DEVELOPMENT AND VALIDATION OF UPLC METHODS FOR SIMULTANEOUS DETERMINATION OF SELECTED ANTIHYPERTENSIVE DRUGS IN THEIR DOSAGE FORMS

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Abstract— Accurate, precise and sensitive UPLC methods were developed and validated for the simultaneous determination of selected antihypertensive drugs; a binary mixture containing Lercanidipine HCl (LER) and Enalapril maleate (ENA), and a ternary mixture containing amlodipine besylate (AML), olmesartan medoxomil (OLM) and hydrochlorothiazide (HCT). Chromatographic separation was achieved using Kinetex™ column – C18 (4.6 x 150 mm, 2.6 µm). Isocratic elution at a flow rate 1 mL/min was performed using a mobile phase of methanol: phosphate buffer pH = 3.0 ± 0.1 (65: 35 v/v), for the binary mixture and acetonitrile: phosphate buffer pH = 3.0 ± 0.1 (45: 55 v/v), for the ternary mixture. The methods developed were satisfactorily applied to the analysis of the pharmaceutical formulations and proved to be specific and accurate for the quality control of the cited drugs in pharmaceutical dosage forms. The obtained results are statistically compared with those obtained by the official or reported methods, showing no significant difference with respect to accuracy and precision at p=0.05.

Index Terms—antihypertensive, binary mixture, ternary mixture, UPLC.

I. INTRODUCTION

Lercanidipine HCl (LER), (Fig. 1a) is a calcium channel blocker, chemically designated as 2-((3,3-diphenyl-propyl) methylamine)-1,1-dimethylethyl, methyl-1,4-dihydro-2, 6-dimethyl-4- (3-nitrophenyl)-3, 5- pyridine dicarboxylate monohydrochloride [1]. LER is an unofficial drug.

Enanlpril maleate (ENA), (Fig. 1b) is a prodrug of the diacid enalaprilat, angiotensin converting enzyme (ACE) inhibitor, chemically designated as (N-{N-[(S)-1-ethoxycrbonyl-3-phenylpropyl]L-alanyl}-proline hydrogen maleate) [2]. ENA is an official drug described in British Pharmacopeia (BP) [3] by potentiometric titration method while in the United State Pharmacopeia (USP) [4] by reversed phase high performance liquid chromatographic methods.

Amlodipine besylate (AML), (Fig. 1c) is a long acting calcium channel blocker, chemically designated as (RS)-3-ethyl 5-methyl 2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate] [5]. AML determination is described in BP [3] and USP [4] by reversed phase high performance liquid chromatographic methods

Olmesartan medoxomil (OLM), (Fig. 1d) is an ester prodrug that is hydrolysed during absorption from gastrointestinal tract to the active form olmesartan, an angiotensin II receptor antagonist. It is chemically designated as 5-methyl-2-oxo-2H-1,3-dioxol-4-yl)methyl 4-(2-hydroxypropan-2-yl)-2-propyl-1-({4-[2-(2H-1,2,3,4-tetrazol-5-yl) phenyl]phenyl}methyl)-1H-imidazole-5-carboxylate [5]. OLM is an unofficial drug.

Hydrochlorothiazide (HCT), (Fig. 1e) is a a thiazide diuretic, chemically designated as 6-chloro-1,1-dioxo-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide [5]. HCT, pure powder is described in British Pharmacopeia [3] by spectrophotometric method. While HCT, dosage form is described in United State Pharmacopeia [6] by high performance liquid chromatographic method.

Zanipress® (LER and ENA) and *Tribenzor*® (AML, OLM and HCT) are recently introduced in the market as a new combined tablet dosage forms, taken once daily for treatment of hypertension.

Literature survey reveals that few methods are available for the simultaneous determination of LER and ENA In their combination. These methods include HPLC [7-10] and spectrophotometric methods [9, 11]

Similarly, few methods were reported for the simultaneous determination of OL, AM and HCT in their ternary mixture. These methods include HPLC [12, 13], one UPLC method [14], chemometric [15, 16] and spectrophotometric methods [16-18].

The aim of this work is to develop sensitive, accurate, precise, reliable, fast and inexpensive analytical methods for the determination of either the binary or ternary mixtures. The proposed method was designed to be suitable for the quality assessment of these mixtures in pharmaceutical preparations.

II. EXPERIMENTAL

A. Apparatus

Ultra HPLC Agilent 1290 Infinity Series Liquid chromatograph, Waldbornn, Germany. It consists of:

- Binary pump-model G 4200 B
- Variable Wavelength Detector (VWD) model - G 1314 E

- Autosampler- model G 4226 A
- Column: Kinetex™ column – (4.6 x 150 mm), particle size (2.6 µm), USA.
- Column heater-model G 1316 A
- SonixTV s-series ultrasonicator (USA)

B. Pure samples

AML was kindly supplied by Al-Hekma Pharmaceutical Company, Cairo, Egypt, its purity was found to be 100.41 ± 0.680 according to the official method [3]. OLM was kindly supplied by Apex Pharmaceutical Company Cairo, Egypt, its purity was found to be 100.07 ± 0.815 according to the reported method [19]. HCT and ENA were kindly supplied by National Organization for Drug Control and Research (NODCAR), Cairo, Egypt. HCT purity was found to be 99.96 ± 0.601 according to the official method [3], while ENA purity was found to be 100.55 ± 0.710 according to the official method [4]. LER was kindly supplied by Pharma Care Company, Cairo, Egypt. Its purity was found to be 99.98 ± 0.933 according to the reported method [20].

C. Pharmaceutical formulations

Tribenzor® tablets (batch number 249720), manufactured by Daiichi Sankyo, Inc. from Vogue Pharmaceuticals Inc., Calgary, Canada. Each tablet was labeled to contain 13.9 mg AML (equivalent to 10 mg amlodipine base), 40 mg OLM and 25 mg HCT.

Zanipress® tablets (batch number CC0CC43), manufactured by Recordati, Inc. from local pharmacy (Union) in Beirut, Lebanon. Each tablet was labeled to contain 10 mg of each LER and ENA.

D. Reagents

Methanol and acetonitrile HPLC grade (SDS, France).
Orthophosphoric acid and triethyl amine (EL-NASR Pharmaceutical Chemicals Co., Abu-Zaabal, Cairo, Egypt).
Deionised water (SEDICO Pharmaceutical Co., Cairo, Egypt).
Phosphate buffer (pH 3.0 ± 0.1, prepared by dissolving 3.4 g potassium dihydrogen orthophosphate and 3.55 g disodium hydrogen phosphate in 1000-mL measuring flask and completing the volume with double distilled water, then the pH was adjusted using orthophosphoric acid or triethyl amine).

E. Standard stock and working solutions

LER, ENA, AML, OLM and HCT stock solutions (1 mg/mL of each in methanol).
LER, ENA, AML, OLM and HCT working solutions (100 µg/mL of each in mobile phase).

F. Chromatographic Conditions

Chromatographic separation was achieved on an Kinetex™ column – C18 (4.6 x 150 mm), particle size (2.6 µm), USA. Isocratic elution was performed using a mobile phase of methanol: phosphate buffer pH = 3.0 ± 0.1 (65: 35 v/v), with UV detection at 209 nm for the binary mixture and acetonitrile: phosphate buffer pH = 3.0 ± 0.1 (45: 55 v/v), with UV detection at 230 nm for the ternary mixture. The buffer solution was filtered through 0.45 µm membrane filter and degassed for 30 min in an ultrasonic bath prior to use. The mobile phase was pumped through the column at a flow rate of 1 mL/min and pressure of 219 Bar. Analyses were performed at column temperature = 50 ^{o}C.

G. Procedures

Linearity

Aliquots of the cited drugs were accurately transferred from their respective working standard solutions (0.1 mg/mL) into separate sets of 10-mL volumetric flasks and the volume was completed then to the mark with the mobile phase to prepare 1.5-45 µg/mL LER and 1-40 µg/mL ENA for the binary mixture and 2.0-27 µg/mL AML, 10-60 µg/mL OLM and 1.5-30 µg/mL HCT, for the ternary mixture. A volume of 5.0 µL volume of each solution was injected in triplicates into the liquid chromatograph under the previously mentioned chromatographic conditions.

Analysis of laboratory-prepared mixtures

Into a set of 10-mL volumetric flasks, accurate aliquots of the cited drugs were separately transferred from their working standard solutions (0.1 mg/ml), then the volume was completed to the mark with mobile phase of methanol: phosphate buffer pH = 3.0 ± 0.1 (65: 35 v/v), for LER and ENA mixture and acetonitrile: phosphate buffer pH = 3.0 ± 0.1 (45: 55 v/v), for AML, OLM and HCT, to prepare mixtures containing the drugs in different ratios. The procedure was then completed as described under linearity. The concentrations of the cited drugs were calculated by substituting in the corresponding regression equation.

Application of the proposed methods for the determination of the drugs in their pharmaceutical dosage forms

Ten tablets of each formulation were accurately weighted and finely powdered. An amount of the powder equivalent to 13.9 mg AM, 40 mg OL and 25 mg HCT of Tribenzor® tablets and 10 mg LER and ENA of Zanipress® tablets was weighted, dissolved in mobile phase of methanol: phosphate buffer pH = 3.0 ± 0.1 (65: 35 v/v), for the binary mixture and acetonitrile: phosphate buffer pH = 3.0 ± 0.1 (45: 55 v/v), for the ternary mixture, by shaking in ultrasonic

bath for 30 minutes. The solution was accurately transferred to a 100-mL measuring flask, the volume was completed with the mobile phase and finally was filtered. An appropriate dilution was made using the same mobile phase to obtain solutions of final concentrations 13.9 µg/mL, 40 µg/mL and 25 µg/mL of AML, OLM and HCT, respectively and 10 µg/mL for both LER and ENA. Then the procedure was completed as described under linearity. The concentrations of the cited drugs were obtained from the computed regression equations and the validity of the methods was further assessed by applying the standard addition technique.

III. RESULTS AND DISCUSSION

In order to optimize the proposed UPLC methods described for the simultaneous determination of the examined drugs, all the experimental conditions were investigated. Several trials were carried out to obtain good and optimum separation of LER and ENA in their binary mixture and AML, OLM and HCT in their ternary mixture. Different composition of mobile phases with different ratios were tried such as acetonitrile: methanol: phosphate buffer pH = 3.0 ± 0.1 (30: 40: 30, by volume), methanol: phosphate buffer pH = 3.0 ± 0.1 (50: 50, by volume), the best resolution was obtained upon using methanol: phosphate buffer pH = 3.0 ± 0.1 (65: 35 v/v), for the binary mixture and acetonitrile: phosphate buffer pH = 3.0 ± 0.1 (45: 55 v/v), for the ternary mixture with a flow rate of 1 mL/min (Figs. 2 and 3).

Linearity was studied for the examined drugs. A linear relationship between relative peak area and corresponding concentration was obtained. The regression equations were also computed. The linearity of the calibration curves were validated by the high value of correlation coefficients.

ICH guidelines for method validation [21] were followed for validation of the proposed UPLC method by measuring concentration range, accuracy, precision, repeatability, intermediate precision and linearity ranges. The results obtained are shown in (Table 1).

The specificity of the developed method was assessed by the analysis of laboratory prepared mixtures containing different ratios of the drugs. Satisfactory results were obtained over the calibration range.

The system suitability testing is based on the concept that, equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as a whole. System suitability is used to ensure the maximum performance of the system before and during the analysis of drugs. System suitability was checked by calculating different parameters such as capacity factor, tailing factor, column efficiency (N), selectivity and resolution factors, where the system was found to be suitable relative to the reference values [22] as shown in (Table 2).

The proposed UPLC method has been successfully applied for the determination of AML, OLM and HCT in Tribenzor® tablets and the determination of LER and ENA in Zanipress® tablets. The validity of the method was further assessed by applying the standard addition technique. The established method proves to be simple, linear, precise, accurate and specific. The total runtime was 2.5 minutes for the binary mixture and 6 minutes for the ternary mixture, within which the drugs were separated.

IV. METHOD VALIDATION

Validation was done according to ICH recommendations [21]. Linearity, accuracy, selectivity, range and precision (repeatability and intermediate precision) were determined. Satisfactory results were obtained within global validation reference values (Table 1).

A. Accuracy

The suggested method has been applied for measuring different samples of the examined drugs within their linearity range and the concentrations were calculated each from their corresponding regression equation. The percentage recoveries for each drug were calculated.

B. Precision

Repeatability: Three concentrations of AML, OLM and HCT (10, 18 and 25 µg/mL each), and three concentrations of LER and ENA (2.5, 12.5 and 30 µg/mL each) were analyzed three times in triplicates in the same day, using the previously mentioned procedure under linearity. RSD values were then calculated for each sample.

Intermediate precision: The above mentioned concentrations of the cited drugs were analyzed on three successive days using the procedure stated under linearity. RSD values were then calculated for each sample.

V. STATISTICAL ANALYSIS

Results obtained from the proposed methods were compared to those obtained by applying the official methods for ENA, AML and HCT and reported methods for LER and OLM, showing no significant difference as observed from the calculated t- and F values (Table 3).

CONCLUSION

From the previous discussion, it could be concluded that the proposed methods are sensitive, selective, accurate, precise and time saving. Thus, the

developed method can be conveniently used by quality control laboratories.

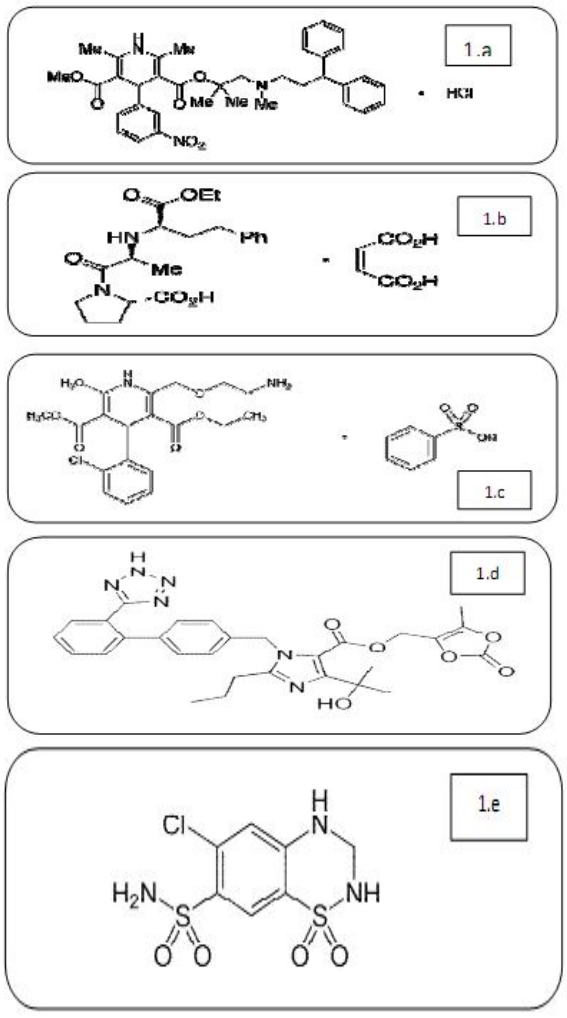


Fig. 1 Chemical structures of (a) LER (b) ENA (c) AML (d) OLM and (e) HCT

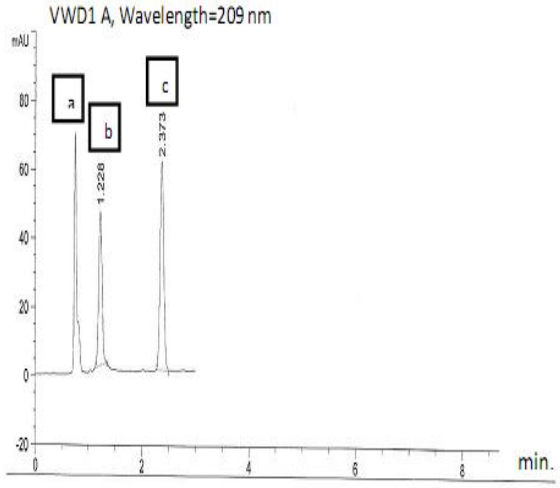


Fig. 2 UPLC chromatogram of a resolved mixture of (a) Maleic acid, (b) Enalapril (t_R = 1.228) and (c) Lercanidipine HCl (t_R = 2.373), using mobile phase of methanol: phosphate buffer pH= 3.0 ± 0.1 (65:35, v/v)

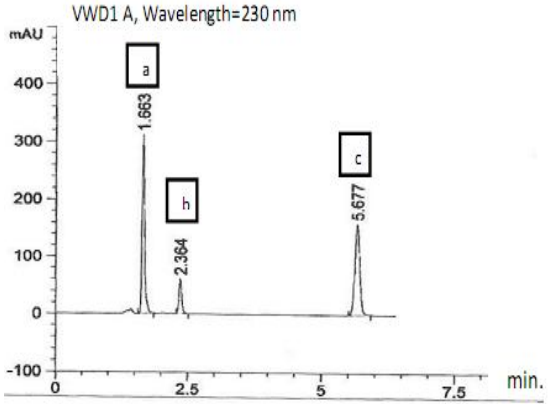


Fig. 3 UPLC chromatogram of a resolved mixture of (a) HCT (t_R= 1.663), (b) AML (t_R= 2.364) and (c) OLM (t_R= 5.677) and using mobile phase of acetonitrile: phosphate buffer pH=3.0 ± 0.1 (45: 55, v/v)

Table 1. Regression and validation parameters for the determination of pure samples by applying the proposed UPLC method

	Zanipress®		Tribenzor®		
Parameters	**LER**	**ENA**	**AML**	**OLM**	**HCT**
Range (µg/mL)	1.5-45	1-40	2-27	10-60	1.5-30
Slope [a]	0.0675	0.0618	0.0725	0.0247	0.0681
Intercept [a]	- 0.0145	0.0710	0.0126	0.1079	- 0.0173
SE of the slope	0.0003	0.0001	0.0004	0.0002	0.0002
SE of the intercept	0.0091	0.0039	0.0069	0.0021	0.0031
Correlation coefficient (r)	0.9998	0.9999	0.9999	0.9998	0.9999
LOD [a] (µg/mL)	0.488	0.320	0.469	0.308	0.213
LOQ [a] (µg/mL)	1.483	0.970	1.421	0.934	0.646
Accuracy					
Mean ± SD	99.49 ± 1.101	99.72 ± 0.718	99.93 ± 1.050	100.08 ± 0.421	100.29 ± 0.945
RSD	1.106	0.720	1.050	0.420	0.942
Precision (RSD)					
Repeatability [b]	0.961	0.959	1.148	0.510	0.752
Intermediate precision [c]	1.164	1.031	1.027	0.984	1.013

[a] Average of three determinations. [b] The intraday (n = 3) standard deviation of concentrations of 10, 18 and 25 µg/mL of AML, OLM and HCT, respectively and 2.5, 12.5 and 30 µg/mL for LER and ENA, respectively, repeated three times within the day. [c] The interday (n = 3) standard deviation of concentrations of 10, 18 and 25 µg/mL of AML, OLM and HCT, respectively and 2.5, 12.5 and 30 µg/mL for LER and ENA, respectively, repeated three times in three successive days.

Table 2. System suitability parameters of the proposed UPLC methods

	Zanipress®		Tribenzor®			Reference values
Parameters	**LER**	**ENA**	**HCT**	**AM**	**OL**	
Resolution (R_s)	75.57	----	87.6	----	92.02	Rs > 1.5
Tailing factor (T)	0.99	0.94	0.81	0.86	1.09	T =1, for a typical symmetrical peak
Capacity factor (K')	6.593	2.929	0.187	0.688	3.05	1 < K' < 10
Selectivity factor (α)	2.259	----	3.67	----	4.43	α > 1
Column efficiency (N)	3925.4	1675.5	2099	5370	12685	Increase with the efficiency of the separation
Height equivalent to theoretical Plate (HETP)	0.038	0.089	0.071	0.027	0.011	The smaller the value the higher the column efficiency

Table 3. Statistical comparison of the results obtained by the proposed method and the official or reported methods for the determination of the examined drugs in their pure form.

Item	Zanipress®				Tribenzor®					
	Proposed UPLC method		Reported method [20]	Official method [4]	Proposed UPLC method			Official method [3]	Reported method [19]	Official method [3]
	LER	ENA	LER	ENA	AML	OLM	HCT	AML	OLM	HCT
Mean	99.49	99.72	99.98	100.55	99.93	100.08	100.29	100.41	100.07	99.96
SD	1.101	0.718	0.933	0.710	1.050	0.421	0.945	0.680	0.815	0.601
Variance	1.212	0.516	0.870	0.504	1.102	0.177	0.893	0.462	0.664	0.361
n	8	8	10	5	7	6	7	5	11	9
Student's t test*	0.839 (3.229)	1.549 (2.201)			0.818 (2.228)	0.026 (2.131)	0.853 (2.145)			
F value*	1.393 (2.120)	1.022 (4.818)			2.384 (6.163)	3.747 (3.999)	2.474 (3.580)			

*These values represent the corresponding tabulated values of t and F at P=0.05

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