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Simultaneous determination of valsartan, amlodipine besylate and hydrochlorothiazide using capillary zone electrophoresis (CZE)

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A capillary zone electrophoresis method was developed for the simultaneous determination of valsartan (VAL), amlodipine besylate (AML) and hydrochlorothiazide (HCZ) in their combined tablets. Separation was achieved on a fused silica capillary by applying a potential of 15 kV (positive polarity) and a running background electrolyte containing 40 mM phosphate buffer at pH 7.5 with UV detection at 230 nm. The samples were injected hydrodynamically for 3 s at 0.5 psi and the temperature of the capillary cartridge was kept at 25 °C. Pyrazinoic acid was used as an internal standard. The method was validated according to ICH guidelines regarding specificity, linearity, limits of detection and quantitation, accuracy and precision, (Supplementary materials, Table S2). The method showed satisfactory linearity in the ranges of 10-200, 2-20 and 2-20 μg mL^{-1} with LODs of 1.82, 0.39, 0.65 μg mL^{-1} and LOQs of 5.51, 1.17, 1.96 μg mL^{-1} for VAL, AML and HCZ, respectively. The proposed method was successfully applied for the analysis of the studied drugs in their laboratory prepared mixtures and co-formulated tablets. The results were compared with reported methods and no significant differences were found. The proposed method can be used for quality control of the cited drugs in ordinary laboratories.

1. Introduction

Valsartan (VAL) chemically, (*S*)-*N*-valeryl-*N*-([2'-(1*H*-tetrazol-5-yl)biphenyl-4-yl]-methyl)-valine (Fig. 1a), is a potent, highly selective, and orally active non peptide antagonist on the AT1-receptor subtype (Criscione et al. 1993). Angiotensin receptor blockers are a major class of antihypertensive agents due to their powerful lowering effects on blood pressure and excellent tolerability (Asmar 2006). Amlodipine besylate (AML), (3-ethyl 5-methyl (4RS)-2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate benzenesulphonate) (Fig. 1b) is a long acting dihydropyridine calcium channel blocker that prevents the transmembrane influx of calcium ions into vascular smooth muscles and cardiac muscles (El-Gindy et al. 2008). Also, it is a direct peripheral arterial vasodilator that reduces peripheral vascular resistance and hence lowers blood pressure (Abdollahpour et al. 2011). Hydrochlorothiazide (HCZ) is a potent diuretic and antihypertensive agent which inhibits active chloride reabsorption and thus increases the excretion of sodium chloride and water (Tengli et al. 2013). It is chemically 6-chloro-1, 1-dichloro-3, 4, dihydro -2*H*-1, 2, 4-benzothiadiazine-7-sulphanomide1, 1-dioxide (Fig. 1c). Triple combination therapy of VAL, AML and HCZ proved to be effective and well tolerated in treating hypertensive high-risk patients (Calhoun et al. 2009). A literature survey revealed methods using HPLC (Daneshtalab et al. 2002; Gonzalez et al. 2002; Kocyigit et al. 2006), LC-MS (Koseki et al. 2007; Li et al. 2007; Senthamil et al. 2007), CE (Hillaert and Bossche 2003; Alnajjar 2011), UV spectrophotometry (Satana et al. 2001; Tatar and Saglik 2002) and spectroflourimetry (Shaalan and Belal 2010) for the estimation of VAL either alone or in combination with other drugs. Methods such as HPLC (Jain et al. 2012; Patel et al. 2012; Sharma et al. 2014), LC/MS/MS (Pilli et al. 2011; Yacoub et al. 2013), CE (Fakhari et al. 2008), UV-spectrophotometry (Rahman and Azmi 2001; Rahman and Hoda 2003; Wankhede et al. 2010) and spectroflourimetry (Shaalan and Belal 2010; Darwish and Backeit 2013) are reported for estimation of AML either alone or in combination with other drugs. Also, methods such as HPLC (Hertzog et al. 2002; Huang et al. 2006; Meyyanathan et al. 2008; Tian et al. 2008; Joshi et al. 2010; Sharma and Pancholi 2012), LC-MS (Rajasekhar et al. 2009; Salvadori et al. 2009; Bharathi et al. 2012), UPLC (Nalwade et al. 2011) and UV- spectrophotometry (El-Gindy et al. 2001) are reported for the estimation of HCZ alone or in combination with other anti-hypertensive agents. There are many methods reported for the simultaneous determination of the three drugs in different matrices using HPLC (Varghese and Ravi 2011; Anandakumar et al. 2012; Sharma and Pancholi 2012), or UV-spectrophotometry (Galande et al. 2012). To the best of our knowledge, no capillary electrophoresis method was reported yet for the simultaneous determination of the studied medications either in pharmaceutical preparations or in biological fluids. CE has a lot of successful applications in the field of pharmaceutical analysis such as assay of drugs, determination of impurities, chiral separation, and in the analysis of pharmaceutical excipients. The advantages of using CE for pharmaceutical analysis include its reliability and low cost of analysis, reductions in solvent consumption and disposal, and the possibility of rapid method development and

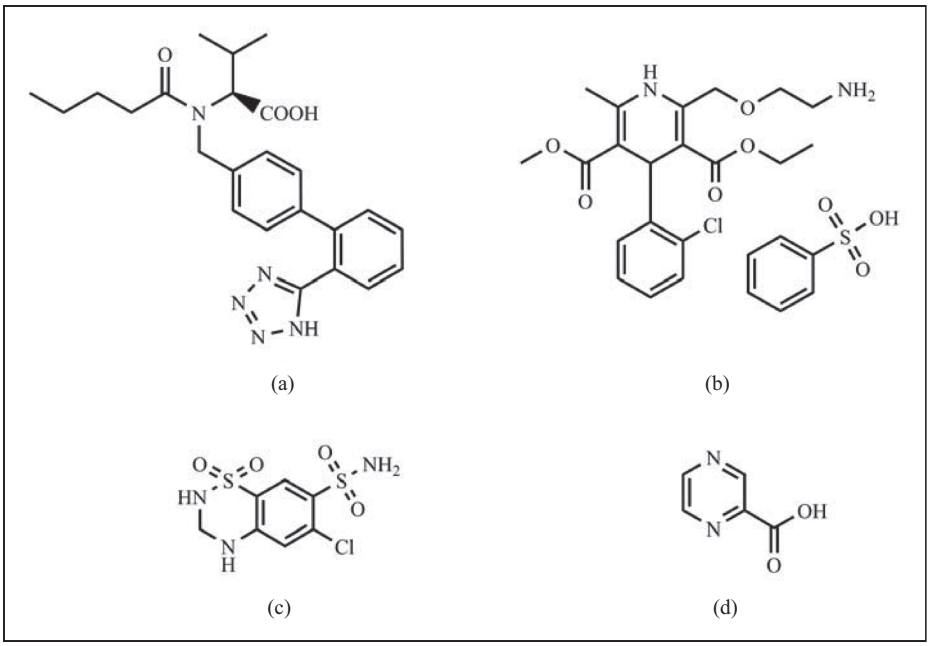

Fig. 1: Structural formulae for the studied drugs; (a) valsartan (VAL), (b) amlodipine besylate (AML), (c) hydrochlorothiazide (HCZ) and (d) Pyrazinoic acid (IS).

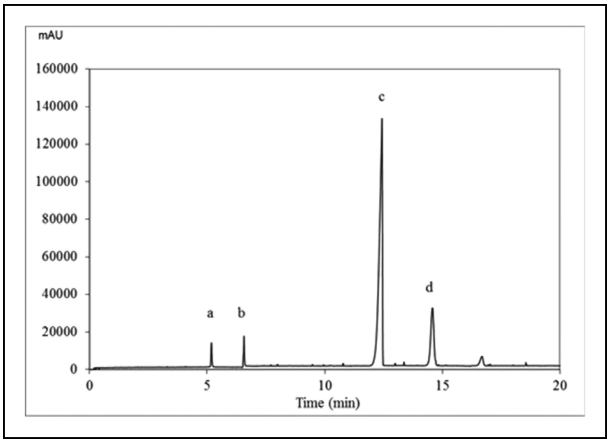

Fig. 2: Typical electropherogram for a laboratory prepared mixture; (a) 11 μg mL^{-1} AML, (b) 10 μg mL^{-1} HCZ, (c) 128 μg mL^{-1} VAL and (d) 100 μg mL^{-1} IS under the described electrophoretic conditions.

feasibility of a high degree of automation. Moreover, CE allows a single set of separation conditions to be applied for a wide range of analyses, introducing efficiency savings (Altria and Filbey 1993; Hendrickx et al. 2011). Recently, CE techniques have been applied for the simultaneous determination of co-formulated drugs in their dosage forms (Fakhari et al. 2008; Alnajjar 2011).

2. Investigation, results and discussion

The aim of this study was to develop and validate a reliable, accurate, inexpensive, selective and efficient method using capillary electrophoresis that could be used for routine quality control analysis of VAL, AML and HCZ simultaneously in their co-formulated tablets. VAL, AML and HCZ are co-formulated in a medicinally recommended ratio of 12.8:1.1:1. Analysis of such a mixture with strong spectral overlapping is challenging. The proposed CZE method allowed the separation and simultaneous quantification of the three medications in a reasonable time less than 15 min with satisfactory accuracy and precision in their co-formulated dosage form.

Figure 2 shows a typical electropherogram for a laboratory prepared mixture of the three drugs under the described electrophoretic conditions. The migration times for VAL, AML and HCZ were 12.74, 5.21 and 6.45 min, respectively.

The proposed CZE method was applied to the simultaneous determination of VAL, AML and HCZ in laboratory prepared mixtures in their medicinally recommended ratio. Furthermore, the proposed method was successfully applied for their determination in their co-formulated tablets. The results shown in Tables 1,2 are in good agreement with those obtained using the reported comparison method (Anandakumar et al. 2012). The comparison method was performed on a RP-C18 column using a mobile phase consisting of acetonitrile: methanol: 50 mM phosphate buffer adjusted to pH 3 with orthophosphoric acid (20: 50: 30, *v/v/v*) at a flow rate of 1.0 mL min^{-1} and the eluents were monitored at 239 nm. Statistical analysis of the obtained results using Student's t-test and variance ratio F-test revealed no significant differences between the performance of the methods regarding accuracy and precision.

2.1. Optimization of the CE conditions

In CZE, the background electrolyte (BGE) type as well as its pH and its concentration are crucial for optimizing the separation of the ionizable analytes as they determine the degree of the analyte ionization, its electrophoretic mobility and the magnitude of the electroosmotic flow (EOF). As AML has pK_a value of 9.5 and HCZ has pK_a values of 8.8 and 9.9, they are positively charged at pH 7.5. On the other hand, VAL has pKa values of 3.6 and 4.7 and it is negatively charged at the same pH. Several capillaries of different length and internal diameter as well as different pHs and molarities of phosphate buffer were tested for CZE analysis and the results were evaluated taking into consideration different parameters such as migration time, resolution, peak shape, height, baseline noise and the electric current produced.

2.1.1. Effect of phosphate buffer pH

The effect of buffer pH was investigated within the range of 5.0–8.0 at a 40 mM phosphate buffer concentration. Electropherograms shown in Fig. 3 demonstrated that the four compounds needed longer time to be separated at pH values lower than 6.5. Also, both resolution and migration times decreased with increasing pH. Taking into consideration the ionization percentage of the analytes, resolution, peak symmetry

Table 1: Accuracy and precision data for the determination of VAL, AML and HCZ by the proposed CZE method

Concentration of VAL ($\mu g\ mL^{-1}$)	Intra-day assay Recovery % ± S.D[a]	Inter-day assay Recovery % ± S.D[a]	Comparison method Recovery %
32.00	101.42 ± 1.40	100.90 ± 0.57	98.04
96.00	99.44 ± 0.32	100.26 ± 1.16	100.64
128.00	99.38 ± 1.35	99.71 ± 1.60	98.66
Mean ± S.D.	100.08 ± 1.16	100.29 ± 0.60	99.11 ± 1.36
Student *t* test	0.94 (2.78)	1.38 (2.78)	
F	1.37 (19.00)	5.21 (19.00)	
Concentration of AML ($\mu g\ mL^{-1}$)	Intra-day assay Recovery % ± S.D[a]	Inter-day assay Recovery % ± S.D[a]	Comparison method Recovery %
2.75	101.60 ± 1.71	99.63 ± 2.96	102.20
8.25	99.56 ± 2.05	99.56 ± 2.48	99.70
13.75	101.52 ± 0.59	100.54 ± 1.49	100.09
Mean ± S.D.	100.89 ± 1.16	99.91 ± 0.55	100.66 ± 1.35
Student *t* test	0.23 (2.78)	0.89 (2.78)	
F	1.37 (19.00)	6.10 (19.00)	
Concentration of HCZ ($\mu g\ mL^{-1}$)	Intra-day assay Recovery % ± S.D[a]	Inter-day assay Recovery % ± S.D[a]	Comparison method Recovery %
2.50	100.31 ± 0.88	98.84 ± 1.35	100.28
7.50	100.84 ± 0.59	99.96 ± 0.78	99.77
12.50	99.65 ± 0.89	100.94 ± 1.16	100.07
Mean ± S.D.	100.27 ± 0.60	99.91 ± 1.05	100.04 ± 0.26
Student *t* test	0.60 (2.78)	0.20 (2.78)	
F	5.26 (19.00)	16.33 (19.00)	

The values between parentheses are the tabulated t and F values at $P = 0.05$
[a] Mean and standard deviation of three determinations

and migration time, pH 7.5 was selected as the optimum pH for separation (Fig. 3).

2.1.2. Effect of phosphate buffer concentration

Buffer concentration has also a significant effect on the separation performance through its influence on the EOF and the current produced in the capillary. The effect of phosphate running buffer concentrations was examined by varying the concentration from 5 to 50 mM with the increase in phosphate buffer concentration, both resolution and migration times increased. A 40 mM concentration of phosphate buffer was chosen in order to achieve a reliable electrolyte background for analysis while maintaining reasonable run time, good resolution and acceptable current ($\approx$ 78 μA).

2.1.3. Effect of applied voltage

The applied voltage effect was investigated under the optimized conditions selected above from 5 to 20 kV. As expected, increasing the applied voltage increases the EOF, leading to shorter separation time and higher efficiencies. However, lowering the applied voltage than 15 kV decreases the EOF so the separation time will be higher and increasing the applied voltage exhibits higher currents and produces Joule's heating. To limit this heating inside the capillary, the maximum applied voltage was chosen from an Ohm's plot (current versus voltage) and it was found to be 15 kV (current $\approx$ 78 μA).

2.1.4. Effect of injection time

Injection time affects the peak width and peak height. Sample solutions were hydrodynamically injected at 0.5 psi while the injection time was varied from 1.0 to 4.0 s. After 3.0 s, the peak widths of VAL, HCZ and IS were increased and the peak shapes were deformed, so 3.0 s was selected as the optimum injection time.

2.1.5. Selection of the internal standard (IS)

The use of IS is recommended to compensate for injection errors, minor fluctuation effects of the migration time and improve the quantitative analysis. Pyrazinoic acid was chosen as it possesses pK_a value of 2.9 (Billes 1980) and its molecular weight is less than VAL so it will be negatively charged under the optimized conditions and as expected it eluted after VAL.

2.1.6. Selection of the detection wavelength

In order to optimize sensitivity and detection limit of the method, a multiwavelength detection system (190 – 300 nm) was used in the CE system. For simultaneous determination of VAL, AML and HCZ, reasonable detection sensitivities were obtained at 230 nm (bandwidth 20 nm).

2.2. Validation of the method

Validation of the proposed CZE method was performed with respect to stability, linearity, range, specificity, limit of detection (LOD), limit of quantitation (LOQ), accuracy and precision according to the ICH Guidelines [http://www.fda.gov/downloads/Regulator%20yInformation/Guidances/UCM128049.pdf].

Table 2: Assay results for the determination of the studied drugs in Exforge HCT® 160/10/12.5 mg tablets

Compound	Proposed method			Comparison method
	Amount taken ($\mu g\ mL^{-1}$)	Amount found ($\mu g\ mL^{-1}$)	Recovery %	Recovery %
VAL	32.00	32.26	100.80	99.41
	64.00	64.97	101.52	99.28
	96.00	97.42	101.48	101.58
	128.00	127.64	99.72	99.32
	160.00	163.18	101.99	99.32
Mean ± S.D.			101.10 ± 0.88	99.78 ± 1.01
Student *t* test			2.21 (2.31)	
F			1.30 (6.39)	
AML	2.75	2.82	102.59	97.93
	5.50	5.58	101.55	99.95
	8.25	8.35	101.21	101.78
	11.00	11.03	100.30	99.97
	13.75	13.96	101.52	99.45
Mean ± S.D.			101.43 ± 0.82	99.82 ± 1.38
Student *t* test			2.26 (2.31)	
F			2.82 (6.39)	
HCZ	2.50	2.57	102.96	97.20
	5.00	5.07	101.37	99.52
	7.50	7.63	101.72	102.67
	10.00	10.06	100.57	100.34
	12.50	12.84	102.71	99.00
Mean ± S.D.			101.87 ± 0.98	99.75 ± 2.00
Student *t* test			2.13 (2.31)	
F			4.15 (6.39)	

Each result is the average of three separate determinations.
The values between parentheses are the tabulated t and F values at $P = 0.05$.

2.2.1. Linearity

A linear relationship was established by plotting the peak area ratio (the studied drug peak area/IS peak area) against the corresponding drug concentration in the range of 10–200, 2-20 and 2-20 $\mu g\ mL^{-1}$ for VAL, AML and HCZ, respectively. Statistical analysis of the data gave high values for the correlation coefficient (r) of the regression equation, small values of the standard deviation of residuals ($S_{y/x}$), of intercept (S_a), and of slope (S_b), and small value of the percentage relative standard deviation (Table 3). These data proved the linearity of the calibration graphs.

2.2.2. Limit of quantitation (LOQ) and limit of detection (LOD)

LOQ and LOD were calculated according to ICH Q2 (R1) recommendations. Results are given in Table 3.

Table 3: Performance data for the determination of the studied drugs by the proposed CZE method

Parameter	VAL	AML	HCZ
Linearity range ($\mu g\ mL^{-1}$)	10-200	2-20	2-20
Intercept (*a*)	-0.0837	-0.0047	0.0154
Slope (*b*)	0.0637	0.0123	0.0453
Correlation coefficient (*r*)	0.9999	0.9998	0.9995
S.D. of residuals ($S_{y/x}$)	0.052	0.002	0.0114
S.D. of intercept (S_a)	0.035	0.001	0.0089
S.D. of slope (S_b)	0.0004	0.0001	0.0007
% RSD	3.362	3.357	3.164
% Error	1.368	1.380	1.304
LOD ($\mu g\ mL^{-1}$)	1.819	0.385	0.647
LOQ ($\mu g\ mL^{-1}$)	5.513	1.166	1.960

2.2.3. Accuracy and precision

To prove the accuracy of the proposed method, the recovery percentage results of the assay of VAL, AML and HCZ were compared with those of the reference methods (USP 2007; BP 2009). Statistical analysis of the results using Student's t-test and variance ratio F-test revealed no significant differences between the performance of the two methods regarding accuracy and precision. The reference methods depend on the analysis of VAL, AML and HCZ using HPLC. Repeatability (intra-day) and intermediate precision (inter-day) were assessed using three concentrations and three replicates of each concentration. The relative standard deviations were found to be very small indicating reasonable repeatability and intermediate precision of the proposed method (Table 1).

2.2.4. Stability

Stability of the standard solutions of VAL, AML and HCZ, stored at 2 °C, were evaluated at various time points over 3 months. The concentrations of freshly prepared solutions and those aged for 2 months were calculated by the method developed and the difference between them was found to be less than 0.3 %. These solutions can therefore be used during this interval of time without the results being affected.

2.2.5. Specificity

The specificity of the method was investigated by observing any interference encountered from common tablet excipients and it was confirmed that the signals measured was caused only by the analytes. Each film-coated tablet of Exforge HCT® contains the following inactive ingredients: colloidal silicon dioxide, crospovidone, hypromellose, iron oxide red, iron oxide yellow, iron oxide black, magnesium stearate, microcrystalline cellu-

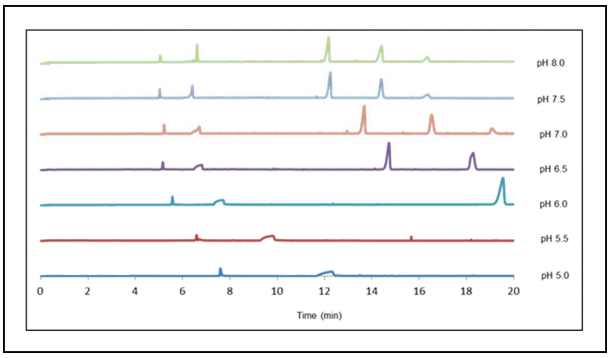

Fig. 3: Effect of phosphate buffer pH; operating conditions: 40 mM phosphate buffer, injection time (3 s), 15 kV, 25 °C, 210 nm (bandwidth 20 nm). (12 μg mL^{-1} AML, 30 μg mL^{-1} HCZ, 192 μg mL^{-1} VAL and 100 μg mL^{-1} IS).

lose, polyethylene glycol, povidone, talc, and titanium dioxide. It was found that the excipients did not interfere with the results of the proposed method. The tablets electropherogram did not show any additional peaks when compared to VAL, AML and HCZ laboratory prepared mixture electropherogram which confirm the specificity of the method.

3. Experimental

3.1. Instruments

This assay was developed and validated using a Beckman P/ACE 5510 system (Fullerton, CA, U.S.A) equipped with an autosampler, a photodiode array (PDA) detector, a temperature controlling system(4 –40 °C) and power supply able to deliver up to 30 kV. Beckman P/ACE^{TM} station software (version 1.2) was used for the instrument control, data acquisition and analysis. Electrophoretic analyses were performed on a fused silica capillary (Polymicro Technologies, Phoenix, AZ, U.S.A) of 57.0-cm-long (50.0-cm effective length) and 75.6 μm i.d. in normal mode, applying a voltage of 15 kV. The wavelength for analysis was 230 nm, hydrodynamic injection of samples for 3 s under a pressure of 0.5 psi and the temperature of the capillary cartridge was 25 °C.
The BGE was filtered using 0.2 μm Anatop 25 Whatman inorganic membrane filter (Maidstone, England) and degassed using Branson Ultrasonic 5510 degasser (Danbury, CT, U.S.A). A SympHonly (SB20) pH-meter (Thermo Orion Beverly, MA, U.S.A) was used for pH measurements. Deionized water was prepared using a Barnstead NANO pure DIamond Analytical (USA) ultrapure water system.

3.2. Materials and reagents

All the chemicals used were of analytical reagent grade, and the solvents were of HPLC grade. Pharmaceutical grade valsartan USP was supplied Zydus, batch Number: VSK1MK A02B (certified to contain 99.40%). Amlodipine besylate (certified to contain 99.75%) was kindly supplied by Global Nabi Co., (Giza -Egypt). Hydrochlorothiazide (certified to contain 99.93%) was supplied by AstraZeneca, Cairo, Egypt (under license of AstraZeneca, Sweden). Methanol and pyrazinoic acid (certified to contain 99%) were obtained from Sigma-Aldrich, MO, U.S.A. Exforge HCT® 160/10/12.5 mg tablets each labeled to contain 160 mg valsartan, 13.9 mg amlodipine besylate and 12.5 mg hydrochlorothiazide were purchased from commercial sources in the local market. Sodium hydroxide, sodium dihydrogen phosphate (J.T. Baker,NJ, U.S.A) and orthophosphoric acid 85% (Fisher Scientific, NJ, U.S.A)) and methanol (Sigma-Aldrich, MO, U.S.A) were used.

3.3. Standard and working solutions

3.3.1. Standard solutions

Stock solutions 2000 μg mL^{-1} of VAL and 1000 μg mL^{-1} of AML, HCZ and IS were prepared in a solvent composed of methanol and water (10: 90, *v/v*). Working solutions were prepared by further dilution of AML and HCZ stock solutions with 10% methanol to give a final concentration of 200 μg mL^{-1}.

3.3.2. Background running electrolyte (BGE)

The optimized BGE solution consisted of 40 mM sodium dihydrogen phosphate buffer adjusted to pH 7.5 with 1 M NaOH and filtered through a 0.2 μm membrane filter.

3.4. Electrophoretic procedure

Before the first use, the capillary was conditioned by flushing with 1 M NaOH for 90 min, then with water for 30 min and BGE for 30 min at 0.5 psi pressure. At the beginning of each working day, the capillary was rinsed with 0.1 M NaOH for 10 min, water for 5 min and then with BGE for 5 min at 20 psi pressure. Before each injection, the capillary was preconditioned with 0.1 M NaOH (5 min), water (5 min) and BGE (5 min) at 20 psi pressure to maintain proper reproducibility of run-to-run injections. Injection was carried out under hydrodynamic pressure at 0.5 psi for 3 s. A diode-array UV detector was set at 230 nm with a bandwidth of 20 nm. The capillary temperature was kept constant at 25 °C and a voltage of 15 kV was applied.

3.5. Procedures

3.5.1. Construction of the calibration graphs

Accurate aliquots of VAL stock solution as well as AML and HCZ working solutions were transferred into a series of 4-mL vials so that the final concentrations were in the range of 10-200 μg mL^{-1} for VAL, 2-20 μg mL^{-1} for AML and 2-20 μg mL^{-1} for HCZ. A constant 100 μL IS stock solution was added and the volume was diluted to 4 mL with 10% methanol. The peak area ratio (peak area of the studied drug/ peak area of IS) was plotted *versus* the final concentration of each drug in μg mL^{-1} to get the calibration graphs. Alternatively, the corresponding regression equations were derived.

3.5.2. Analysis of VAL/AML/HCZ laboratory prepared mixture

Accurate aliquots of VAL stock solution as well as AML and HCZ working solutions keeping the pharmaceutical ratio of 12.8:1.1:1 were transferred into a series of 4-mL vials, diluted with 10% methanol after addition of 100 μL IS and mixed well. The procedure described under "section 3.5.1" was then applied. The percentage recoveries were calculated using the corresponding regression equations.

3.5.3. Analysis of the studied drugs in their co-formulated tablets

Ten tablets of Exforge HCT® 160-10-12.5 were weighed and then crushed to a fine powder. An accurately weighed amount of the finely powdered tablets, equivalent to 50.00 mg VAL, 4.30 mg AML and 3.91 mg HCZ, was transferred to 25 mL volumetric flask. Twenty milliliters of methanol were added and the solution was sonicated for 30 min. The solution was completed to volume with the same solvent and then filtered through Whatman filter paper and filtered again using 0.2 μm Whatman inorganic membrane filter. For analysis, different aliquots from the prepared sample solution, spiked with 100 μL IS stock solution, were diluted to 4 mL using 10% methanol. The procedure described under "section 3.5.1" was then applied. The percentage recoveries were calculated by referring to the corresponding regression equations.

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