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UPLCMS Method Development and Validation of Amlodipine, Hydrochlorthiazide and Losartan in Combined Tablet Dosage Form

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Abstract

A simple, rapid, sensitive and specific UPLCMS method was developed and validated following ICH guidelines for simultaneous estimation of tablet dosage form containing amlodipine (AMLO) hydrochlorothiazide (HCT) and losartan (LOSAT) using telmisartan (TELMI) as an internal standard (IS). The separation was achieved using Waters ACQUITY BEH C18 (1.7 µm, 2.1 × 50 mm) column with gradient mode, mobile phase containing acetonitrile (A) & 1% ammonium acetate (B) pH adjusted to 2.8 with trifluoro acetic acid with gradient mode. The flow rate was 0.4 mL·mL^{-1} and the injection volume 2 µl. The retention time for amlodipine, hydrochlorothiazide and losartan was found to be 3.7, 2.5 and 3.9 min, respectively. The developed method was found to be linear over the concentration range of 50 - 300 ng·mL^{-1}, 125 - 750 ng·mL^{-1} and 500 - 3000 ng·mL^{-1} for AMLO, HCT and LOSAT respectively. The signal intensities obtained in ion mode for amlodipine, hydrochlorothiazide, losartan and telmisartan (IS) they were found to be much higher positive ion mode (M+)– parent ion at *m/z*, 409.02, 297.97, 422.91 and 515.03, respectively, in QUATTROZQ full scan mass spectra.

Keywords

UPLCMS, Amlodipine, Hydrochlorothiazide, Losartan, Telmisartan, Simultaneous Estimation

1. Introduction

Cardiovascular diseases (CVDs) are the disorders of heart and blood vessels and primarily include coronary

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heart disease, hypertension, cerebrovascular disease, peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure. CVDs are the major cause of death in developed countries and also are rapidly emerging as a main cause of death in the developing world. An estimated 17.5 million people died from CVDs till 2005, representing almost 30% of all the global deaths. It is projected that almost 20 million people will die from CVDs by 2015. The major risk factors involved in CVDs are high low density lipoprotein (LDL) cholesterol, raised blood pressure, increased serum homocysteine level and platelet aggregation, which are primarily caused by unhealthy diet, physical inactivity and tobacco use.

Ultra-performance liquid chromatography (UPLC) is a new category of separation technique based upon well-established principles of liquid chromatography, which utilizes sub-2 µm particles for stationary phase. These particles operate at elevated mobile phase linear velocities to affect dramatic increase in resolution, sensitivity and speed of analysis. Owing to its speed and sensitivity, this technique is gaining considerable attention in recent years for pharmaceutical and biomedical analysis. Besides that, the combination of UPLC with a tandem mass spectrometer (MS/MS) appears to be a suitable approach that fulfills key requirements in terms of sensitivity and selectivity for the rapid determination of analytes at low concentrations in complex matrices. In the present work, this technology has been applied to the method development and validation amlodipine, hydrochlorothiazide and losartan in bulk drug and in pharmaceutical dosage form.

Amlodipine besylate (AMLO), chemically described as 3-ethyl-5-methyl-(4RS)-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate benzenesulfonate is an antihypertensive agent. AMLO is a calcium antagonist that inhibits the transmembrane influx of calcium ions into vascular smooth muscles and cardiac muscles, which in turn affects their contractile process and results in reduced blood pressure, lower heart rate, reduce chest pain (angina), and to reduce the risk of recurrent heart attacks. Literature review reveals various methods for estimation of amlodipine alone and its combination are by liquid chromatography coupled with UV [1] [2], voltametric [3], mass spectrophotometric [4] [5], HPLC [6]-[8], gas chromatography [9], HPTLC [10] [11].

Hydrochlorothiazide (HCT), 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulphonamide-1,1-dioxide is the potent orally diuretic and antihypertensive agent related to hydrochlorothiazide. This inhibits active chloride reabsorption and thus increases the excretion of sodium chloride and water. There are several methods for the determination hydrochlorothiazide in tablet dosage forms by using spectrophotometric [12]-[14], fluorodensistometric [15], gas and liquid chromatographic [16]-[18], LCMSMS [19] polarographic techniques [20].

Losartan potassium is monopotassium salt of 4-butyl-4-chloro-1-[[2'-(1H-tetrazole-5-yl)[1,1'-biphenyl]-4-yl] methyl]-1H-imidazole-5-methanol. It is a selective, compitative angiotensin II receptor type 1 (AT_1) receptor antagonist. Losartan administration results in a decrease in total peripheral resistance and cardiac venous return. However, several methods have been described for the determination of Losartan potassium drug substance in tablets. Various methods developed are HPLC [21] [22], spectrophotometric [23] [24], capillary electrophoresis (CE) [25]-[28], HPTLC [29] [30] voltamatric [31] and liquid chromatography electrospray ionization tandem mass spectrometry [32].

Market is flooded with combination of drugs in various dosage forms. The multicomponent formulations have gained a lot of importance nowadays due to greater patient acceptability, increased potency, multiple action, fewer side effects and quicker relief. There is a plethora of analysis of such formulations without prior separation. Simultaneous analysis procedures are now being used more frequently for estimation of drugs in multicomponent pharmaceutical formulations due to their inherent advantages viz. avoid time consuming extraction and separation, economical in the sense that use of expensive reagents is minimized are equally accurate and precise.

It is known several patients with hypertension require two or more antihypertensive drugs with complementary mechanisms of action to lower their blood pressure. The angiotensin II type 1-receptor antagonist (amlodipine, losartan, atenolol and telmisartan) and the diuretic Hydrochlorothiazide are two antihypertensive agents that have a well recognized clinical efficacy. Their combination was shown in randomized, controlled trials to be more effective than each agent alone in lowering blood pressure, due to a dual and synergistic mechanism.

Although various HPLC-MS/MS, HPLCMS methods have been reported for the simultaneous estimation of antihypertensive drugs in tablet dosage form in ternary and binary formulation of HCT, AMLO and LOST; UPLCMS method for simultaneous estimation these drugs in tablet dosage using internal standard has not been reported.

A simple, rapid and reliable UPLCMS method has been established for simultaneous determination of HCT, AMLO and LOST. The method has several advantages, including rapid analysis, a simple mobile phase, simple sample preparation and improved sensitivity and relatively short time. It is suitable for analysis of these antihypertensive agents in their ternary formulations in a single isocratic run, in contrast with previous methods. This makes the method suitable for routine analysis in quality-control laboratories.

2. Experimental

2.1. Materials and Reagents

Working standards, Amlodipine (AMLO), Hydrochlorothiazide (HCT) Losartan (LOSAT) Telmisartan (TELMI), were procured from Ranbaxy laboratories, New Delhi, India. Ammonium acetate AR grade, HPLC grade Acetonitril, Methanol, trifluoro acetic acid (AR grade) and water from Ranchem, Mumbai. The pharmaceutical dosage form containing 12.5 mg HCT, 5 mg AMLO and 50 mg LOSAT, Triclopace (Sun Pharmaceuticals Ltd.), 20 tablets purchased from a local drug store. Telmisartan which was employed as an internal standard (IS) was obtained from Ranbaxy Laboratories, New Delhi.

2.2. Instrumentation

UPLC Condition

The development and validation of the assay was performed on a Waters Aquity UPLC Micromass Quattro Micro API Mass spectrometer (Waters Corp., MS, USA), equipped with a binary solvent delivery system, auto sampler, photodiode array and ELSD detector. The chromatography was performed on a Waters ACQUITY BEH C18, 1.7 µm, 2.1 × 50 mm column (Waters Corp., Ireland) with mobile phase containing acetonitrile (A) & 1% ammonium acetate (B) pH 2.8 adjusted with trifluoro acetic acid [Gradient mode (2 min: 98% A : 2% B, 2 - 4 min: 24% A : 76% B, 4 - 5 min, 50% A : 50% B, 8 - 10 min, 2% A: 98% B)]. The flow rate was 0.4 mL·min^{-1} column maintained at 25˚C and the injection volume was 2 µl.

2.3. Standard Preparation

Separate stock solutions of 10,000 ng·mL^{-1} of amlodipine, hydrochlorothiazide, losartan and telmisartan were prepared by dissolving in 25 mL HPLC grade methanol and diluted to 100 mL with acetonitrile, then 10 mL of stock solution into a 100 mL of standard volumetric flask and diluted with acetonitrile (stock solution contains 1000 ng·mL^{-1}). The prepared stock solutions were stored at 25˚C protected from light.

2.4. Preparation of Calibration Plot

Standard solutions were freshly prepared from the stock solution by diluting with acetonitrile as 50, 100, 150, 200, 250 & 300 ng·mL^{-1} amlodipine, 125, 250, 375, 500, 625 & 750 ng·mL^{-1} of hydrochlorothiazide, and 500, 1000, 1500, 2000, 2500 & 3000 ng·mL^{-1} losartan respectively. Each solution was injected in triplicate and chromatographed under the chromatographic conditions specified above. Telmisartan (20 ng·mL^{-1}) was used as internal standard for determination of mixtures of amlodipine, hydrochlorothiazide & losartan with telmisartan. Linear relationships were obtained when drug to internal standard peak-area ratios were plotted against the corresponding concentrations for each drug.

2.5. Sample Preparation

Average weight was calculated by weighing 20 tablets. The tablets were crushed into homogenous powder. A quantity of powder equivalent to one tablet containing 2.5 mg of amlodipine, 12.5 mg of hydrochlorothiazide and 50 mg of losartan was transferred into a 100 mL volumetric flask. To this flask, 25 mL of methanol were added, and the solution was sonicated for 30 min with intermittent shaking. The solution was cooled to ambient temperature. Then the volume was made up with acetonitrile and centrifuged at 10,000 rpm for 10 min. The centrifuged solution filtered through a 0.45 µm nylon filters (Millipore, Milford, MA, USA). From the filtered solution, aliquots of appropriate volume were transferred to 10 mL volumetric flasks and diluted to volume with acetonitrile to furnish the concentration range listed in **Table 1**.

Table 1. Results of system suitability study.

	Amlodipine		Hydrochlorothiazide		Losartan		Telmisartan (IS)	
	Retention time (min)	Peak area	Retention time (min)	Peak area	Retention time (min)	Peak area	Retention time (min)	Peak area
Mean	3.7	114,310	2.5	21,898	3.9	227,792	3.8	12,463
SD	0.0209	1783.6	0.0263	322.362	0.0268	1454.681	0.0081	165.480
RSD	0.566	1.560	1.039	1.472	0.6845	0.6385	0.2152	1.3277

2.6. Method Validation

2.6.1. Linearity & Range

The linearity of the method was evaluated by analyzing different concentration of the drugs. According to ICH recommendations, at least five concentrations must be used. In this study six concentrations were chosen, in the ranges 50, 100, 150, 200, 250 & 300 ng·mL^{-1} amlodipine, 125, 250, 375, 500, 625 & 750 ng·mL^{-1} of hydrochlorothiazide, and 500, 1000, 1500, 2000, 2500 & 3000 ng·mL^{-1} losartan respectively.

2.6.2. Accuracy and Precision

The accuracy of the method was determined by recovery experiments using the standard addition method. Each solution was injected in triplicate and percentage recovery was calculated. The precision of the method was assessed by studying intra-day and inter-day variation. In the intra-day studies, standard and sample solutions were analysed in triplicate on the same day and percentage RSD was calculated. In the inter-day studies, standard and sample solutions were analysed in triplicate on three consecutive days and percentage RSD were calculated.

2.6.3. Limits of Detection (LOD) and Limit of Quantitation (LOQ)

In accordance with ICH recommendations the approach based on the standard deviation of the response and the slope of the calibration plots was used to determine detection and quantification limits. LOD and LOQ values were estimated as [(standard deviation of repeatability)/(slope of the regression equation)] by multiplying with 3.3 and 10 respectively. The values obtained are given in **Table 2**.

2.6.4. Selectivity

The selectivity of the method was evaluated by assessing whether excipients present in the pharmaceutical formulations interfered with the analysis. A placebo for each tablet was prepared by mixing the respective excipients, and solutions were prepared by following the procedure described in the section on sample preparation. The commonly used tablet excipients did not interfere with the method.

2.6.5. Robustness

Robustness is a measure of capacity of analytical methods to remain unaffected by small but deliberate variation of the operating conditions. This was tested by studying the effect of changing mobile phase pH by ±0.2, the amount of buffer in the mobile phase by ±2%, and detector wavelength by ±2 nm.

3. Results and Discussion

It is important to optimize the UPLC conditions and develop UPLCMS method and validate in pharmaceutical formulations, preliminary tests were performed with the objective of selecting optimum chromatographic conditions. The separation was tried using different columns described previously in the literature or alternative stationary phases. The main problems encountered during these investigations were lack of resolution between amlodipine, hydrochlorothiazide and losartan with IS. To solve these problems, three columns, HSS C18, BEH C8, and BEH Phenyl, BEH C18 were used for simultaneous determination of the drugs. The best resolution and peak shape, without excessive tailing, were obtained by use of the BEH C18 column. The effect of mobile phase composition, flow rate and pH were also studied. The best resolution with reasonable retention time was obtained with mobile phase containing acetonitrile and 1% ammonium acetate buffer (pH adjusted with trifluoro acetic

Table 2. Linearity range, LOD & LOQ.

Name of the drug	Linearity range (ng·mL^{-1})	LOD (ng·mL^{-1})	LOQ (ng·mL^{-1})	Regression equation
AMLO	50 - 300	0.1	1.0	y = 0.0858x + 0.0928 R^2 = 0.9997 (n = 6)
HCT	125 - 750	0.6	1.0	y = 0.0067x R^2 = 0.9985 (n = 6)
LOSAT	500 - 3000	2.0	5.0	y = 0.0178x R^2 = 0.9994 (n = 6)

acid) in gradient elution mode with 0.5 mL·mL^{-1} flow rate. A major reason for using a concentration of 1% was to achieve maximum sensitivity of PDA detection at low wavelengths. The specificity of the method is illustrated in **Figure 1**, which indicates separation of the compounds was complete. Average retention times ± standard deviation for IS, AMLO, HCT and LOSAT were 3.7 ± 0.02092.5 ± 0.0263, 3.9 ± 0.0268 min, respectively, for six replicate analyses. In determination of accuracy and precision, recovery was 99.90 ± 1%, which indicates the method is accurate, and intra-day and inter-day variation, as RSD, were no more than 0.653%, indicating the method is precise. In determination of the robustness of the method, slight variation of mobile phase pH, amount of buffer, in the mobile phase, and detector wavelength had no significant effect on chromatographic resolution.

Mass spectrometry parameters to develop and validate a selective and rapid assay method for simultaneously determination of amlodipine, hydrochlorothiazide and losartan in pharmaceutical dosage form. MS parameters were optimized by infusing standard analyte solution of 500 ng·mL^{-1} into the mass spectrometer having atmospheric pressure electrospray as the ionization source. The signal intensities obtained in ion mode for amlodipine, hydrochlorothiazide and losartan was found to be much higher positive ion mode (M+)− parent ion at *m/z*, 409.02, 297.97 and 422.91 and telmisartan (internal standard) were higher in positive ion mode (M + H)+ parent ions at *m/z* 515.03, respectively in QUATTROZQ full scan mass spectra. Fragmentation was initiated using sufficient nitrogen for collision-activated dissociation and by applying 8 V collision energy to break the parent ions. It was observed that higher nebuliser gas pressure (70 psi) had a better impact on spectral response. The intensity was further enhanced after acidifying the solution, as it increases the ionization (protonation) resulting in high response in positive ion mode. Cone voltage, capillary voltage, source temperature and desolvation gas temperature did not have much impact on behavior of compounds and were maintained at 3.50˚C, 30.00˚C, 120˚C and 350˚C, respectively. There was no cross talk between the MRMs of analytes and IS. Fine tuning of gas 1 (nebuliser gas), gas 2 (heater gas) and CAD gas was done to get a consistent and stable response with high signal to noise ratio. **Figures 1-3** show the mass spectra of parent and product ions for analytes and IS, respectively. An APEI single probe was selected as the ionization source as it gave high spectral response for both the analytes and the regression curves obtained were linear. The APEI source provided reliable data on method validation and for quantitation of drugs in pharmaceutical dosage form. Since amlodipine, hydrochlorothiazide, losartan and telmisartan have different physicochemical properties, it was difficult to set chromatographic conditions that produced sharp peak shape and adequate response. This included mobile phase selection, flow rate, column type and injection volume. Acetonitrile and methanol were tried in different ratio with buffers like ammonium acetate, ammonium formate as well as acid additive like acetic acid in varying strength. It was observed that 1% ammonium acetate: acetonitrile as the mobile phase in gradient mode was most appropriate to give best sensitivity, efficiency and peak shape. Acidic buffer helped to improve the peak shape and spectral response. 50% aqueous part was adequate to retain the polar compound amlodipine, hydrochlorothiazide, and losartan. The use of a short chromatography column Waters ACQUITY BEH C18, 1.7 µm, 2.1 × 50 mm helped in the separation and elution of all four compounds including internal in a very short time. The total chromatographic run time was 5 min for each run. Simultaneous recovery of the amlodipine, hydrochlorothiazide and losartan were 98.73% - 99.9%, 99.46% - 99.98% & 98.7% - 99.9% respectively. Overall, this method is fast and simple in terms of chromatography and sample preparation, respectively, which helped in giving a high turn around for routine sample analysis. The developed method was validated in accordance with ICH guidelines. Since all three drugs viz amlodipine, hydrochlorothiazide, losartan and telmisartan (internal standard) had similar chromatographic behaviour. Moreover, there was no significant matrix effect of IS on the analytes. Also, the validation results obtained from this UPLC-MS/MS methodology encouraged its selection as an IS for the present study.

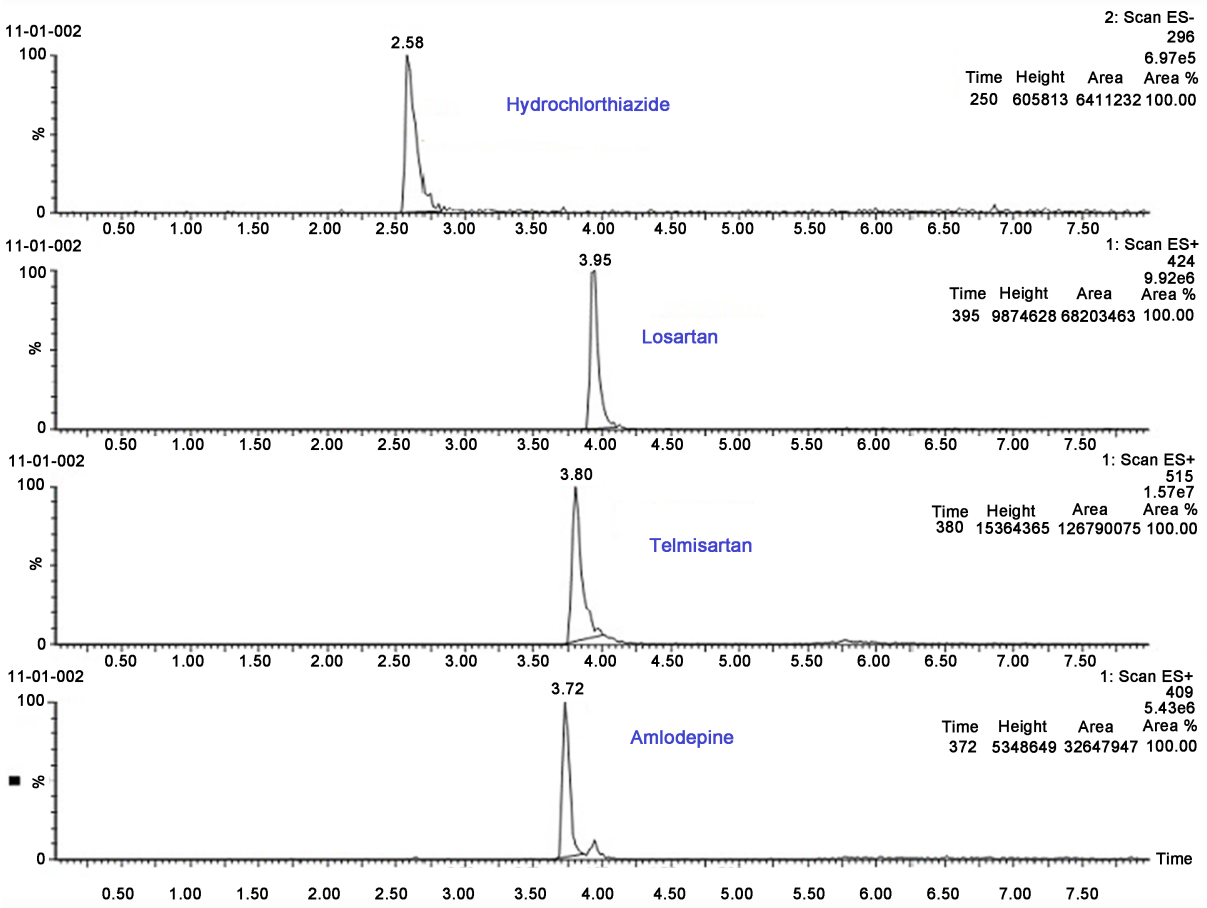

Figure 1. UPLC chromatogram of HCT, LOSAT, TELMI and AMLO.

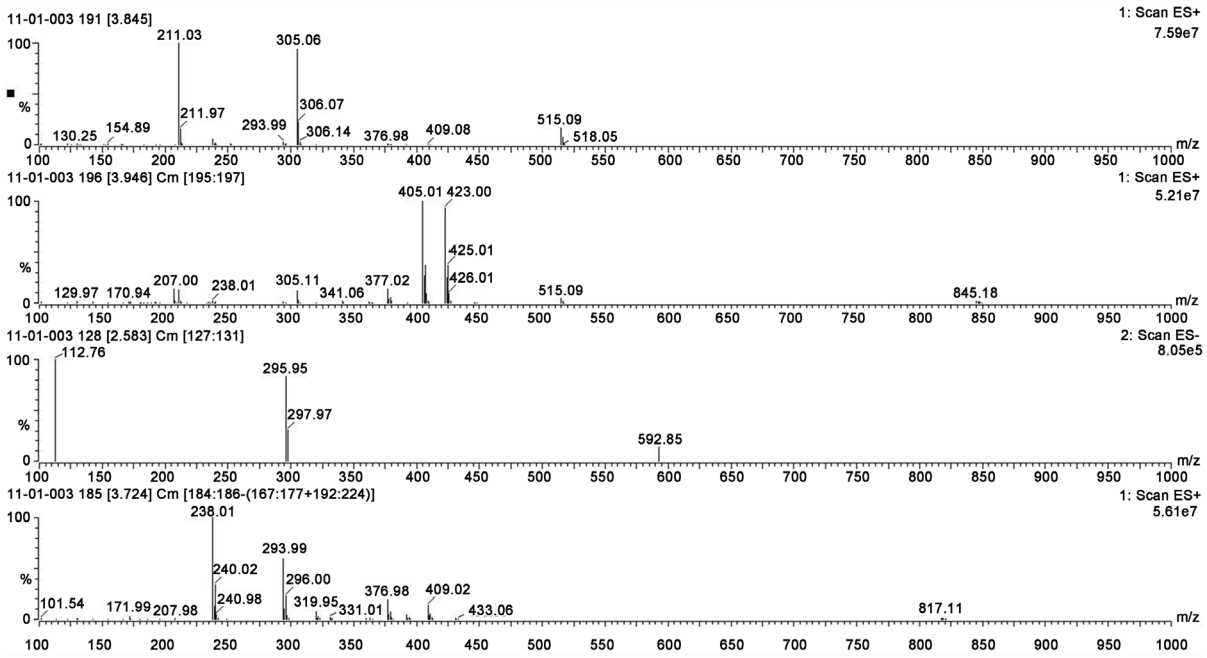

Figure 2. Mass spectra of HCT, LOSAT, TELMI and AMLO.

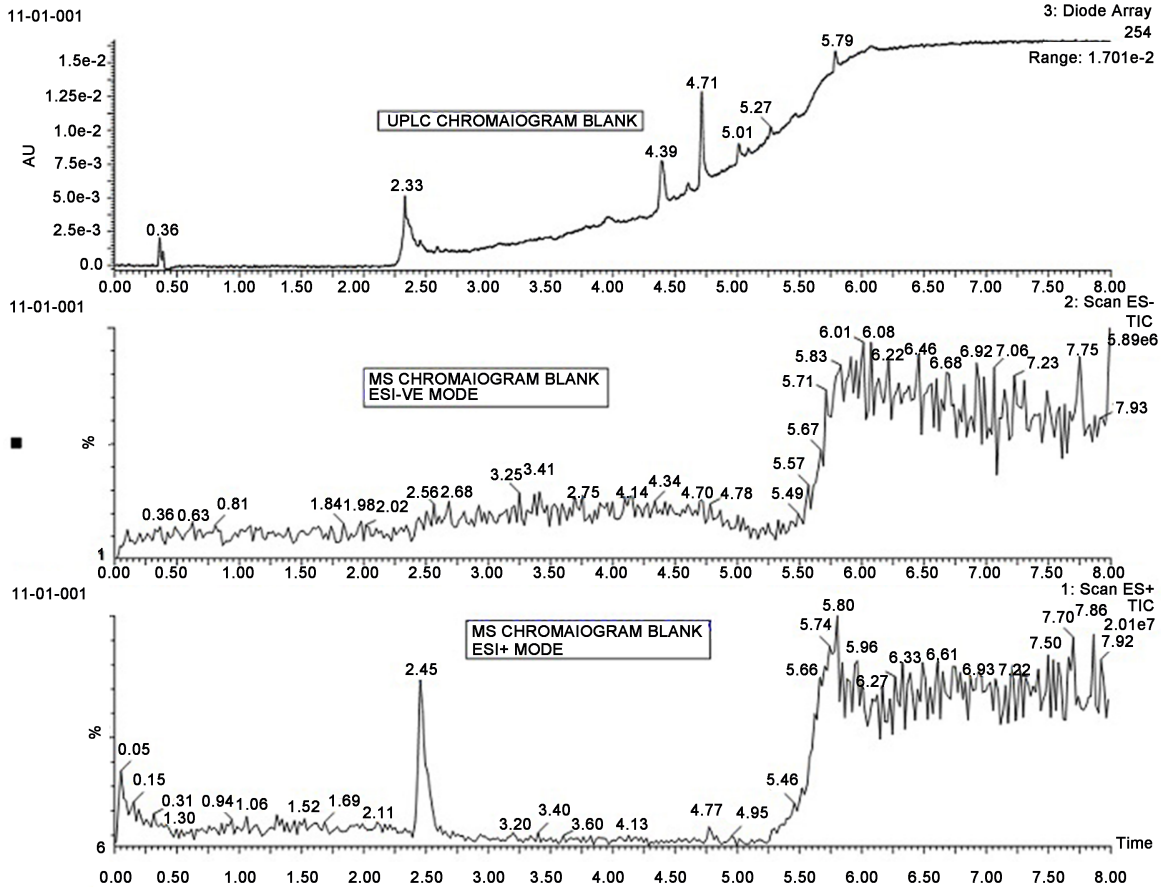

Figure 3. Blank UPLCMS chromatogram.

3.1. Method Validation

3.1.1. System Suitability

The RSD values of peak area and retention time for drugs and IS are within 2% indicating the suitability of the system (**Table 3**).

3.1.2. Linearity

The calibration curves were prepared by plotting the peak areas of the drug to IS which were linear in the range of 50 - 300 ng·mL^{-1}, 125 - 750 ng·mL^{-1} and 500 - 3000 ng·mL^{-1} of AMLO, HCT, and LOSAT respectively. Peak area ratios and concentrations were subjected to least square linear regression analysis to calculate the calibration equations and correlation coefficients. The mean regression equations were found as y = 0.0858x + 0.0928 R^2 = 0.9997 (n = 6), y = 0.0067x R^2 = 0.9985 (n = 6) and y = 0.0178x R^2 = 0.9994 (n = 6) for AMLO, HCT and LOSAT respectively. Y = ax + b where "y" is the peak area ratio of drugs, "a" is the slope, "b" is the intercept and "x" is the concentration of the measured solution in ng·mL^{-1}. The result shows that there is an excellent correlation between the peak area ratios and the concentrations of drugs in the range tested.

3.1.3. LOD and LOQ

The LOD was 0.1 ng·mL^{-1} for AMLO, 0.6 ng·mL^{-1} for HCT and 2 ng·mL^{-1} for LOSAT at a signal to noise ratio of 3.3. The limit of quantification was determined as 1 ng·mL^{-1} for AMLO, 1 ng·mL^{-1} HCT and 5 ng·mL^{-1} for LOSAT at a signal to noise ratio of 10.

3.1.4. Precision

Intra-day precision was performed by relative standard deviation of six repeated assays of samples at the three

Table 3. Intra-day and inter-day precision and accuracy of AMLO, HCT and LOSAT.

Name of the drug	Actual concentration ($ng \cdot mL^{-1}$)	Intra-day			Inter-day		
		Found concentration ($ng \cdot mL^{-1}$) ± SD	RSD (%)	RME (%)	Found concentration ($ng \cdot mL^{-1}$) ± SD	RSD (%)	RME (%)
	100	99.760 ± 0.202	0.202	0.090	99.323 ± 0.648	0.653	0.292
AMLO	150	149.538 ± 0.298	0.199	0.089	149.54 ± 0.332	0.222	0.099
	200	199.672 ± 0.290	0.145	0.065	199.302 ± 0.518	0.260	0.116
	250	249.79 ± 0.169	0.067	0.030	249.72 ± 0.144	0.057	0.025
HCT	375	374.89 ± 0.381	0.101	0.045	374.85 ± 0.431	0.115	0.051
	500	499.89 ± 0.109	1.291	0.021	499.76 ± 0.146	0.029	0.013
	1000	999.835 ± 0.129	0.013	0.006	999.03 ± 0.441	0.044	0.020
LOSAT	1500	1499.890 ± 0.079	0.005	0.002	1497.05 ± 6.478	0.433	0.194
	2000	1999.148 ± 0.533	0.027	0.012	1999.44 ± 0.645	0.032	0.014

concentration levels. Inter-day precision was determined by analyzing the same set of samples of five different days. The RSD values were found to be 0.145% - 0.653% for AMLO, 0.021% - 0.1152% for HCT and 0.005% - 0.433% for LOSAT respectively, indicating good precision (**Table 3**).

3.1.5. Recovery

To examine the accuracy of the method, recovery studies were carried out by standard addition method. The percent recovery of the added standard to the assay samples was calculated from:

$$\text{Recovery } \% = \left[\left(C_1 - C_u \right) / C_a \right] \times 100$$

were C_1 is the total concentration of analyte found; C_u is the concentration analyte present in the formulation; and C_a is the concentration added to the formulation. The average percent recoveries recoveries obtained as 99.28% - 99.97% indicate good accuracy of the method (**Table 4**).

3.1.6. Specificity

The specificity of the UPLCMS method was determined by the complete separation of AMLO, HCT, LOSAT and IS as show in (**Figure 1**) with parameters like retention time (t_R), resolution (R_s) and tailing factor (T). The peaks obtained for AMLO, HCT, LOSAT and IS were sharp and have a clear baseline separation.

3.1.7. Robustness

To ensure the insensitivity of the HPLC method to minor changes in the experimental conditions it is important to demonstrate robustness of the method. None of the modifications caused a significant change in the resolution between the drugs and IS, peak area RSD, USP tailing factor, peak width or theoretical plates.

4. Conclusion

A simple, rapid, and reliable UPLCMS method has been established for simultaneous determination of AMLO, HCT, LOSAT either alone or in their ternary formulations. The method has several advantages, including rapid analysis, a simple mobile phase, simple sample preparation, and improved sensitivity. It is suitable for analysis of these antihypertensive agents in their ternary formulations in a single isocratic run, in contrast with previous methods. This makes the method suitable for routine analysis in quality-control laboratories.

Table 4. Results of recovery studies by standard addition method.

Name of the drug	Amount of drug in tablet (ng)[a]	Amount of pure drug added (ng)	Total found (ng)[b] (Mean ± SD[c])	RSD (%)	Recovery of pure drug added (%)
	99.11	100	198.21 ± 0.402	0.203	99.10
AMLO	99.11	150	248.40 ± 0.510	0.205	99.50
	99.11	200	297.93 ± 0.604	0.203	99.40
	249.14	250	499.46 ± 0.493	0.099	99.86
HCT	249.14	375	623.00 ± 0.636	0.102	99.70
	249.14	500	746.81 ± 2.501	0.335	99.54
	998.76	1000	1995.12 ± 4.675	0.234	99.60
LOSAT	998.76	1500	2490.50 ± 7.036	0.283	99.40
	998.76	2000	2995.47 ± 0.587	0.020	99.84

[a]TERAM-H5 20 tablets purchased from a local drug store (containing 12.5 mg HCT, 5 mg, RAMI, 40 mg TELMI); [b]Five independent analyses; [c]Standard deviation.

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