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Different spectrophotometric and TLC-densitometric methods for determination of olmesartan medoxomil and hydrochlorothiazide and their degradation products

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ABSTRACT

In this work, multivariate calibration models and TLC-densitometric methods have been developed and validated for quantitative determination of olmesartan medoxomil (OLM) and hydrochlorothiazide (HCZ) in presence of their degradation products, olmesartan (OL) and salamide (SAL), respectively. In the first method, multivariate calibration models including principal component regression (PCR) and partial least square (PLS) were applied. The wavelength range 210-343 nm was used and data was auto-scaled and mean centered as pre-processing steps for PCR and PLS models, respectively. These models were tested by application to external validation set with mean percentage recoveries 99.78, 100.01, 100.41 and 100.46% for OLM, HCZ, OL and SAL, respectively, for PLS model and also, 100.22, 100.40, 102.25 and 100.13% for them, respectively, for PCR model. The second method is TLC-densitometry at which the chromatographic separation was carried out using silica gel $60F_{254}$ TLC plates and the developing system consisted of a mixture of ethyl acetate:chloroform:methanol: formic acid:tri-ethylamine (60:40:4:4:1, by volume) with UV-scanning at 254 nm. The developed methods were successfully applied for determination of OLM and HCZ in their pharmaceutical dosage form. Also, statistical comparison was made between the developed methods and the reported method using student's-t test and F-test and results showed that there was no significant difference between them concerning both accuracy and precision.

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1. Introduction

Olmesartan medoxomil chemically is (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl 5-(2-hydroxypropan-2-yl)-2-propyl-3-[[4-[2-(2*H*-tetrazol-5-yl)phenyl] phenyl]methyl]imidazole-4-carboxylate [1] (Figure 1). It is considered as a prodrug, which is hydrolyzed to the active form, olmesartan during absorption from the gastrointestinal tract. Olmesartan (OL) chemically is 5-(2-hydroxypropan-2-yl)-2-propyl-3-[[4-[2-(2*H*-tetrazol-5-yl)phenyl]phenyl]methyl]imidazole-4-carboxylic acid [1] (Figure 1). It is a selective AT_1 subtype angiotensin II receptor antagonist, and also it is considered as a hydrolytic degradation product for olmesartan medoxomil [2].

Hydrochlorothiazide chemically is 6-chloro-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide [1] (Figure 1). It is a thiazide diuretic works by inhibiting water reabsorption in the nephron. Salamide chemically is 4-amino-6-chlorobenzene-1,3-disulfonamide [1] (Figure 1). It is reported to be process impurity of HCZ [1]. Additionally, SAL was found to be HCZ hydrolytic and photolytic degradation product [3,4], respectively.

Combination of OLM and HCZ is an effective and highly tolerated antihypertensive combined therapy. This combination was reported to reduce systolic blood pressure (SBP) and diastolic blood pressure (DBP) to higher extent than other compound alone. This combined antihypertensive therapy was observed to compare favorably with other antihypertensive combined therapies [5].

After reviewing the literature extensively, different methods have been published for the determination of OLM and HCZ in their mixture. The binary mixture was analyzed by differrent spectrophotometric [6-12], spectrofluorimetric [13], electrophoretic [14], HPTLC [15-18], HPLC [15,19-31], and UPLC [32,33] methods. The studied mixture was also determined in plasma by LC-MS [34-36]. On the other hand, the drugs were determined in presence of their impurities and related substance by different HPLC [37-39], and UPLC [40] methods.

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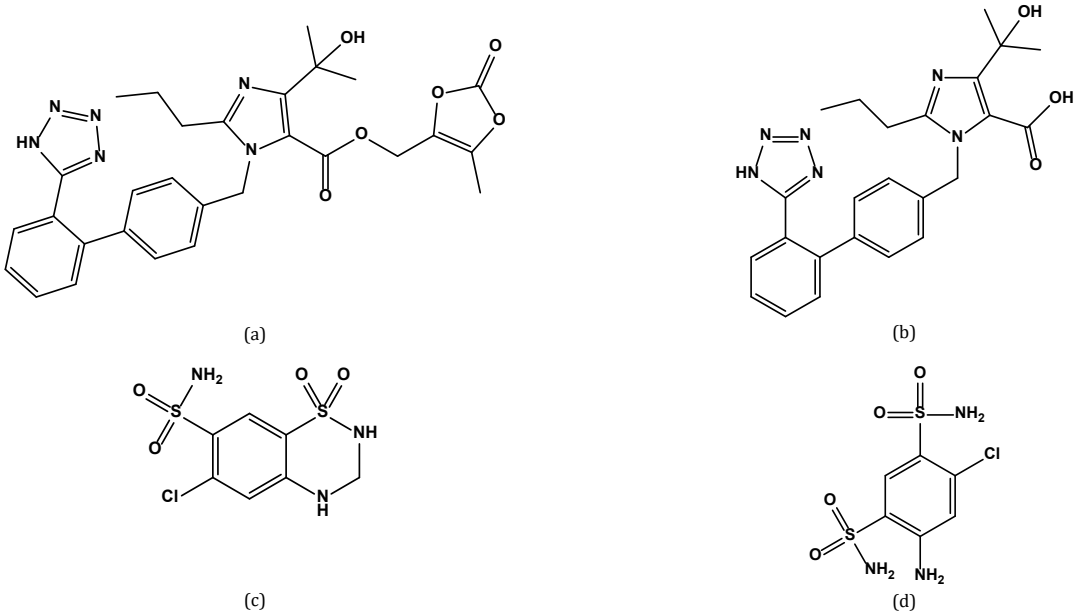

Figure 1. Chemical structure of olmesartan medoxomil (a), olmesartan (b), hydrochlorothiazide (c) and salamide (d).

In the same way, OLM and HCZ combination was analyzed in presence of only the degradation products of olmesartan medoxomil by HPTLC [41] and HPLC [38,42,43] methods.

The drug products' manufacturers should examine the degradation process of the drug products before their comercial release to ensure the integrity of the manufacturing process. From the previous collected literature, it becomes clear that there isn't any published method used for simultaneous determination of OLM and HCZ in presence of the studied degradation products, OL and SAL, respectively. So, this work aims to develop for the first time rapid, sensitive, efficient and validated chemometric and TLC-densitometric methods for simultaneous determination of OLM, HCZ, OL and SAL. The developed methods have advantages of high selectivity, being time and cost effective methods.

2. Experimental

2.1. Instrumentation

2.1.1. For chemometric models

A double beam UV-Visible spectrophotometer (Shimadzu, Japan), model UV-1601 PC with one cm path length, quartz cell was used and connected to IBM compatible computer. UVPC personal spectroscopy software version 3.7, of Matlab version 2007b was used for the proposed models of multivariate calibration, PCR and PLS.

2.1.2. For TLC-Densitometric method

Aluminum foil plates specially designed for High Performance Thin layer chromatography that pre-coated with 0.25 mm silica gel $60F_{254}$ (Merck, Germany) with diameters of 13×20 cm which were cut from 20×20 cm initial plates were used. CAMAG TLC Scanner 3 S/N 130319 operated with WINCATS software was used. The used scanning mode was absorbance mode, and scanning speed was 20 mm/s. TLC Linomat IV sample applicator that its syringe is of 100-µL (CAMAG, Muttenz, Switzerland) was used with spraying rate of 10 µL/s. Radiation source was deuterium lamp, band width was 6 mm, and slit dimensions were 3×0.45 mm. The outputs appeared as chromatogram and integrated peak area. Sonix TV ss-series ultrasonicator (USA) was used for complete dissolution while preparing stock solutions.

2.2. Samples

2.2.1. Pure samples

Olmesartan medoxomil and hydrochlorothiazide were gently provided by BIG Pharma (Sabaa Co., Cairo, Egypt). Their purity was found to be 100.03 and 100.15%, respectively, according to manufacturer certificates of analysis. Salamide with claimed purity of 98% was purchased from Cornal Lab Co., Cairo, Egypt (Batch No. 163715), while olmesartan medoxomil degradation product was laboratory prepared from hydrolysis of olmesartan medoxomil.

Forced degradation study of olmesartan medoxomil: Different degradation conditions including hydrolysis, oxidation and photo degradation were studied.

Alkaline hydrolysis of olmesartan medoxomil: It was carried out by weighing about 0.5 g of OLM in 100 mL conical flask and then dissolving in 10 mL methanol then add 15 mL 0.1 N NaOH. The prepared sample was kept at room temperature for 30 minutes.

Acidic hydrolysis of olmesartan medoxomil: It was studied by weighing two portions of OLM, each equivalent to 0.5 g into two separated 100 mL conical flasks. Each weighted powder was then dissolved in 10 mL methanol and then add 15 mL 0.1 N HCl. One of the prepared samples was kept at room temperature for two hours while the other was refluxed at 80 °C for two hours.

Oxidative degradation of olmesartan medoxomil: It was tested in 100 mL conical flask by dissolving 0.5 g of OLM in 10 mL of methanol then add 15 mL 30 % H_2O_2 then keeping the solution at room temperature for two hours.

Photo degradation of olmesartan medoxomil: It was tested by dissolving 0.5 g of OLM in 25 mL methanol and exposing to day light for about 24 hours. All degradation pathways were followed via TLC using ethyl acetate: chloroform: methanol: formic acid: tri-ethylamine (6:4:0.4:0.4:0.05; by volume) as developing system.

Separation of the forced degradation products: The brown solution obtained from hydrolytic degradation (either acidic or alkaline hydrolysis) was neutralized by using either 0.1 N

NaOH or 0.1 N HCl where a brown precipitate was formed. The resulted precipitate was then washed with water 3 times each with 10 mL and then was filtered through a filter paper. The obtained degradation product was dried at 60 °C till dryness. The separated powder was identified by IR and MS analyses. On the other hand for oxidative and photo-degradation pathways, no degradation products were observed when followed on TLC.

2.2.2. Marketed samples

Medosartan (40/12.5) tablets (Batch No. 183615) manufactured by BIG Pharma (Sabaa Co., Cairo, Egypt) labeled to contain 40 mg of OLM and 12.5 mg of HCZ per tablet. Angiosartan plus (40/25) tablets (Batch No. 147032) manufactured by Chemipharm, Cairo, Egypt labeled to contain 40 mg of OLM and 25 mg of HCZ per tablet. Erastapex plus (20/12.5) tablets (Batch No. 168217) manufactured by Multi-Apex for pharmaceutical Industries, S.A.E, Badr City, Egypt labeled to contain 20 mg of OLM and 12.5 mg of HCZ per tablet.

2.3. Chemicals and solvents

All the used solvents were of HPLC grade while the other used chemicals were of analytical grade. Methanol and ethanol were purchased from Sigma-Aldrich Company (Germany) through the Egyptian International Center for import and export (EIC, Egypt). Purified water for injection manufactured by FIPCO Company, Borg Alarab, Alexandria, Egypt. Ethyl acetate, formic acid, tri-ethylamine solution, chloroform, sodium hydroxide, 30% hydrogen peroxide solution and hydrochloric acid were obtained from El-Nasr Pharmaceutical Chemicals Co., Abu-Zabaal, Al Qalyubiyah 28, Talaat Harb St, Cairo, Egypt.

2.4. Solutions

Stock solutions of OLM, HCZ, OL, and SAL were prepared in methanol in the concentration of 1 mg/mL. The working solutions of OLM, HCZ (0.1 mg/mL) and OL, SAL (0.05 mg/mL) for chemometric methods were prepared by appropriate dilutions of their respective stock solutions using ethanol as a solvent. The working solutions of OLM, HCZ (0.2 mg/mL), OL (0.1 mg/mL) and SAL (0.05 mg/mL) for TLC-densitometric method were prepared by appropriate dilutions of their respective stock solutions using methanol as a solvent.

2.5. Laboratory prepared mixtures

Different mixtures with variable ratios of OLM, HCZ, OL, and SAL (including the marketed pharmaceutical formulation ratio) were prepared using their corresponding working solutions and using ethanol as a solvent (for chemometric method), while using methanol as a solvent (for TLC-densitometric method).

3. Procedure

3.1. Multivariate calibration methods

Construction of the calibration and validation sets was performed by Multi-levels multi-factors design. The five-levels, four-factors calibration design was applied to prepare 25 laboratory prepared mixtures consisting of variable ratios of the four studied components, the used concentrations are shown in Table 1. The absorption spectra were recorded in the range of 200-400 nm and the chosen wavelength range to construct models was 210-343 nm, the spectral data was collected with 1 nm interval and then data processing was performed using Matlab® 2007b [44]. For the construction of the calibration model, fifteen mixtures were used, while the selected ten mixtures were repeated to be used as an external validation set.

3.2. TLC-densitometric method

Different concentrations in the ranges of 40-300, 20-200, 10-100 and 10-100 µg/mL of OLM, HCZ, OL, and SAL, respectively, were prepared in four series of 10 mL volumetric flasks using their respective stock solutions and methanol for dilution. 10 µL of each sample was applied in triplicates on TLC plates. The chromatographic development was carried out on a glass reservoir saturated for half an hour with developing system mixture of ethyl acetate: chloroform: methanol: formic acid: tri-ethylamine solution (60:40:4:4:1, by volume). The developed plates were air dried and then scanned using UV scanner at 254 nm. Then for each component, the integrated peak area was determined and a calibration curve was constructed by plotting the mean integrated peak area against the corresponding concentration and finally the regression equation of each component was obtained.

3.3. Application to pharmaceutical formulation

For each pharmaceutical formulation, ten tablets were separately weighed, crushed and blended accurately. An amount of Erastapex plus®, Angiosartan plus® or Medosartan® tablet formulation powder equivalent to [20 mg of OLM (contains also 12.5 mg of HCZ)], [40 mg of OLM (contain also 25 mg of HCZ)] or [40 mg of OLM (contain also 12.5 mg of HCZ)], respectively, was transferred carefully and separately into four 25 mL volumetric flasks. Methanol (15 mL) was added and the prepared samples were sonicated for 15 min then cooled, and filtered. The volume was adjusted with methanol. Then, working solutions (0.1 mg/mL) were prepared by suitable dilutions of the previously prepared sample solutions using either ethanol (for multivariate calibration models) or methanol (for TLC-densitometric method). Different final dilutions were prepared in 10mL volumetric flasks and then the previously illustrated procedure for construction of calibration curves for each method was applied on the prepared samples. Concentrations of OLM and HCZ in each pharmaceutical formulation were then calculated using the corresponding regression equation and then the percentage recoveries were calculated.

3.4. Application of standard addition technique

It depends on the addition of variable known concentrations of pure OLM and HCZ separately to the prepared pharmaceutical formulation samples and then the proposed methods were followed as illustrated previously.

4. Results and discussion

Olmesartan medoxomil and HCZ combination is very effective and useful in treatment of hypertension. OL and SAL are considered to be OLM and HCZ degradation products, respectively [2-4]. As drug degradation may appear into the pharmaceutical formulations during different processes of manufacturing, packing, or storage. So, it is very important to supervise their limits which are based on known safety data or pharmaceutical studies [45]. From the collected literature review; till now there aren't any published spectrophotometric or TLC-densitometric methods for determination of OLM, HCZ, OL, and SAL in their quaternary mixture. So, this work aims to develop and validate new, sensitive, precise, and selective methods of analysis for proper determination of the drugs in presence of their degradation products in their laboratory prepared mixtures and for determination of the active drugs in their pharmaceutical formulations.

Table 1. Concentrations of olmesartan medoxomil, hydrochlorothiazide, olmesartan, and salamide by µg/mL used in the calibration and validation sets.

Sample no	OLM (µg/mL)	HCZ (µg/mL)	OL (µg/mL)	SAL (µg/mL)
1	9.0	3.0	3.0	1.5
2 *	9.0	2.0	2.0	0.5
3	3.0	1.0	2.5	2.5
4 *	6.0	5.0	2.0	2.5
5	3.0	5.0	4.0	1.5
6 *	15.0	3.0	4.0	1.0
7	15.0	2.0	3.0	2.5
8	9.0	5.0	2.5	1.0
9 *	6.0	2.0	4.0	2.0
10	15.0	4.0	2.5	2.0
11 *	6.0	4.0	3.5	1.5
12	12.0	3.0	3.5	2.5
13 *	12.0	5.0	3.0	2.0
14	9.0	4.0	4.0	2.5
15	15.0	5.0	3.5	0.5
16 *	12.0	1.0	4.0	0.5
17	15.0	1.0	2.0	1.5
18 *	3.0	3.0	2.0	2.0
19	3.0	4.0	3.0	0.5
20 *	9.0	1.0	3.5	2.0
21	12.0	4.0	2.0	1.0
22	3.0	2.0	3.5	1.0
23 *	12.0	2.0	2.5	1.5
24	6.0	3.0	2.5	0.5
25	6.0	1.0	3.0	1.0

* The selected mixtures used for validation set.

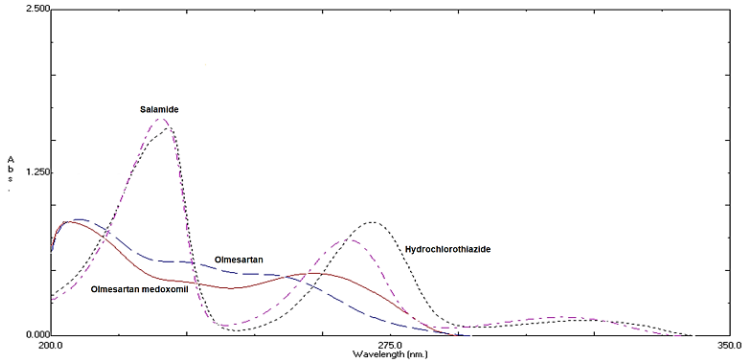

Figure 2. Zero order absorption spectra of 10 µg/mL each of olmesartan medoxomil (—), hydrochlorothiazide (····), olmesartan (—) and salamide (·–·–) using ethanol as a solvent.

4.1. Structure elucidation of olmesartan medoxomil degradation product

Olmesartan medoxomil contain medoxomil ester group, which is liable to either acidic or alkaline hydrolytic degradation (like that happening during its absorption from Gastrointestinal tract) to give active drug (Olmesartan). The obtained acidic and alkaline brown degradation products were elucidated by TLC which showed that both degradation pathways gave the same degradation product (Olmesartan). Then the prepared degradation product powder (OL) was identified by infrared spectroscopy and mass spectrometry, where the IR spectrum of OL, showing appearance of stretching broad peak at 3444 cm^{-1} which represent carboxylic OH group and only one sharp peak of the carbonyl C=O group of the carboxylic acid at 1633 cm^{-1} while the IR spectrum of OLM, showing presence of two sharp peaks of the two carbonyl C=O groups in medoxomil ester moiety at 1832 and 1740 cm^{-1}. This give the evidence of the cleavage of the medoxomil ester moiety and complete degradation of OLM drug and yield OL this happened at the previous stated conditions. Also mass spectrums of OLM and OL, gave confirmation about their identities due to mass molecular ion peaks at m/z were 559.4 for the intact drug (OLM) and 447.4 for its degradation product (OL).

4.2. Method development and optimization

4.2.1. Multivariate calibration methods

Chemometrics is the science of conducting information in chemical problems by data-driven means. Also, it is performed to solve both types' of chemical problems predictive and descriptive types. It has a lot of advantages like, high productivity with minimum cost, improved precision and truthful of results, increased affirmation for carrying out laboratory operations, easier validation of the variable steps of an analytical technique [46]. Wide applications of multivariate calibretion methods were seen for different multi-components mixtures as in [47-52].

The absorption spectra of OLM, HCZ, OL, and SAL showed severe overlap where the application of direct spectrophotometry, derivative, and derivative ratio spectrophotometric methods could not resolve this overlapping (Figure 2). By applying multivariate calibration methods (PCR and PLS), OLM, HCZ, OL, and SAL concentrations could be estimated without any interference.

As it is important to develop and validate highly selective methods, so factors affecting method selectivity were studied and optimized in order to reach for the best results.

Table 2. Determination results of olmesartan medoxomil, hydrochlorothiazide, olmesartan, and salamide in the validation set using multivariate calibration methods.

Mixture no	Recovery %							
	PLS method				PCR method			
	OLM	HCZ	OL	SAL	OLM	HCZ	OL	SAL
1	101.39	98.76	100.96	100.97	100.02	101.12	103.28	100.43
2	99.93	101.61	100.47	99.82	102.03	98.84	102.71	98.36
3	98.70	99.32	99.94	102.56	98.88	99.50	101.98	102.48
4	97.11	99.72	100.72	98.44	100.42	101.70	102.28	98.70
5	99.47	101.77	100.68	101.77	100.68	101.94	102.50	100.45
6	98.79	100.54	98.80	99.21	100.78	100.39	100.48	99.21
7	101.04	99.34	99.85	101.68	100.18	98.75	101.69	101.81
8	101.15	101.54	102.17	99.92	100.96	100.84	102.55	99.00
9	98.08	100.49	100.08	99.98	98.26	101.50	102.87	100.57
10	100.47	97.02	100.40	100.29	100.00	99.38	102.16	100.32
Mean±standard deviation	99.78±0.93	100.01±1.49	100.41±0.87	100.46±1.27	100.22±1.06	100.40±1.20	102.25±0.77	100.13±1.34
RMSEP *	0.0956	0.0436	0.0228	0.0165	0.0895	0.0358	0.0706	0.0198

* Root Mean Square Error of Prediction.

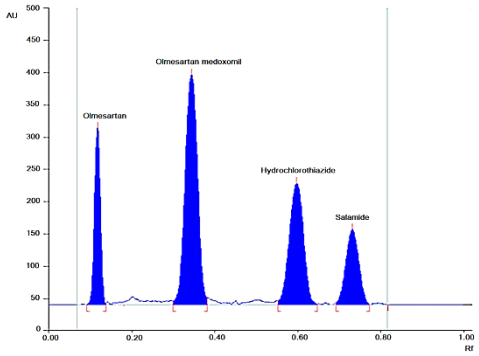

Figure 3. TLC-densitogram of olmesartan, olmesartan medoxomil, hydrochlorothiazide and salamide, using ethyl acetate: chloroform: methanol: formic acid: tri-ethylamine solution (60:40:4:4:1, by volume) as a developing system and 254 nm as a scanning wavelength.

Different solvents were tried (methanol, ethanol, distil water, 0.05 N HCl, and 0.05 N NaOH), concerning selectivity, it was found that ethanol was the most appropriate solvent for the deve-loped methods.

The first step in the analysis of the studied components by using multivariate calibration methods was building the calibration set for the quaternary mixture (OLM, HCZ, OL, and SAL). Calibration set was obtained by using five levels, four factors calibration design to prepare 25 laboratory mixtures containing different ratios from each of OLM, HCZ, OL, and SAL (Table 1). The absorption region was selected in the range of 210-343 nm, and then acquisition of the spectral data was collected with 1 nm interval. Fifteen mixtures were used to build the calibration set. In this method, as a pre- processing step for PLS and PCR models the data was mean centered and auto- scaled, respectively. Five latent variables were selected to be the optimum number of latent variable. The second step is to check the ability of the suggested model to predict the concentrations of the studied components in an external validation set which consisting of another repeated ten mixtures and the root mean squared error of prediction (RMSEP) values were calculated, (Table 2), where the obtained results ensures the great predictive ability of the obtained models.

4.2.2. TLC-densitometric method

TLC method consider as very common method with used several times in solving different mixtures as in references [53-56]. In order to separate the four studied components several trials were performed to choose the most appropriate developing systems. Firstly, different developing systems consisting of ethyl acetate: chloroform (in different ratios), and methanol: chloroform (in different ratios) were tested. Suitable resolution among HCZ and SAL was obtained upon using developing system mixture of ethyl acetate: chloroform (6:4, *v*:*v*), but with highly retained peaks for OLM and OL. Different amounts of tri-ethylamine, formic acid, acetic acid, and ammonia solution were added. It was observed that 0.4 mL formic acid is necessary to elute OLM while OL was eluted after using of 0.1 mL tri-ethylamine. In order to enhance the separation between OLM and OL, methanol was added to the mobile phase where 0.4 mL methanol was sufficient to improve the resolution between OLM and OL without affecting the chromatographic separation between HCZ and SAL. Finally, good separation among the four components was obtained on using a developing system mixture of ethyl acetate: chloroform: methanol: formic acid: tri-ethylamine (6:4:0.4:0.4:0.1, by volume), where the obtained R_F values were 0.12, 0.35, 0.60, and 0.73 for OL, OLM, HCZ, and SAL, respectively, (Figure 3). Scanning at different wave lengths was also studied by testing different detection wavelengths (215, 225 and 254 nm). Chosen UV scanning wavelength of 254 nm resulted in good sensitivity with minimum detector noise for all the studied components. Also, slit dimensions of scanning wavelength and interspace between bands were optimized where slit dimensions were 3×0.45 mm and bands were separated by 5 mm from each other and 10 mm apart from the bottom margin of the plate.

4.3. Application to pharmaceutical formulations

After methods development and optimization, they were used for determination of OLM and HCZ in Erastapex plus®, Angiosartan plus®, and Medosartan® tablet formulations. Then, results were shown as percentage recoveries and it was observed that results were in the acceptable limits (90-110%), (Table 3). Also, the results of standard addition technique proved accuracy of the developed methods (Table 4) and also confirmed that the tablets excipients made no interference with the measurement of the studied components.

Table 3. Determination of olmesartan medoxomil and hydrochlorothiazide in their tablets by different developed methods and application of standard addition technique.

Pharmaceutical formulation	Method		Component	Taken (µg/mL)	Recovery% ± SD [a]	Standard addition technique			
						Added (µg/mL) or (µg/band)	Found (µg/mL) [b] or (µg/band)	Recovery %	Mean±SD
Angiosartan plus tablets labeled to contain 40 mg of OLM and 25 mg of HCZ (Batch No. 147032)	Multivariate calibration models	PLS model	OLM	6.4	95.08±1.39	-	-	-	-
			HCZ	4.0	90.92±1.02	-	-	-	-
		PCR model	OLM	6.4	96.98±1.17	-	-	-	-
			HCZ	4.0	91.71±1.39	-	-	-	-
	TLC-densitometry		OLM	2.4	97.60±1.13	-	-	-	-
			HCZ	1.5	92.97±1.63	-	-	-	-
Erastapex plus tablets labeled to contain 20 mg of OLM and 12.5 mg of HCZ (Batch No. 168217)	Multivariate calibration models	PLS model	OLM	6.4	103.86±0.57	-	-	-	-
			HCZ	4.0	92.66±1.33	-	-	-	-
		PCR model	OLM	6.4	102.40±0.66	-	-	-	-
			HCZ	4.0	93.67±1.27	-	-	-	-
	TLC-densitometry		OLM	2.4	101.14±1.00	-	-	-	-
			HCZ	1.5	92.18±1.84	-	-	-	-
Medosartan tablets labeled to contain 40 mg of OLM and 12.5 mg of HCZ (Batch No. 183615)	Multivariate calibration models	PLS model	OLM	6.4	90.66±0.45	5.0	5.16	103.20	101.58±1.69
						6.0	5.99	99.83	
						7.0	7.12	101.71	
			HCZ	2.0	91.91±1.62	1.0	1.01	101.00	100.56±1.39
						2.0	1.98	99.00	
						3.0	3.05	101.67	
		PCR model	OLM	6.4	91.43±1.39	5.0	4.98	99.60	99.98±0.90
						6.0	5.96	99.33	
						7.0	7.07	101.00	
			HCZ	2.0	93.10±1.87	1.0	1.00	100.00	99.39±0.67
						2.0	1.99	99.50	
						3.0	2.96	98.67	
	TLC-densitometry		OLM	1.6	93.79±1.56	1.0	1.008	100.80	100.21±0.52
						1.2	1.197	99.75	
						1.4	1.401	100.07	
			HCZ	0.5	92.65±1.35	1.0	1.016	101.6	100.22±1.22
						1.2	1.194	99.50	
						1.4	1.394	99.57	

[a] Average of six determinations.
[b] Average of three determinations.

Table 4. Regression and analytical parameters of the obtained methods for determination of olmesartan medoxomil, hydrochlorothiazide, olmesartan, and salamide [a].

Parameters	Multivariate calibration models								TLC-densitometry			
	PLS				PCR							
	OLM	HCZ	OL	SAL	OLM	HCZ	OL	SAL	OLM	HCZ	OL	SAL
Calibration range µg/mL	3.0-15.0	1.0-5.0	2.0-4.0	0.5-2.5	3.0-15.0	1.0-5.0	2.0-4.0	0.5-2.5	0.4-3.0	0.2-2.0	0.1-1.0	0.1-1.0
Slope	1.0069	0.979	1.0063	1.0127	1.0107	1.0009	0.9849	1.0206	0.2673	0.249	0.2669	0.1498
Intercept	-0.0338	0.0482	-0.0286	-0.0211	-0.0973	-0.0122	-0.0191	-0.0271	-0.4839	-0.1999	-0.2462	-0.0651
Correlation coefficient	0.9997	0.9998	0.9997	0.9998	0.9997	0.9997	0.9997	0.9998	0.9999	0.9999	0.9999	0.9999
Accuracy	99.29	99.99	100.02	99.89	99.91	100.02	100.00	99.97	100.08	100.03	99.98	99.74
Repeatability (%RSD) [b]	0.52	0.63	0.46	0.95	0.87	0.83	0.61	1.21	1.70	1.28	1.21	0.92
Intermediate precision (%RSD) [c]	1.16	1.28	0.65	1.12	1.06	1.14	0.71	1.24	1.85	1.53	1.51	1.93
LOD [d]	-	-	-	-	-	-	-	-	0.13	0.09	0.04	0.03
LOQ [e]	-	-	-	-	-	-	-	-	0.39	0.25	0.14	0.09

[a] N.B. from predicted *vs.* known concentration plot, the slope, intercept and correlation coefficient for multivariate calibration methods were obtained.
[b] The intra-day precision (n = 9), average of three different concentrations repeated three times within day.
[c] The inter-day precision (n = 9), average of three different concentrations repeated three times in three successive days.
[d] LOD = (SD of the response/slope)×3.3.
[e] LOQ = (SD of the response/slope)×10.

5. Method validation

According to International Conference on Harmonization (ICH) guidelines [57], method validation was performed.

5.1. Linearity

The developed methods linearity was ensured by analyzing variable concentrations of OLM, HCZ, OL, and SAL in triplicates. It was achieved in the range of 3.0-15.0, 1.0-5.0, 2.0-4.0, and 0.5-2.5 µg/mL for OLM, HCZ, OL, and SAL for multivariate calibration methods, respectively, and in the range of 0.4-3.0, 0.2-2.0, 0.1-1.0, and 0.1-1.0 µg/band for OLM, HCZ, OL, and SAL for TLC-densitometric method, respectively. The regression parameters like the correlation coefficients, slope, and intercept were presented in Table 4, while these linear ranges were used for construction of both calibration and validation sets for multivariate calibration methods.

5.2. Accuracy

The accuracy was checked for the proposed multivariate calibration models (PLS and PCR) by applying the method to predict concentration of validation set (10 laboratory prepared mixtures), where good percentage recoveries were obtained, (Table 3). And also, it was checked for the developed TLC-densitometric method by applying the method for determination of different concentrations of the pure samples from the studied components within their ranges of linearity. By using the corresponding regression equation, the concentrations were calculated then the recoveries percentage were calculated and presented in Table 4. Also, technique of standard addition was made to ensure method accuracy; their results presented in Table 3 access the accuracy of the developed methods.

Table 5. System suitability testing parameters of the developed TLC-densitometric method.

Parameters	TLC-densitometric method				Reference values [58]
	OL	OLM	HCZ	SAL	
Symmetry factor	1.00	1.00	1.03	1.03	~1
Resolution (R_s)	5.14	3.80	2.00	-	>1.5
Selectivity (α)	4.18	1.82	1.25	-	>1
Retension factor (R_f)	0.12	0.35	0.60	0.73	-

Table 6. Experimental results of robustness for determination of olmesartan medoxomil, hydrochlorothiazide, olmesartan and salamide by the developed TLC-densitometric method.

Parameters	OLM	HCZ	OL	SAL
	(% RSD) [a]			
0.4 mL Methanol ±0.05 mL	0.74	0.52	0.25	0.24
0.4 mL Formic acid ±0.05 mL	0.41	0.11	0.12	0.18
0.1 mL Tri-ethylamine ±0.02 mL	0.67	0.10	0.65	0.08
Saturation time ±5 min	0.55	0.48	0.16	0.07
Scanning wavelength ±2 nm	0.09	0.08	0.10	0.12

[a] %RSD relative standard deviation of the change in R_f.

Table 7. Results were obtained from the statistical comparison which was made between the developed methods and the reported method for the determination of olmesartan medoxomil and hydrochlorothiazide in pure powder form.

Component	Multivariate calibration models				TLC-densitometry		Reported method [a]	
	PLS model		PCR model					
	OLM	HCZ	OLM	HCZ	OLM	HCZ	OLM	HCZ
Mean	100.00	100.19	99.99	99.90	100.08	100.03	99.98	99.85
SD	0.87	1.59	0.98	1.25	0.97	1.16	0.70	1.15
Variance	0.76	2.53	0.96	1.56	0.94	1.35	0.49	1.32
N	7	7	7	7	7	7	7	7
t-Test (2.447) [b]	0.776	0.692	0.381	0.949	0.822	0.770	-	-
f-Test (4.284) [b]	1.295	1.902	1.795	1.375	1.209	1.974	-	-

[a] RP-HPLC method to estimate OLM and HCZ using acetonitrile: phosphate buffer (50:50, *v:v*, pH = 4.7 adjusted with diluted phosphoric acid) as mobile phase, and 250×4.6 mm, 5 µm particle size, C8 Qualisil BDS column was used as stationary phase. Also adjusting the flow rate to be 1 mL/min and a detection wavelength was 225 nm [19].

[b] The numbers between parentheses represent the corresponding tabulated values of t and F at probability (0.05).

5.3. Precision

It was studied by testing repeatability and intermediate precision. Repeatability was preformed through analysis of different three concentrations of the pure samples from studied components in triplicates at the same day. The chosen concentrations for multivariate calibration models were 3.0, 9.0, and 15.0 µg/mL for OLM, 1.0, 3.0, and 5.0 µg/mL for HCZ, 2.0, 3.5, and 4.0 µg/mL for OL, and 0.5, 1.5, and 2.0 µg/mL for SAL, while these for TLC-densitometric method were 0.4, 1.6, and 3.0 µg/band for OLM, 0.5, 1.5, and 2.0 µg/band for HCZ, 0.1, 0.6, and 0.9 µg/band for OL, and 0.2, 0.4, and 0.9 µg/band for SAL. For determination of the intermediate precision, the experiment was repeated on three consecutive days using the same mentioned concentrations. The obtained relative standard deviation values (RSD %) were within the acceptable values and given in Table 4.

5.4. Limits of detection and limits of quantitation (LOD and LOQ)

For determination of the limits of detection and quantification, OLM, HCZ, OL and SAL concentrations present in the lower part of the calibration curves and the following equations were used; LOD = 3.3 × N/B and LOQ = 10 × N/B, where N was the standard deviation of the response and B is the slope of the obtained calibration curve. The resulted values of LOD and LOQ are shown in Table 4 which proved that TLC-densitometric method have high sensitivity.

5.5. Specificity

Specificity of both multivariate calibration models and TLC-densitometric method were assessed by their application to validation set or to different laboratory prepared mixtures containing different concentrations of OLM, HCZ, OL, and SAL. Good results were obtained as given in Table 2 and also, good resolution was obtained as shown in Figure 3. Moreover, specificity was confirmed by application of these methods to pharmaceutical formulations containing OLM and HCZ and result obtained ensured that there was no any interference from additives (Table 3).

5.6. System suitability testing parameters

This parameter was made for TLC-densitometric method which used to test the performance of the system before or during the analysis of the studied components. It was evaluated by calculating some parameters like resolution, selectivity and symmetry factors. Good results were obtained as given in Table 5 [58].

5.7. Robustness

This parameter was determined for TLC-densitometric method which used to ensure that the method was unaffected by small deliberate variations in parameters of the method. The studied parameters were: methanol volume (±0.05 mL), formic acid volume (±0.05 mL), tri-ethylamine volume (±0.02 mL), and also saturation time (±5 min). Then the effects of these changes on R_f values were studied and represented as %RSD value. The results given in Table 6 ensured the robustness of the developed method.

6. Statistical analysis

Statistical comparison was made between the results obtained by analysis of pure samples of the studied components by the developed methods and those obtained by reported HPLC method for OLM and HCZ [19]. By using student's-t test and F-ratio test, there was no significant difference between them was attained (Table 7).

7. Conclusion

The developed methods are the first developed ones for analysis of OLM, HCZ, and their degradation products OL and SAL, respectively. Multivariate calibration methods have advantages over other spectrophotometric methods of high selectivity due to the implication of multiple spectral inten-

sities which enhance the resolution power of the method. On the other hand, TLC-densitometric method has advantages of high sensitivity and low analysis time since several samples could be analyzed on the same time. Additionally, the validation of the methods was carried out and the obtained values confirmed there validity. The developed methods can be used in quality control laboratories for monitoring the stability of the chosen drugs. They can be considered as alternative tools for the high cost HPLC method.

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