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RAPID AND SENSITIVE TLC AND HPLC WITH ON-LINE WAVELENGTH SWITCHING METHODS FOR SIMULTANEOUS QUANTITATION OF AMLODIPINE, VALSARTAN AND HYDROCHLOROTHIAZIDE IN PHARMACEUTICAL DOSAGE FORMS

HANY W. DARWISH, SAID A. HASSAN*, MAISSA Y. SALEM AND BADR A. EL-ZEANY

Department of Analytical Chemistry, Faculty of Pharmacy, Cairo University, Kasr El-Aini Street, 11562, Cairo-Egypt.

ABSTRACT

Two RP-HPLC and TLC methods were developed and validated according to the ICH guidelines for the simultaneous determination of Amlodipine, Valsartan and Hydrochlorothiazide in tablet dosage form. The two methods are simple, rapid and selective. Complete HPLC separation was achieved using Nucleosil C_{18} column and acetonitrile/methanol/isopropyl alcohol (55:41:4 by volume) mixture as the mobile phase, the pH was adjusted to 8 ± 0.1 with triethylamine and the flow rate was 1.2 mL/min. The detection wavelengths were chosen to be 238, 248 and 271 nm for Amlodipine, Valsartan and Hydrochlorothiazide, respectively. The linearity of the proposed method was established over the ranges, 2.0–28.0, 10.0–120.0 and 0.6–32.0 µg/mL for Amlodipine, Valsartan and Hydrochlorothiazide, respectively. For the densitometric TLC method, silica gel 60 F_{254} plates were used and ethyl acetate/toluene/methanol/ammonia (50.5:23.5:23.5:2.5 by volume) mixture as the developing solvent. Detection and quantification were performed densitometrically at 252 nm. The linearity of the proposed method was established over the ranges, 0.5-9.0, 4.0-18.0 and 3.0-11.0 µg/band for Amlodipine, Valsartan and Hydrochlorothiazide, respectively.

KEYWORDS: **HPLC; Densitometry; TLC; Amlodipine; Valsartan; Hydrochlorothiazide.**

SAID A. HASSAN
Department of Analytical Chemistry, Faculty of Pharmacy, Cairo University, Kasr El-Aini Street, 11562, Cairo,Egypt.
e-mail: Saidmonem_84@yahoo.com
Tel.: +201000994542

Corresponding author

INTRODUCTION

Amlodipine (AML), 2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridine carboxylic acid 3-ethyl 5-methyl ester) [1] (Fig. 1a) is a dihydropyridine derivative with calcium channel blocker activity. It is used in the management of hypertension, chronic stable angina pectoris and prinzmetal's variant angina [2]. Valsartan (VAL) is chemically described as N-[p-(o-1H-Tetrazol-5-ylphenyl)benzyl]-N-valeryl-L-valine [1] (Fig. 1b), is a potent and specific competitive antagonist of the angiotensin-II AT_1-receptor. VAL exerts its pharmacological effects by blocking the vasoconstricting and aldosterone-secreting effects of angiotensin-II by selectively preventing binding of angiotensin-II to the AT_1 receptor in many tissues. The drug is used for treatment of hypertension, heart failure, and post-myocardial infarction [3]. Hydrochlorothiazide (HCT), 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulphonamide-1,1-dioxide [1] (Fig. 1c), is a benzothiadiazines diuretic widely used in antihypertensive pharmaceutical formulations, alone or in combination with other drugs, which inhibits NaCl transport in distal convoluted tubule and decreases blood pressure [4].

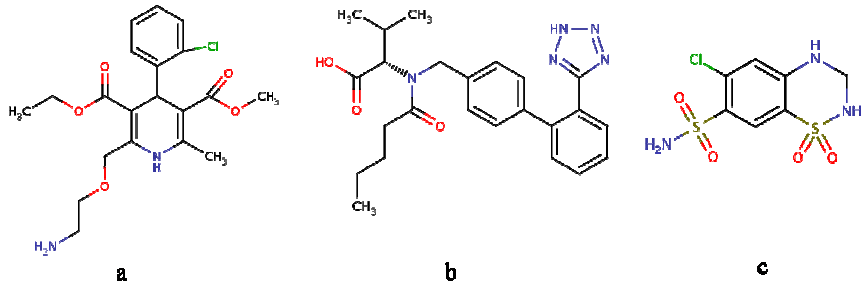

Figure 1.
Structural formulae for a) Amlodipine b) Valsartan and c) Hydroclorothiazide

The triple combination of AML, VAL and HCT is intended for oral administration for the treatment of hypertension and is available in several different strength combinations. Literature survey revealed that Amlodipine besylate and Hydrochlorothiazide are official drugs in British Pharmacopoeia [5], Valsartan, Hydrochlorothiazide and their mixture are official in US Pharmacopoeia [6]. Several methods were reported for the determination of AML, VAL or HCT in different dosage forms [7-10], or in their binary mixtures [11-13], but only few methods were reported for the simultaneous estimation of AML, VAL and HCT in their ternary mixture [14-17]. This manuscript describes the development and the subsequent validation of an isocratic RP-HPLC with on-line wavelength switching method and a simple densitometric TLC method for the simultaneous quantification of AML, VAL and HCT mixture in bulk powders and in pharmaceutical dosage forms. The linearity of the response, the accuracy, the intermediate precision and the robustness of the described methods has been checked as well.

MATERIALS AND METHODS

1. Chemicals and reagents

– ***Pure samples***

Pure Amlodipine besylate; was kindly supplied by Al-Hekma Pharmaceutical Company, Cairo, Egypt, its purity was certified to be 99.89 ± 0.691. Pure Valsartan; was kindly supplied by Novartis Pharmaceutical Company, Cairo, Egypt, its purity was certified to be 99.69 ± 0.231. Pure Hydrochlorothiazide;was kindly supplied by Al-Hekma Pharmaceutical Company, Cairo, Egypt, its purity was certified to be 99.78 ± 0.364.

– ***Market samples***

Three different concentration combinations of EXFORGE HCT® tablets were purchased

from the US market, labeled to contain AML, VAL and HCT in concentrations of 5/160/12.5 mg respectively (batch number 5002125), 5/160/25 mg (batch number 5002141) and 10/320/25 mg (batch number 5002159). The samples were manufactured by Novartis Pharmaceuticals Corporation, USA.

- *Acetonitrile and methanol* of HPLC grade was supplied by Sigma-Aldrich (Germany).
- *Isopropyl alcohol and triethylamine* of Analytical grade were purchased from Sigma-Aldrich (Germany).
- *Methanol, ethyl acetate, toluene* of Analytical grade were purchased from El-NASR Pharmaceutical Chemicals Co., Cairo, Egypt.

2. Instrumentation

For HPLC

An Agilent 1100 Series liquid chromatograph consisted of a dual pneumatic pumping system (model G1310A), an ultra-violet variable wavelength detector (model G1314A) and a Rheodyne injector (model 7725 I) equipped with 20-µL injector loop, Agilent (USA).

For TLC

20 X 20 cm plates (Sigma-Aldrich, Germany) coated with 0.2 mm silica gel 60 F_{254}. The sample was applied to the plates using Camag Linomat 5 autosampler with Camag micro syringe (100 µl). TLC scanner 3 densitometer model 3 S/N 130319, and Camag TLC scanner model 3 S/N 1302139 with win CATS software for densitometric evaluation (Camag, Muttenz, Switzerland).

3. Chromatographic conditions

HPLC method

The mobile phase was prepared by mixing acetonitrile/methanol/isopropyl alcohol (55:41:4 by volume), pH was adjusted to 8 ± 0.1 with triethylamine. The mobile phase was degassed by ultrasonic vibrations for 30 minutes prior to use. All determinations were performed in an air-conditioned laboratory atmosphere (18±2 ºC) using the following chromatographic conditions:

- Column: Nucleosil C_{18} 10U (250 mm x 4.6 mm I.D)
- Flow rate: 1.2 mL/min
- Injection volume: 20 µL
- Wavelength: the spectrophotometric detector was set at 248 nm. The detection wavelength was switched on-line to 271 after 1.8 minutes and to 238 nm after 3 minutes. This switch was performed on-line by HPLC System Manager Chromatography Data Station Software.

TLC method

The plates were first washed with the mobile phase mixture consisting of ethyl acetate/toluene/methanol/ammonia (50.5:23.5:23.5:2.5 by volume), followed by activating for 15 minutes in an oven at 100^{o}C before use. For detection and quantitation, aliquots were applied as separate compact bands 30 mm apart and 15 mm from the bottom of the plates, 6 mm band length and 150 nL/S dosage speed. The chromatographic tank was saturated with the mobile phase for 30 minutes prior to use. The plates were developed over a distance of 80 mm in an ascending manner, air-dried, and were scanned under the following conditions:

- Source of radiation: deuterium lamp
- Scan mode: absorbance mode
- Slit dimension: 3 mm x 0.45 mm
- Scanning speed: 20 mm/s
- Output: chromatogram and integrated peak area
- Wavelength: 252 nm

4. Procedures

a) Standard solutions

- Standard stock solutions of AML, VAL and HCT 10 mg/mL in methanol.
- Working standard solutions for HPLC 100 µg/mL of AML and HCT and 500 µg/mL of VAL were prepared from stock solutions by appropriate dilution with the mobile phase.
- Working standard solutions for TLC 500 µg/mL of AML, VAL and HCT were prepared from stock solutions by appropriate dilution with methanol.

b) Construction of calibration curves

For HPLC

Suitable aliquots of AML, VAL and HCT were accurately transferred from their respective standard working solutions (100 and 500 µg/mL) into three separate series of 10-mL volumetric flasks then completed to the volume with the mobile phase to prepare 2.0-

28.0 µg/mL AML, 10.0-120.0 µg/mL VAL and 0.6-32.0 µg/mL HCT. A volume of 20 µL of each solution was injected in triplicate into the liquid chromatograph under the previously mentioned chromatographic conditions. The average peak areas obtained for each concentration of AML, VAL and HCT were compared to that obtained from the external standard (10 µg/mL VAL) and were plotted versus the corresponding concentrations. The regression equations were computed.

For TLC

Aliquots equivalent to (0.5-9.0 µg) AML, (4.0-18.0 µg) VAL and (3.0-11.0 µg) HCT were transferred from their working standard solutions (500 µg/mL) and were spotted on TLC plates, using Camag Linomat autosampler with micro syringe (100 µL), the previously mentioned chromatographic conditions were implied. The scanning profiles for AML, VAL and HCT were obtained. The calibration curves relating the area under the peak to the corresponding concentration were constructed and the regression equations were computed.

c) Application of HPLC and TLC methods for the determination of AML, VAL and HCT in EXFORGE HCT® tablets

For HPLC

Seven tablets of each Exforge HCT® formulation were accurately weighed and finely powdered. An amount of the powder equivalent to 16 mg VAL was weighed, dissolved in methanol by shaking in ultrasonic bath for 30 minutes. The solutions were filtered into three 100-mL measuring flasks, and the volume was completed with methanol. Six mL aliquots were transferred from these solutions into three 100-mL measuring flasks. The volume was completed with the mobile phase and analyzed as described.

For TLC

Seven tablets of each Exforge HCT® formulation were accurately weighed and finely powdered. An amount of the powder equivalent to 200 mg VAL was weighed, dissolved in methanol by shaking in ultrasonic bath for 30 minutes. The solutions were filtered into three 100-mL measuring flasks, and the volume was completed with methanol. Aliquots of four µL were spotted on TLC plates for the determination of VAL and aliquots of 32 were spotted on TLC plates for the determination of AML and HCT. The same procedure described under linearity was applied and the concentrations of AML, VAL and HCT were calculated from the corresponding regression equations.

RESULTS AND DISCUSSION

HPLC method

The main criteria for development of a successful HPLC method for the determination of AML, VAL and HCT in the tablet form are that the method should be able to determine the suggested drugs in a single run. It should be accurate, reproducible, robust, free from excipients interference and simple enough for routine use in the quality control laboratory as well.

Two types of columns were investigated, Zorbax Eclipse C_8 (150 mm x 4.6 mm I.D) and Nucleosil C_{18} 10U (250 mm x 4.6 mm I.D). Zorbax C_8 did not show a good retention or resolution of the suggested drug mixture. Nucleosil C_{18} resulted in a perfect separation of the three drugs with different retention times and excellent resolution. In order to develop and optimize a suitable mobile phase for the separation of AML, VAL and HCT, the chromatographic behavior was examined using mobile phase mixtures of different polarity. Aqueous methanol and aqueous acetonitrile solutions were used in various ratios for preliminary study. A clear separation could not be obtained unless equal ratio of methanol/acetonitrile was used. The mixture resolved the peaks giving broad peaks with long retention time.

Considering that VAL is acidic in nature, while AML and HCT are basic, the use of phosphate buffer systems of different pH values was investigated. The retention behavior was studied in the pH range of 3-8, using different combinations of acetonitrile/methanol/buffer adjusting the pH using triethylamine or phosphoric acid. At pH 3, the peaks were resolved in the order of AML-HCT-VAL, but were broad with long retention time. By increasing the pH gradually using triethylamine from 3-8, a better resolution and shorter retention time were obtained. At pH

8.00 the best improvement was achieved, the peaks were eluted in the order of VAL-HCT-AML but still have long retention time. Isopropyl alcohol was tested as well and its effect on the resolution was studied, where 1 - 5% of buffer was replaced with isopropyl alcohol, the best resolution was obtained by using 4% but the elution time was still long. Replacing buffer system by acetonitrile/methanol at the same pH 8, improved the results. The effect of acetonitrile was studied, where 5-10% methanol was replaced with acetonitrile, the best result was obtained by using 7% acetonitrile. From the previous results, a combination of acetonitrile/methanol/isopropyl alcohol (55:41:4 by volume) achieved the best results, the pH was adjusted to 8 ± 0.1 with triethylamine. The UV detection at 254 nm, and on-line wavelength switching between the three different λ_{max} were studied at 248, 271 and 238 nm. Best sensitivity and selectivity were obtained by on-line wavelength switching, which allowed the analysis of the dosage form in a single run. Different flow rates were tested as well (0.8, 1.0, 1.2 and 1.5 mL/min), the best resolution, peak symmetry and elution time were obtained by adjusting the flow rate at 1.2 mL/min. By using these optimized conditions, a good separation of the three suggested drugs was obtained, (Fig. 2).

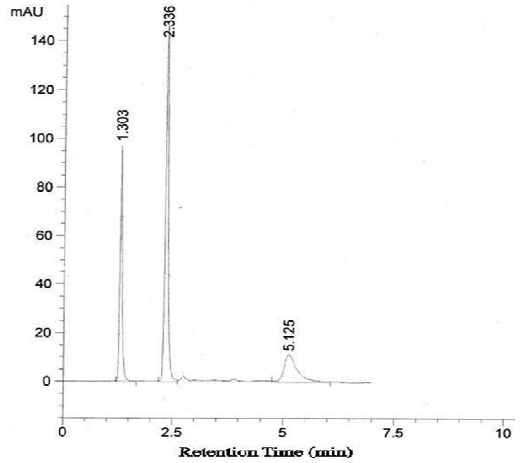

Figure 2.
HPLC chromatogram of a resolved mixture VAL (10 µg/mL, Rt = 1.303), HCT (10 µg/mL, Rt = 2.336) and AM (10 µg/mL, Rt = 5.125), mobile phase methanol/ acetonitrile/ isopropyl alcohol (55/41/4 by volume), pH was adjusted to 8 ± 0.1 using triethylamine.

Under the specified experimental conditions a linear relationship was obtained between the relative peak areas at the selected wavelengths and the corresponding concentrations of the drugs in the range of 2.0–28.0 µg/mL, 10.0–120.0 µg/mL and 0.6–32.0 µg/mL for AML, VAL and HCT, respectively. Using the external standard method for calibration, 10.0 µg/mL of VAL was used as external standard. These ranges allowed the analysis of the dosage form in a single run.

The regression equation were computed and found to be

$$P_{AML} = 0.0704\ C - 0.0612 \quad r = 0.9999$$
$$P_{VAL} = 0.1178\ C - 0.2694 \quad r = 0.9998$$
$$P_{HCT} = 0.1686\ C + 0.1500 \quad r = 0.9999$$

Where P is the relative peak area, C is the concentration in µg/mL and r is the correlation coefficient.

TLC

The TLC procedure was developed and optimized for quantification of AML, VAL and HCT in the tablet formulation. In order to develop and optimize the mobile phase used for separation of the suggested drugs, an initial mixture of toluene/methanol in different ratios was tried, but the separation was not achieved efficiently. Ethyl acetate was added to this mixture in different ratios to achieve a better resolution for the peaks. The mobile phase consisting of ethyl acetate/toluene/methanol (50.5:23.5:26 by volume) showed a good separation with a very small R_f for AML, broad peaks were obtained as well. By replacing 1% methanol with acetic acid, AML did not elute from baseline while upon replacing with ammonia, a better elution of AML was observed. The effect of the ammonia solution was studied, where 1-5% methanol was replaced with ammonia and the best separation and peak shape were obtained by 2.5% ammonia. Based on the previous results, the best system to apply was ethyl acetate/toluene/methanol/ammonia (50.5:23.5:23.5:2.5 by volume). Well-defined bands were obtained when the chamber was saturated with the mobile phase for 30 min at room temperature. The instrumental conditions for the densitometric measurement such as the scan mode and the wavelength detection were optimized (Fig. 3). The scanning wavelengths 233 and 252 nm were investigated, a better band shape and sensitivity were observed at 252 nm. AML, VAL and HCT were completely resolved from each other and their R_f values were 0.42, 0.68 and 0.19, respectively. This allowed the quantitative determination of the drugs without any interference. The normal phase TLC plate (20 x 20 cm, coated with 0.25 mm silica gel 60 F_{254}) was used as it gave best separation with no tailing.

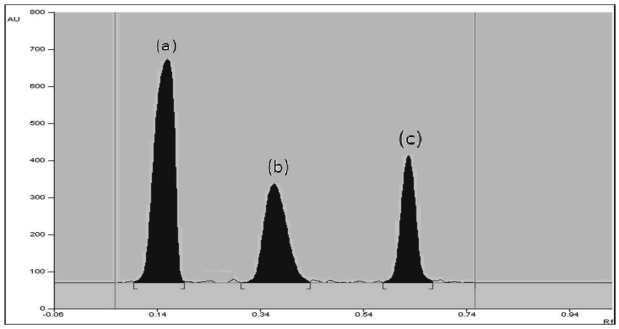

Figure 3.

TLC chromatogram of a resolved mixture (a) VAL (12 µg/band, Rf = 0.19), (b) AML (6 µg/band, Rf = 044) and (c) HCT (2 µg/band, Rf = 0.67), mobile phase ethyl acetate/toluene/methanol/ammonia (50.5 : 23.5 : 23.5 : 2.5 by volume).

Under the specified experimental conditions, the relationships between the concentrations of the suggested drugs and the peak areas of the spots were investigated. These relationships were best represented by a polynomial fit, because it gave random distribution of the residuals with no outline, in the range of 0.5-9.0 µg/band, 4.0-18.0 µg/band and 3.0-11.0 µg/band for AML, VAL and HCT, respectively.

The regression equations were computed and found to be

$P_{AM\,L} = -136.9\,C^2 + 2{,}760.0\,C - 205.1$ r = 0.9998
$P_{VAL} = -31.5\,C^2 + 1{,}672.8\,C + 5{,}389.8$ r = 0.9997
$P_{HCT} = -120.3\,C^2 + 2{,}544.9\,C + 2{,}766.1$ r = 0.9999

Where P is the peak area, C is the concentration in µg/band and r is the correlation coefficient. The suggested methods are valid and applicable for the analysis of EXFORGE HCT® tablets with no interference of the excipients as shown in

(Table 1). The validity of the proposed procedure was assessed by applying the standard addition technique. This was achieved by spiking different known concentrations of the pure drug to the formulation followed by applying the procedure described under the determination of pharmaceutical formulations as shown in (Table 2).

Table 1
Determination of AML, VAL and HCT in Exforge HCT® tablets by the proposed chromatographic methods.

Product		Drug	HPLC Method	TLC Method	Reported method [a]
Exforge 5/160/12.5	**HCT®**	AML	99.60±1.015	99.64±1.136	100.11±0.890
		VAL	99.65±1.381	100.67±0.684	100.43±1.500
		HCT	100.74±0.839	100.01±1.180	99.00±1.056
Exforge 5/160/25	**HCT®**	AML	98.70±0.882	100.03±1.075	100.72±1.609
		VAL	100.38±0.784	99.31±1.497	98.39±0.941
		HCT	100.69±0.909	99.86±0.887	100.10±0.868
Exforge 10/320/25	**HCT®**	AML	99.52±1.196	101.05±0.561	99.83±1.451
		VAL	99.71±1.128	100.72±0.640	99.13±0.940
		HCT	100.60±0.927	100.34±1.186	100.39±0.531

[a] HPLC method using Luna C_{18} column, a mobile phase consisting of methanol – phosphate buffer (30 mM, pH 5.5) (62:38 by volume) at a flow rate of 1 mL/min and UV detection at 234 nm.

Table 2
Application of standard addition technique to the determination of AML, VAL and HCT in Exforge HCT® tablets by the proposed chromatographic methods.

Product	Drug	Standard Addition							
		HPLC				TLC			
		Taken [b] (µg/mL)	Added (µg/mL)	Found (µg/mL)	Recovery% [c]	Taken [b] (µg/band)	Added (µg/band)	Found (µg/band)	Recovery% [c]
Exforge HCT® 5/160/12.5	AML	3	2	1.982	99.11	2	1	0.988	98.79
			3	3.003	100.12		2	1.964	98.20
			4	4.067	101.68		3	3.003	100.10
				Mean±RSD	**100.30±1.289**			**Mean±RSD**	**99.03±0.982**
	VAL	16	14	13.99	99.93	8	6	5.905	98.41
			16	15.746	98.41		8	7.878	98.48
			18	18.091	100.51		10	10.099	100.99
				Mean±RSD	**99.62±1.085**			**Mean±RSD**	**99.29±1.480**
	HCT	7.5	5	4.954	99.08	5	4	3.924	98.09
			7.5	7.456	99.41		5	4.937	98.75
			10	10.054	100.54		6	5.969	99.48
				Mean±RSD	**99.67±0.766**			**Mean±RSD**	**98.77±0.705**
Exforge HCT® 5/160/25	AML	3	2	1.987	99.37	2	1	0.985	98.49
			3	2.965	98.82		2	1.968	98.39
			4	4.026	100.65		3	3.000	100.00
				Mean±RSD	**99.62±0.944**			**Mean±RSD**	**98.96±0.909**
	VAL	16	14	13.915	99.39	8	6	6.026	100.44
			16	16.005	100.03		8	8.234	102.93
			18	18.326	101.81		10	10.228	102.28
				Mean±RSD	**100.41±1.248**			**Mean±RSD**	**101.88±1.267**
	HCT	15	13	13.233	101.79	5	4	4.987	99.68
			15	14.96	99.73		5	5.091	101.82
			17	17.025	100.15		6	5.900	98.33
				Mean±RSD	**100.56±1.083**			**Mean±RSD**	**99.95±1.762**
Exforge HCT® 10/320/25	AML	3	2	1.982	99.11	2	1	1.006	100.60
			3	3.026	100.87		2	1.965	98.27
			4	4.006	100.14		3	3.047	101.57
				Mean±RSD	**100.04**±0.885			**Mean±RSD**	**100.15±1.698**

		14	13.991	99.94		6	5.937	98.95
VAL	16	16	15.77	98.56	8	8	8.080	101.00
		18	18.163	100.91		10	10.077	100.77
			Mean±RSD	**99.80±1.180**			**Mean±RSD**	**100.24±1.118**
		5	4.939	98.78		4	4.000	99.99
HCT	7.5	7.5	7.47	99.61	5	5	5.074	101.48
		10	10.114	101.14		6	5.921	98.69
			Mean±RSD	**99.84±1.197**			**Mean±RSD**	**100.05±1.397**

[a] HPLC method using Luna C_{18} column, a mobile phase consisting of methanol – phosphate buffer (30 mM, pH 5.5) (62:38 by volume) at a flow rate of 1 mL/min and UV detection at 234 nm.
[b] For application of standard addition technique, AML and HCT were analysed in a single run while VAL was analysed in another run for suitability of the linear ranges.
[c] average of three determinations

System suitability was checked by calculating the tailing factor, capacity factor, selectivity factor, resolution, column efficiency (N) and the plate height (HETP), where the system was found to be suitable, (Table 3 and 4).

Table 3
System suitability parameters and robustness for the HPLC method.

Drug	*Robustness parameter*	*T*[a]	*K*[a]	*α*[b]	*Rs*[b]	*N*[c]	*HETP*	*%Assay*
	No change (repeatability)	0.95	3.07	----	----	3540	0.0071	100.03
	% Acetonitrile (+5%)	0.98	3.24	----	----	3255	0.0077	100.79
	% Acetonitrile (-5%)	1.00	3.20	----	----	2945	0.0085	99.21
	pH (+0.3 units)	1.02	3.03	----	----	3289	0.0076	100.47
VAL	*pH (-0.3 units)*	1.01	3.38	----	----	2515	0.0099	101.31
	Flow rate (+0.2 mL/min)	1.00	2.34	----	----	2903	0.0086	99.70
	Flow rate (-0.2 mL/min)	1.06	3.67	----	----	2591	0.0096	97.50
	Zobrax ODS (250 x 4.6 mm)	1.04	3.25	----	----	2703	0.0092	99.52
	Prodigy ODS (150 x 4.6 mm)	0.98	1.64	----	----	2179	0.0069	100.03
	No change (repeatability)	1.05	6.30	2.05	9.90	4547	0.0055	99.57
	% Acetonitrile (+5%)	0.96	6.34	1.96	7.93	4364	0.0057	99.06
	% Acetonitrile (-5%)	0.96	6.32	1.98	8.34	4463	0.0056	97.97
	pH (+0.3 units)	1.00	6.10	2.01	10.89	3760	0.0066	100.26
HCT	*pH (-0.3 units)*	1.25	6.49	1.92	7.60	3002	0.0083	99.68
	Flow rate (+0.2 mL/min)	0.98	5.46	2.33	8.57	3377	0.0074	100.08
	Flow rate (-0.2 mL/min)	1.10	8.01	2.18	9.42	4318	0.0058	100.48
	Zobrax ODS (250 x 4.6 mm)	0.90	5.30	1.63	3.36	3929	0.0064	100.63
	Prodigy ODS (150 x 4.6 mm)	0.95	2.93	1.78	4.97	2095	0.0072	98.25
	No change (repeatability)	1.25	15.02	2.38	9.58	2935	0.0085	98.44
	% Acetonitrile (+5%)	1.20	12.29	1.94	5.37	2178	0.0115	100.22
	% Acetonitrile (-5%)	1.18	12.10	1.91	6.94	2575	0.0097	99.17
	pH (+0.3 units)	1.11	13.94	2.29	11.43	3356	0.0074	99.84
AML	*pH (-0.3 units)*	1.29	15.95	2.46	8.29	2593	0.0096	99.62
	Flow rate (+0.2 mL/min)	1.10	13.04	2.39	8.35	2422	0.0103	99.51
	Flow rate (-0.2 mL/min)	1.30	18.27	2.28	8.30	2363	0.0106	98.86
	Zobrax ODS (250 x 4.6 mm)	1.89	15.82	2.99	5.42	2413	0.0104	99.64
	Prodigy ODS (150 x 4.6 mm)	1.09	4.17	1.43	4.37	2108	0.0071	100.01

[a] Tailing factor and capacity factor determined for individual peak.
[b] Selectivity factor and resolution factor determined between VAL and HCT peaks and between HCT and AML peaks.
[c] Column efficiency expressed as number of theoretical plates for VAL, HCT and AML.

Table 4
System suitability parameters and robustness for the TLC method

Drug	Robustness parameter		T [a]	K' [a]	α [b]	Rs [b]	%Assay
VAL	No change (repeatability)		0.82	5.25	----	----	100.66
	Mobile phase ratio	(48.0:26.0:23.5:2.5)	0.89	6.55	----	----	99.68
		(48.0:23.5:26.0:2.5)	1.10	5.30	----	----	99.61
		(53.0:21.0:23.5:2.5)	0.91	5.45	----	----	100.23
		(53.0:23.5:21.0:2.5)	1.20	5.35	----	----	99.71
		(50.5:21.0:26.0:2.5)	0.96	5.20	----	----	99.48
		(50.5:26.0:21.0:2.5)	1.16	6.34	----	----	100.26
	Mobile phase amount	(+10 mL)	1.04	5.72	----	----	100.22
		(-10 mL)	0.98	5.61	----	----	99.23
	Development distance	(+5 mm)	1.06	6.14	----	----	99.01
		(-5 mm)	1.02	6.41	----	----	99.05
	Duration of saturation	(+10 min)	0.81	5.67	----	----	100.60
		(-10 min)	0.87	5.96	----	----	101.27
	Temperature	(+5°C)	1.23	6.84	----	----	100.84
		(-5°C)	1.10	5.25	----	----	101.52
AML	No change (repeatability)		1.15	1.70	3.08	3.00	100.30
	Mobile phase ratio	(48.0:26.0:23.5:2.5)	0.96	1.99	3.30	2.48	99.06
		(48.0:23.5:26.0:2.5)	0.86	1.51	3.51	2.94	97.97
		(53.0:21.0:23.5:2.5)	1.09	1.52	3.58	3.03	99.02
		(53.0:23.5:21.0:2.5)	1.21	1.49	3.59	3.59	99.70
		(50.5:21.0:26.0:2.5)	0.83	1.49	3.49	3.25	99.42
		(50.5:26.0:21.0:2.5)	1.10	1.90	3.34	2.50	101.84
	Mobile phase amount	(+10 mL)	0.90	1.79	3.20	3.00	100.82
		(-10 mL)	0.85	1.87	3.01	2.60	98.76
	Development distance	(+5 mm)	1.06	1.86	3.31	2.77	99.04
		(-5 mm)	0.98	1.75	3.66	2.56	98.78
	Duration of saturation	(+10 min)	1.10	1.68	3.38	2.62	100.01
		(-10 min)	0.82	1.66	3.59	2.49	99.87
	Temperature	(+5°C)	0.79	1.87	3.66	3.25	100.60
		(-5°C)	1.02	1.79	2.94	3.10	99.15
HCT	No change (repeatability)		1.10	0.59	2.90	4.18	98.21
	Mobile phase ratio	(48.0:26.0:23.5:2.5)	1.28	0.57	3.47	4.43	100.48
		(48.0:23.5:26.0:2.5)	0.98	0.60	2.53	3.25	99.05
		(53.0:21.0:23.5:2.5)	0.88	0.58	2.62	3.47	98.54
		(53.0:23.5:21.0:2.5)	1.09	0.57	2.62	4.04	101.12
		(50.5:21.0:26.0:2.5)	0.87	0.59	2.54	3.56	99.23
		(50.5:26.0:21.0:2.5)	1.29	0.59	3.22	4.56	98.78
	Mobile phase amount	(+10 mL)	0.89	0.57	3.13	4.46	99.53
		(-10 mL)	1.09	0.56	3.32	4.28	100.01
	Development distance	(+5 mm)	0.79	0.62	3.01	4.07	99.94
		(-5 mm)	0.88	0.64	2.75	3.35	99.42
	Duration of saturation	(+10 min)	0.92	0.55	3.03	3.97	100.49
		(-10 min)	1.23	0.58	2.85	3.38	100.53
	Temperature	(+5°C)	1.04	0.61	3.06	4.00	99.72
		(-5°C)	0.99	0.62	2.90	4.61	99.45

[a] *Tailing factor and capacity factor determined for individual peak.*
[b] *Selectivity factor and resolution factor determined between VAL and AML peaks and between AML and HCT peaks.*

Robustness of the proposed methods was assessed with respect to the effect of small but deliberate variation in chromatographic conditions. The degree of reproducibility and system suitability parameters of the results proved that the methods are robust, (Table 3 and 4). The robustness of the methods was assessed at three different concentrations for each analyte (12, 16 and 20 µg/mL) for HPLC and (4, 6, and 8 µg/band) for TLC. The results obtained for the analysis of AML, VAL and HCT in EXFORGE HCT® tablets by the proposed methods were statistically compared with those obtained by applying the reported method [14], where no significant difference was obtained as shown in (Table 5). A validation sheet according to the ICH guidelines [18] is also presented in (Table 6). Comparing the proposed HPLC method to the reported methods [14,15], the proposed method could

separate the three drugs in shorter time, thus reduced solvent consumption. Also the proposed method does not require preparation of buffer that may be harmful to the column due to its content of mineral salts which by improper wash will precipitate and cause damage to the column. The on-line wavelength switching increased the sensitivity of the proposed method compared to the reported method [14]. The proposed method is simple isocratic method without need for sophisticated gradient pump as that used in the reported method [14]. Comparing the proposed TLC method to the reported one [14], the proposed method utilized simple TLC plates without the need for sophisticated HPTLC and did not involve the use of hepatotoxic chloroform in the developing solvent.

Table 5

Statistical comparison for the results obtained by the proposed chromatographic methods and the reported method [14] for the analysis of AML, VAL and HCT in Exforge HCT® tablets

Value	HPLC Method			TLC method			Reported Method[a]		
	AML	VAL	HCT	AML	VAL	HCT	AML	VAL	HCT
Mean	99.27	99.91	100.59	100.24	100.23	100.07	100.16	99.71	99.83
SD	0.994	1.035	0.985	1.042	1.121	0.972	0.908	1.016	0.966
RSD%	1.001	1.036	0.979	1.040	1.118	0.971	0.906	1.019	0.967
N	9	9	9	9	9	9	9	9	9
Variance	0.987	1.071	0.970	1.087	1.256	0.944	0.824	1.032	0.932
Student's t test [b]	1.982 (2.12)	0.415 (2.12)	1.655 (2.12)	0.162 (2.12)	1.033 (2.12)	0.530 (2.12)	-----	-----	-----
F value [b]	1.198 (3.438)	1.037 (3.438)	1.040 (3.438)	1.319 (3.438)	1.217 (3.438)	1.012 (3.438)	-----	-----	-----

[a] HPLC method using Luna C_{18} column, a mobile phase consisting of methanol – phosphate buffer (30 mM, pH 5.5) (62:38 by volume) at a flow rate of 1 mL/min and UV detection at 234 nm.

[b] The values in the parenthesis are the corresponding theoretical values of t and F at P= 0.05.

Table 6

Assay validation sheet of the proposed chromatographic methods for the simultaneous determination of AML, VAL and HCT

Parameter	*HPLC Method*			*TLC-Densitometry Method*		
	AML	*VAL*	*HCT*	*AML*	*VAL*	*HCT*
Accuracy (mean ± RSD)	100.90±1.014	101.11±0.905	100.41±1.137	100.24±1.409	100.08±0.699	100.40±0.895
Precision						
– *Repeatability[a]*	1.396	0.846	0.842	0.589	0.682	0.749
– *Intermediate precision[b]*	1.354	0.976	1.289	0.900	0.766	1.563
Robustness [c]	0.441	1.166	1.024	1.002	0.793	0.740
Linearity						
– *Slope 1[d]*	0.0704	0.1178	0.1686	-136.9	-31.5	-120.3
– *Slope 2[d]*				2,760.0	1,672.8	2,544.9
– *Intercept*	0.0612	-0.2694	0.1500	-205.1	5,389.8	2,766.1
– *Correlation coefficient (r)*	0.9999	0.9998	0.9999	0.9999	0.9998	0.9999
– *Range*	2-28 µg/mL	10-120 µg/mL	0.6-32 µg/mL	0.5-9.0 µg/band	4.0-18.0 µg/band	3.0-11.0 µg/band

a. The intraday (n = 3), average of three concentrations for the three drugs (12,16 and 20 µg/ml) for HPLC and (4, 6 and 8 µg/band) for TLC repeated three times within the day.

b. The interday (n = 3), average of three concentrations for the three drugs (12,16 and 20 µg/ml) for HPLC and (4, 6 and 8 µg/band) for TLC repeated three times in three days.

c. Robustness (n = 3), average of three concentrations for the three drugs (12,16 and 20 µg/ml) for HPLC and (4, 6 and 8 µg/band) for TLC analyzed in different conditions mensioned before.

d. Slope 1 and 2 are the coefficients of X^2 and X, respectively. Following a polynomial regression $A = ax^2 + bx + c$ Where, A is the integrated peak area, x is the concentration of AML, VAL or HCT (µg/band), a and b are coefficients 1 and 2, respectively and c is the intercept.

CONCLUSION

From the previous discussion, it could be concluded that the proposed HPLC and TLC methods are precise, accurate and simple. The two methods have the advantage of being simple, sensitive and do not require calculations as the spectrophotometric methods applied for the analysis of this ternary mixture. Also the proposed methods showed advantages over the reported chromatographic ones as discussed, and can be used for the routine analysis of AML, VAL and HCT, in pharmaceutical formulations or in bulk powder. ICH guidelines were followed throughout the study for method validation.

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