1 ratio (400:200 µL) sequentially to the spiked plasma samples placed in the Eppendorf tube. After vortexing the resultant mixture, it was centrifuged for five minutes at 14,000 rpm. Enough of the supernatant was then pipetted in the autosampler's vial insert. Finally, the vials were capped and placed into the auto-sampler vial rack for LC-MS/MS analysis.

Analytical method validation, The developed analytical method for the concurrent quantitation of the three drugs in human plasma and solution was validated based on the Bioanalytical Method Validation Guideline of the European Medicines Agency (EMA, 2011). The method was evaluated for lower limits of quantification, linearity, selectivity, accuracy, and precision.

Selectivity (specificity), evaluating the method's selectivity assesses how well the analytical method can quantify and distinguish the target analyte(s) and IS from any endogenous components and potential other constituents in the sample.

The Lower limit of quantification (LLoQ) is the lowest concentration of an analyte present in a sample. LLoQ was quantified with satisfactory precision and accuracy by the developed method and was considered the lowest calibration standard point. Furthermore, the signal of the LLoQ sample analyte must be at least five times greater than that of the blank sample.

For linearity, we used 7 calibration points along with a blank. The standard form of the linear equation was y=mx+c, where m represents the slope and c represents the intercept of the straight line. The coefficient of determination (R^2) of the calibration curve's linearity was used for assessment purposes. The accuracy was determined using the equation:

$$\text{Accuracy} = \frac{\text{Determined Value}}{\text{True (Nominal) Value}} \text{ x } 100\%$$

Within-run accuracy was performed in a single run using six samples per QC concentration level. This covered the whole range of the calibration curve. Between-run accuracy was performed for all the QC samples in three runs over three days.

The precision of an analytical method reflects the degree of scatter among a sequence of repeated measurements. It is illustrated as the coefficient of variation (CV) and determined using the equation:

$$Precision\ (CV\%) = \frac{Standard\ Deviation}{Mean}\ x\ 100\%$$

Within-run precision was determined using 6 samples for every QC concentration level in a single run. Between-run precision was determined using four QC samples from 3 runs analyzed on 3 different days.

RESULTS AND DISCUSSION

The LC-MS/MS analytical method for the concurrent quantitation of the three drugs (AML, ROS, and HC) in solution and plasma was ascertained to be sensitive, selective, linear, precise, and accurate by the Bioanalytical Method Validation Guideline of the European Medicines Agency (EMA, 2011).

AML, ROS, HC, and ondansetron (the internal standard (IS)) in solution were efficiently separated. The three drugs were efficiently separated from ondansetron and other endogenous constituents such as plasma proteins. No interferences at the retention times among AML, ROS, HC, and ondansetron were observed in solution and plasma. The peaks obtained were of acceptable shape, entirely resolved from the plasma constituents. Figure 1. shows the optimized chromatogram upon determining the three drugs in solution *versus* the initially obtained chromatogram before optimization.

The LLoQ for AML, ROS, and HC was 0.5 µg/mL in plasma. Accuracy for this limit was within the accepted range of (80–120%) according to the European Medicines Agency (2011) and lay in the range from (88.4–118.0%), (91.60–153.60%), and (88.60–112.20%), with an average of 100.07, 104.64, and 98.09% for AML, ROS, and HC, respectively.

Moreover, the precision was within the accepted criteria of the CV value within-run and between-run tests for the LLOQ, which must be within 20%, according to the European Medicines Agency (2011). Results lay in the range from (8.08-

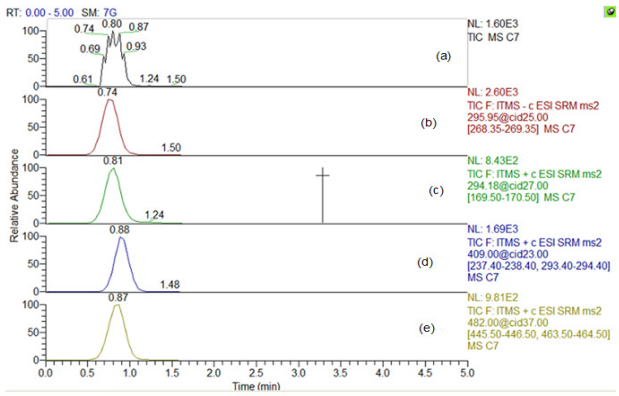

Figure 1: The optimized chromatogram was obtained upon the concurrent determination of the three drugs. (a) Total ion chromatogram, (b) HC peak, (c) IS peak, (d) AML peak and (e) ROS peak

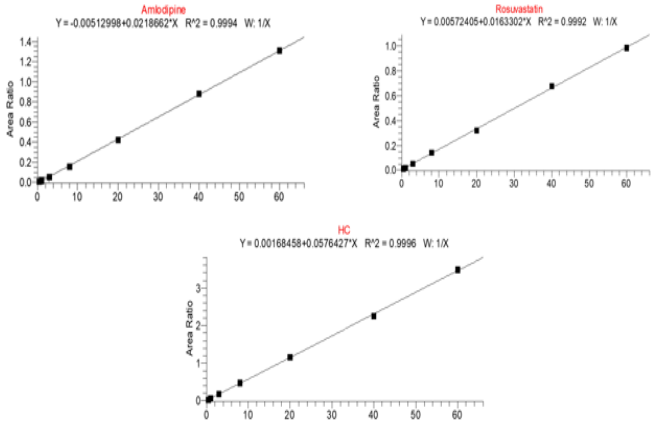

Figure 3: Calibration curves for AML, ROS, and HC in plasma

10.15%), (7.12–20.0%), and (6.039–16.26%), with an average of 8.45, 10.84, and 12.54% for AML, ROS and HC, respectively.

The linearity for AML, ROS, and HC in solution, the curves corresponding to the drugs were linear with R^2 values of 0.9993, 0.9992, and 0.9988, respectively, Figure 2. The LLoQ (STD1 point) was found to be within the acceptance range of (80-120%) and all the calibration points were within (85–115%) by the European Medicines Agency (2011) guidelines as shown in Table 2.

Regarding drugs in plasma, the obtained curves were found to be linear with R^2 of 0.9994, 0.9992, and 0.9996, for AML, ROS, and HC, respectively, Figure 3. Nevertheless, the STD1 (LLoQ point) of AML, STD2 point of ROS, and the STD3 point of HC were out of the acceptance range of (80–120%) for the LLoQ and (85–115%) for the other calibration points, Table 3. However, six calibration points on each curve were within the acceptance range. It was deemed acceptable following the European Medicines Agency (2011) guidelines.

Accuracy results obtained upon the LC/MS-MS simultaneous determination of AML, ROS, and HC in solution and plasma were found to be within the acceptance range of (85–115%) for low (L), medium (M), and high (H) quality

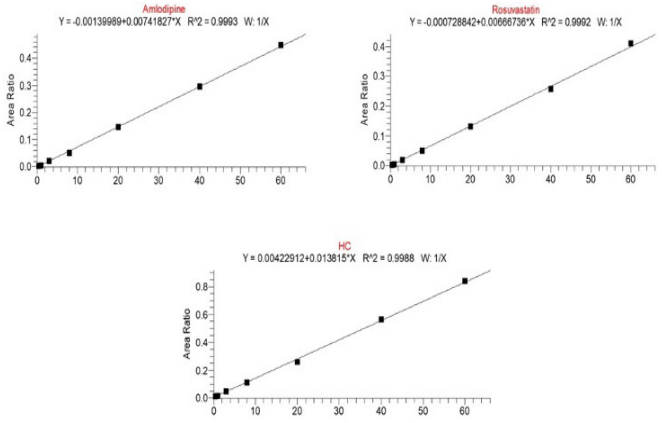

Figure 2: AML, ROS, and HC calibration curves in solution (area ratio against concentration (µg/mL))

control (QC) samples, and (80–120%) for the LLOQ, according to the European Medicines Agency (2011) guidelines. Accuracy showed to be in the range of (88.8–110.2%), (90.4–111.6%), and (86.7–109.5%), for AML, ROS, and HC, respectively, in solution and (88.4–118%), (86.2–109%), and (88.2–113%), for the three drugs, respectively, in plasma. Average accuracy % results over three days are presented in Table 3.

According to the European Medicines Agency's (2011) guidelines, the precision results fell within the acceptance criteria of the percentage coefficient of variation (%CV). The %CV did not exceed the maximum of 15% for low (L), medium (M), and high (H) quality control (QC) samples and 20% for the LLoQ. The %CV ranged from (2.87–7.60%), (3.63–10.40%), and (2.12–7.63%), for AML, ROS, and HC, respectively, in solution and (3.55–10.15%), (3.06–11.41%), and (2.70–16.26%), for the three drugs, respectively, in plasma. The results showed a precise, acceptable degree of scattering for the drugs in solution and plasma. Average results of precision over three days are summarized in Table 4.

In the current study, we developed and verified an analytical LC-MS/MS method for the simultaneous measurement of the three medications. The currently reported methods in the literature are either for the individual determination of each drug alone in solution,[22,29-30] in plasma,[23,31-32,] or for the determination of each drug in combination with others.[33-35]

Owing to the physical and chemical properties of the three drugs, it was challenging to optimize the conditions of a liquid chromatography method and a sample extraction procedure that ensured the proper retention, separation, and thus the simultaneous determination of the three analytes in a single run. The composition of the mobile phase and the selection of the analytical column are the crucial parameters that determine the accuracy and precision of the developed method. In the present work, we started a method development of a mobile phase consisting of acetonitrile and methanol in varying volume ratios (with 0.1% acetic acid) along with either C8 (thermos) or C18 (ACE) columns. It was eventually turned out that an isocratic mixture of 0.1% CH_3COOH and methanolCH_3OH 1:4 v/v (pH adjusted by adding 0.02% trimethylamine) with C18 ACE column are optimum to provide

Table 2: hypertension calibration points linearity results upon the LC/MS-MS concurrent determination of the three drugs.

Calibration point	*Concentration (µg/mL)*	*Calculated Amount (µg/mL)*	*Accuracy (%)*	*Concentration (µg/mL)*	*Calculated Amount (µg/mL)*	*Accuracy (%)*
	AML in solution			*AML in plasma*		
STD[a] 1	5	5.7	114	0.5	0.895	179
STD 2	10	9.4	94	1	1.025	103
STD 3	30	30	100	3	3.087	103
STD 4	80	73	91	8	8.349	104
STD 5	200	198	99	20	19.095	95
STD 6	400	400.6	100	40	39.657	99
STD 7	600	608.1	101	60	60.815	101
	ROS in solution			ROS in plasma		
STD 1	5	5.3	106	0.5	0.554	111
STD 2	10	99.6	99.6	1	0.755	75.5
STD 3	30	29	97	3	2.986	99.5
STD 4	80	79	99	8	7.834	98
STD 5	200	19.8	99	20	17.444	87
STD 6	400	387	97	40	42.224	105.5
STD 7	600	616	103	60	60.466	101
	HC in solution			HC in plasma		
STD 1	5	4.9	99.5	0.5	0.531	106
STD 2	10	10.1	101	1	0.983	98
STD 3	30	31.5	105	3	4.179	139
STD 4	80	79	98.8	8	8.057	101
STD 5	200	185	92.6	20	19.617	98
STD 6	400	407	102	40	39.492	98.7
STD 7	600	607	101	60	59.847	99.7

[a]STD is standard.

the required linearity of the calibration curves and acceptable peak shape of the three analytes and the internal standard.

Besides being the first LC-MS/MS method to simultaneously determine AML, ROS, and HC in solution and plasma, the method presented in this study offers some other advantages over the currently available analytical methods in the literature. In this work, the developed method used the protein precipitation technique (acetonitrile) to extract the analytes from the plasma samples. This is a quicker and less labor-intensive extraction process with fewer steps. Consequently, the presented method is cost-effective and produces less variation in the results of some previously published methods that used other extraction techniques. For example, as shown in Table 5, the analyte was extracted using the organic solvents diethyl ether and dichloromethane, which was reported by Ramakrishna *et al*.[23] In another study by Jaglinska *et al*.,[31] a lengthy and costly five-stage solvent front position extraction was adopted. As shown by Hull *et al*.[36] and Rajasekhar *et al*.[37] studies, the solid-phase extraction procedures were time-consuming and resulted in the more expensive multi-step.

Moreover, optimizing the mobile phase by adding 0.02% trimethylamine improved resolution and increased the method‘s intensity. This reduced the runtime to less than 2 minutes. This was much shorter than the run time compared to other reported methods that was as long as 5 minutes.[22,25,30,31,36] The flow rate in the presented method was 0.5 mL/min and 0.2 mL/min for the analysis in plasma and solution, respectively. Such a low flow rate reduces the backpressure on the column and minimizes the potential segregation of the packed stationary phase. Noteworthy, the flow rate was lower than that implemented in methods in the literature.[22,23,25,30,33,34,36]

In addition, the injection volume in our developed method was 10 µL, which is considered relatively lower compared to 20 µL in some other reports.[20,22,25,30,33] Concerning the mobile phase, we employed an isocratic elution technique using a mixture of 0.1% acetic acid and methanol 1:4 (v/v) to separate the analytes. There is no need for column reconditioning compared to the gradient mobile phase used by Ramakrishna *et al*.[31] Therefore, the isocratic elution is favored over the gradient one. The mobile phase‘s composition was thoughtfully planned and optimized to effectively ensure the separation of the analytes at a reasonable cost. The extensive use of acetonitrile in LC-MS/MS-based analysis led to severe shortages in the solvent‘s supply in 2008 and 2009.[38] In the presented method. We avoided using either acetonitrile or any

Table 3: Hypertension hypertension average accuracy % results over three days upon the three drugs' LC/MS-MS concurrent determination

QC[a] sample	*Day*	*Average accuracy (%)*	*3-Day Average Accuracy (%)*	*QC sample*	*Day*	*Average accuracy (%)*	*3-Day Average Accuracy (%)*
AML in solution				AML in plasma			
L[b] (15 µg/mL)	1	98.65			1	94.76	
	2	99.96	99.81	L (1.5 µg/mL)	2	97.93	97.81
	3	100.81			3	100.75	
M[c] (300 µg/mL)	1	99.37			1	102.69	
	2	100.52	99.15	M (30 µg/mL)	2	98.49	101.02
	3	97.57			3	101.89	
H[d] (500 µg/mL)	1	101.68			1	97.15	
	2	102.56	101.31	H (50 µg/mL)	2	97.22	98.61
	3	99.69			3	101.46	
ROS in solution				ROS in plasma			
L (15 µg/mL)	1	100.40			1	97.45	
	2	100.39	99.73	L (1.5 µg/mL)	2	102.56	98.68
	3	98.39			3	96.03	
M (300 µg/mL)	1	98.47			1	95.51	
	2	100.64	99.88	M (30 µg/mL)	2	99.13	100.25
	3	100.53			3	106.12	
H (500 µg/mL)	1	101.07			1	97.11	
	2	100.91	101.34	H (50 µg/mL)	2	101.62	98.94
	3	102.05			3	98.08	
HC in solution				HC in plasma			
L (15 µg/mL)	1	100.71			1	99.84	
	2	101.17	101.26	L (1.5 µg/mL)	2	104.93	102.11
	3	101.91			3	101.56	
M (300 µg/mL)	1	99.53			1	100.23	
	2	102.34	100.26	M (30 µg/mL)	2	101.75	103.81
	3	98.91			3	109.44	
H (500 µg/mL)	1	102.03			1	98.81	
	2	99.14	98.90	H (50 µg/mL)	2	98.76	98.11
	3	95.53			3	96.75	

[a]QC is quality control, [b]L is low, [c]M is medium, and [d]H is high.

costly components used in the mixture of the mobile phases developed in recent studies such as formic acid,[25,31,33,36] ammonium acetate,[23,33,37] ammonium formate,[20,34] phosphate buffer,[30] and ortho-phosphoric acid.[22] The optimized mobile phase led to the shortest retention time for this investigation compared to others as shown in Table 5.

of 0.5–60 and 5–600 µg /mL in plasma and solution, respectively, and with coefficient of determination (R^2) value greater than 0.99. All calibration points and LLoQ points were within the accepted range of (85–115%) and (80–120%), respectively, according to the European Medicines Agency (2011) guidelines.

The LLoQ for AML, ROS, and HC was set at 0.5 µg /mL in plasma and 5 µg /mL in solution. Samples at 0.5 µg/mL concentration in plasma were tested and evaluated for precision and accuracy. At this concentration, the precision, concerning the %coefficient of variation (CV), was found to be 8.45%, 5.61%, and 12.54%, and the accuracy was found to be 100.07, 101.16 and 98.09% for AML, ROS, and HC, respectively.

The quality control samples of the three drugs in plasma

Table 4: Average precision results over three days upon the LC/MS-MS concurrent determination of the three drugs

QC[a] sample	*Day*	*Average Concentration (µg/mL)*	*%CV*	*3-Day Average %CV*	*QC sample*	*Day*	*Average concentration (µg/mL)*	*%CV*	*3-Day Average %CV*
AML in solution					AML in plasma				
L[b] (15 µg/mL)	1	14.80	4.87	5.50	L (1.5 µg/mL)	1	1.421	10.18	7.17
	2	14.99	7.60			2	1.469	5.67	
	3	15.16	4.03			3	1.440	5.65	
M[c] (300 µg/mL)	1	298.11	4.85	4.20	M (30 µg/mL)	1	30.808	3.96	4.85
	2	301.57	4.87			2	29.548	7.03	
	3	298.29	2.87			3	31.836	3.55	
H[d] (500 µg/mL)	1	508.41	5.73	5.98	H (50 µg/mL)	1	48.573	8.41	7.63
	2	512.82	6.23			2	48.612	5.27	
	3	489.35	5.97			3	49.039	9.22	
ROS in solution					ROS in plasma				
L (15 µg/mL)	1	15.10	5.62	7.17	L (1.5 µg/mL)	1	1.509	11.41	7.81
	2	15.78	10.31			2	1.538	6.38	
	3	14.76	5.58			3	1.440	5.65	
M (300 µg/mL)	1	316.62	10.40	6.26	M (30 µg/mL)	1	30.023	10.15	7.08
	2	301.91	4.76			2	29.796	7.54	
	3	301.60	3.63			3	31.836	3.55	
H (500 µg/mL)	1	514.39	5.64	6.22	H (50 µg/mL)	1	48.554	3.58	5.29
	2	504.57	4.93			2	50.874	3.06	
	3	510.25	8.09			3	49.039	9.22	
HC in solution					HC in plasma				
L (15 µg/mL)	1	15.10	3.95	3.10	L (1.5 µg/mL)	1	1.498	8.82	12.76
	2	15.18	3.23			2	1.649	14.54	
	3	15.39	2.12			3	1.544	14.92	
M (300 µg/mL)	1	298.60	7.63	5.98	M (30 µg/mL)	1	30.070	7.96	7.42
	2	307.92	4.89			2	30.504	10.04	
	3	296.75	5.41			3	32.210	4.27	
H (500 µg/mL)	1	510.20	5.02	4.85	H (50 µg/mL)	1	49.407	2.70	4.78
	2	495.69	2.66			2	49.123	3.16	
	3	477.65	6.86			3	50.599	8.48	

[a]QC is quality control, [b]L is low, [c]M is medium, and [d]H is high.

were analyzed intra- and inter-day. The results showed that the percentages of codine (10.56, 4.85, and 7.63%), ROS (10.71, 7.08, and 5.29%), and HC (12.76, 7.42, and 4.78%) were for AML, ROS (7.17, 6.26, and 6.22%), and HC (3.10, 5.98, and 4.84%) for each drug in solution. In the same context, the intra- and inter-day accuracy results were found to be (97.81, 101.02, and 98.61%) for AML, (99.16, 100.25, and 98.94%) for ROS, and (103.78, 103.81, and 98.11%) for HC in plasma and (99.81, 99.15, and 101.31%) for AML, (99.73, 99.88, and 101.34%) for ROS and (101.26, 100.26, and 98.90%) for HC in solution. Noteworthy, the obtained intra- and inter-day %CV and accuracy results were found to comply with the guidelines of the European Medicines Agency (2011), which stipulate a maximum threshold of 15% for the %CV and (85-115%) accuracy range upon analyzing the L-QC, M-QC, and H-QC samples using the developed analytical method.

Table 5: Comparison of the current study's findings with those from earlier publications in the literature

Method type	*Drug analyzed*	*Drug extraction (if any)*	*Mobile phase*	*Injection volume (µL)*	*Flow rate; run time*	*Retention time*	*LLoQ*	*Linearity (R^2)*	*Precision*	*Accuracy*	*Reference*
LC-MS/MS	AML, ROS, and HC in solutions and plasma	precipitating agent (acetonitrile) containing 1 µg/mL of IS (ondansetron) was added in 2:1 ratio (400:200 µL) to the sample	isocratic mixture of 0.1% acetic acid and methanol 1:4 (v/v) (pH adjusted by adding 0.02% trimethylamine)	10	0.5 mL/ min and 0.2 mL/min for measuring the analytes in plasma and in solution; 2 min	0.88, 0.87, and 0.81 min. for AML, ROS, and HC, respectively.	500 ng/mL	Min 0.9988	2.87-11.41%	86.2-113%	This study
LC-MS/MS	AML in human plasma	Analytes were quantitatively extracted using solid phase extraction on Oasis HLB cartridges from 1000 µL of human plasma.	Isocratic elution using methanol and ammonium formate (pH 4.5, 10.0 mM; 80:20)	20	0.5 mL/min; 3.2 min	1.82 min	0.1 ng/mL	Min. 0.9990	Intra-run and inter-run precision was ≤ 8.94%.	93.53–103.3%.	20
LC-UV	AML in powdered tablets/ capsules	10 mg was transferred to a 100 mL volumetric flask. After addition of 50 mL diluting solution and sonication (15 min), the samples were made up to volume with same solvent and centrifuged. An aliquot (2 mL) of the clear supernatant liquid was transferred to a 10 mL volumetric flask and diluted with water.	Isocratic elution using 0.1% (v/v) ortho-phosphoric acid (pH 3.0) and acetonitrile (60 : 40, v/v)	20	1.0 mL/min; 10 min.	4.6 min	1 µg/mL	0.9999	Intra-day and inter-day precision was ≤ 2%.	98.0–102.5%.	22
LC-MS/MS	HC in plasma.	The analyte was extracted using diethyl ether and dichlorometh-ane (70:30, v/v) from 500 µL plasma.	Isocratic elution using 10 mm Ammonium acetate–methanol (15:85, v/v).	20	1.0 mL/min; 2.5 min.	1.6 min	0.5 ng/mL	Min. 0.99	Intra-batch and inter-patch precision was ≤9.0%.	93.3–106%.	23
LC-MS/MS	ROS in human plasma.	ROS was extracted from a 0.5 mL plasma sample using 4 mL ethyl acetate, acidified with 100 µL of 0.1 M HCl.	Isocratic elution using acetonitrile and methanoic acid (0.1%) (60:40, v/v) mixture.	20	0.8 mL/min; 6 min.	3.0 min.	0.1 ng/mL	> 0.99	Intra-day and inter-day precision was ≤ 7.7%.	89.9– 97.2%.	25

RP-HPLC	HC in tablets	N/A	Isocratic elution using Phosphate buffer (pH 2.5) and acetonitrile (50:50, v/v) mixture.	20	0.6 mL/min; 7 min.	3.47 min.	20 µg/mL	0.9999	Intra-day and inter-day precision was ≤ 0.20%.	100.08–100.33%.	30
LC-MS/MS	HC in plasma.	Solvent front position extraction technique, implying five stages, was adopted. The drug was extracted using a prototype horizontal chamber for TLC/HPTLC with a moving pipette, powered by a 3D mechanism, to distribute the solvent on the adsorbent layer.	Gradient elution of solvent A (0.1% formic acid in water) and solvent B (100% acetonitrile)	N/A	0.4 mL/min; 8 min.	2.1 min	4.41 ng/mL	0.984	Intra-day and inter-day precision was ≤ 9.74%.	88.90–79.88%.	31
LC-MS/MS	ROS and AML in human plasma	Liquid–liquid extraction of the drugs from 100 µL aliquots of plasma using ethyl acetate and n-hexane mixture (80:20, v/v)	Isocratic elution using 0.1% formic acid in 5 mM ammonium acetate, methanol, and acetonitrile (20:20:60, v/v/v) mixture.	20	0.75 mL/min; < 2.5 min.	1.3, and 1.7 min, respectively	0.52, 0.10 ng/mL, respectively	0.9999	Intra-day and inter-day precision was ≤ 4.81% for all analytes.	97.05–100.68%, and 99.00–102.45%.	33
LC-MS/MS	AML, valsartan, and HC in plasma.	Analytes were quantitatively extracted using solid phase extraction on Oasis HLB cartridges from 100 µL of human plasma.	Isocratic elution using acetonitrile and 2 mM ammonium formate, pH 4.0 (90:10, v/v).	5	1.2 mL/min; 2.5 min.	1.80, 1.08, 1.43 min, respectively	0.02, 5, 0.2 ng/mL, respectively	1.0083, 0.9738, 0.9760, respectively	Intra-batch and inter-batch precision was ≤ 5.56% for all the analytes.	93.4–99.6% for all the analytes.	34
LC-MS/MS	ROS in plasma.	Analytes were quantitatively extracted using solid phase extraction on Oasis HLB cartridges from 500 µL of human plasma.	Isocratic elution using methanol and 0.2% formic acid in water (70:30 v/v).	100	1.0 mL/min; 5 min.	3.76 min	0.1 ng/mL	0.998	Intra-batch and inter-batch precision was ≤ 15%.	93–107%.	36
LC-MS/MS	HC in plasma.	Analytes were quantitatively extracted using solid phase extraction on Oasis HLB cartridges from 3000 µL of human plasma.	Isocratic elution using 2 mM ammonium acetate with acetonitrile in the ratio of 10 : 90 (v / v).The rinsing solution was prepared by mixing acetonitrile with HPLC water in the ratio of 50 : 50 (v / v)	20	0.5 mL/min; 2.5 min	1.50 min	2.036 ng/mL	> 0.99	Intra-day and inter-day precision was ≤ 5.17%.	98.43–106.05%.	37

Given our results, it could be suggested that the developed LC-MS/MS method for the concurrent quantification of the three drugs (AML, ROS, and HC) in solution and plasma is selective, accurate, and precise, which makes it very beneficial in bioavailability /bioequivalence studies and for therapeutic drug monitoring purposes.

CONCLUSION

This study is the first to employ LC-MS/MS to determine HC, ROS, and AML simultaneously in solution and human plasma. An affordable and economical extraction was possible using acetonitrile as a precipitating agent. From extraction to analysis, the process is straightforward, having a short run time, which is suitable for high-throughput analysis. The presented approach was valid, simple, precise, and accurate. Because of the dynamic range of linearity, the plasma concentrations of the three drugs can be quantified in therapeutic drug monitoring and clinical investigations for pharmacokinetic, bioavailability, and bioequivalence research.

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