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Simultaneous determination of aliskiren and hydrochlorothiazide in tablets and spiked human urine by ion-pair liquid chromatography

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An alternative method for analysis of aliskiren (ALI) and hydrochlorothiazde (HCT) in combined dosage forms by ion-pair reversed phase high performance liquid chromatography was developed and validated. The pharmaceutical preparations were analyzed using a C18 column (250 mm × 4.6 mm, 3 μm) with a mobile phase consisting of 25% methanol, 50% sodium monobasic phosphate aqueous solution containing 6 mM tetrabutylammonium bromide and 25% water at pH 7.2. Isocratic analysis was performed at a flow rate of 1 mL/min and a column temperature of 30 °C under direct UV detection at 210 nm. Paracetamol was used as internal standard. The validation was performed according to the ICH guidelines. The proposed method was linear over the concentration range of 0.250 to 60 and 0.1 to 10 μg/mL for ALI and HCT, respectively. The limits of detection and quantitation (LOD and LOQ) were 0.075 and 0.198 μg/mL, respectively, for ALI and 0.04 and 0.062 μg/mL, respectively, for HCT. The method proved to be specific, sensitive, precise and accurate with mean recovery values of 101.1 ± 0.32 % and 100.9 ± 0.41 % for ALI and HCT, respectively. The method robustness was evaluated by means of an experimental design. The proposed method was applied successfully to spiked human urine samples with mean recoveries of 98.8 ± 0.36 % and 98.1 ± 0.21 % for ALI and HCT, respectively.

1. Introduction

Hypertension is a major risk factor in the development of cardiovascular disease, heart attack and stroke and one of the most important public health problems worldwide (Kearney et al. 2005). The majority of patients with hypertension require two or more agents from different drug classes to lower blood pressure (Cushman et al. 2002; Mancia et al. 2007). Aliskiren (ALI), is the first in a new class of orally effective direct renin inhibitors for the treatment of hypertension (Fig. 1). Hydrochlorothiazide (HCT), is an orally active thiazide diuretic that proved to be effective for the treatment of hypertension (Fig. 1). A combination of ALI and HCT provide more optimal blood pressure control than the monotherapies (Brown 2008, Baldwin et al. 2009). A number of analytical methods have been reported in the literature for the individual determination of ALI or HCT. These methods include: spectrophotometry (Razak 2004; Wrasse-Sangoi et al. 2010), reversed phase liquid chromatography (LC) (Papadoyannis et al. 1998; Lefevre et al. 2000; Belal et al. 2001; Takubo et al 2004; Tagliari et al. 2008; Dousa et al. 2012), thin-layer chromatography (Stumph et al. 1984) and spectrofluorimetry (Aydoğmuş et al. 2011). ALI is not official in any Pharmacopoeia while HCT is official in International Pharmacopoeia (I.P.), British Pharmacopoeia (B.P) and United States Pharmacopoeia (U.S.P.). Only one HPLC method was reported (Sangoi et al. 2011) for the simultaneous determination of ALI and HCT in tablets using a monolithic column which is more expensive than conventional C18 columns and could not be applied in biological fluids as the unavoidable matrix effects would affect the accuracy of determination due to the reductions in retention times of both drugs. In addition, a micellar electrokinetic chromatography (MEKC) method was recently reported (Sangoi et al. 2011), but this technique is less available in pharmaceutical companies than HPLC-UV and more expensive. This it is preferable that the developed method is HPLC-UV, the most widespread apparatus in pharmaceutical companies and more sensitive than CE. In this study, an ion-pair LC method for this purpose was developed and validated.

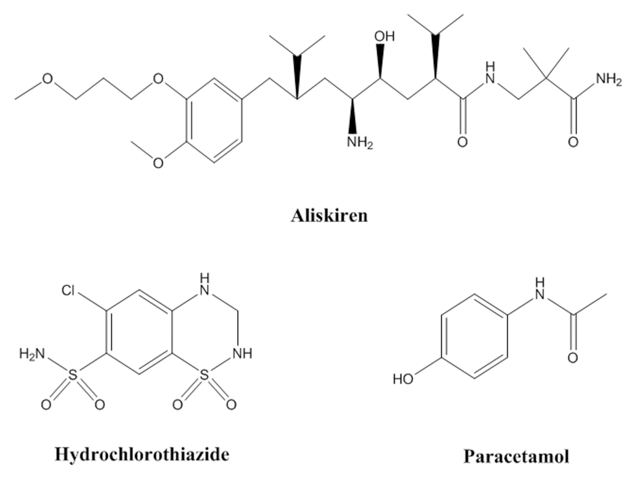

Fig. 1: Chemical structures of aliskiren (ALI), hydrochlorothiazide (HCT) and the internal standard paracetamol (PAR).

Taking into account that ion-pairing chromatography is an established and reliable technique which improves separation providing reduced retention times, highly reproducible results and sharper peak shapes in the separation of charged analytes (Weiss 1995; Snyder et al. 1979, 1997), the objective of this work was to develop such a method for the optimal separation of ALI and HCT. Important factors affecting the selectivity and efficiency of the chromatographic separation were studied, including mobile phase composition and pH, ion pairing reagent concentrations and column temperature. The proposed method allows the quantitative determination of ALI and HCT in pharmaceutical dosage forms as a routine quality control procedure and in spiked human urine samples.

2. Investigations, results and discussion

2.1. Method development and optimization

The experimental results which were tried on a C 18 column (250 mm × 4.6 mm i.d., 3 μm particle size) indicated that, the retention time of ALI was too short to get adequate resolution between ALI and solvent peak, regardless of the composition of the mobile phases. HCT has a retention time of 3.9 min on using the mobile phase composition above except containing the ion-pairing reagent. In order to achieve satisfactory results, ion-pairing reagent was added to the mobile phase.

Three ion pairing reagents, including triethylamine (TEA), tetrabutylammonium bromide (TBAB) and cetyltrimethylammonium bromide (CTAB) were attempted. After several trials, it was found that, TEA could not effectively separate ALI from solvent peak, whereas CTAB resulted in longer analysis time with lowest sensitivity for the two drugs. Tetrabutylammonium bromide (TBAB) was, capable of retaining both drugs within low retention times and more efficiency; it was, therefore, selected for further method optimization.

Trials were performed using BDS HYPERSIL Phenyl column (250 mm × 4.6 mm i.d., 5 μm particle size), Thermo Electron Corporation and Shim-pack CLC- C8 column (250 mm × 4.6 mm i.d., 5 μm particle Size), Shimadzu. The phenyl column resulted in separation of both drugs but with bad peak shape and low resolution between ALI and solvent peak. On the other hand using C8 column resulted in separation of both drugs but with low sensitivity for ALI ($t_R = 4.1$ min) and HCT ($t_R = 5.4$ min). As a consequence, the C18 column was used for further method development and optimization.

The effect of pH of the mobile phase on the retention times and the number of theoretical plates for the tested solutes was investigated using mobile phases of pH values ranging from 3.5–7.5. It was found that, the resolution and efficiency increased steadily over the tested range. At a pH of 3.0–6.0, the resolution was less than 2.0 with less symmetrical peaks. The best resolution and sensitivity was at pH of 6.5–7.5, as both sulfonimide and carboxyimide groups in HCT and ALI are susceptible to be completely ionized permitting their full interaction with tetrabutylammonium cations and enhancing resolution (Thanawiroon et al. 2003). Based on these experiments, pH 7.2 was used to further optimize mobile phase composition.

Besides pH, the salt concentration is another parameter controlling the retention of ALI and HCT. The salt concentration was investigated using mobile phases containing concentrations of 10- 50 mM of phosphate buffer. Buffer of ionic strength less than 10 mM caused overlapping between the two drugs. With increasing the ionic strength, the retention times of ALI and HCT decreased. As a compromise, 25 mM phosphate buffer was chosen as the most suitable one giving good resolution with more symmetrical peaks.

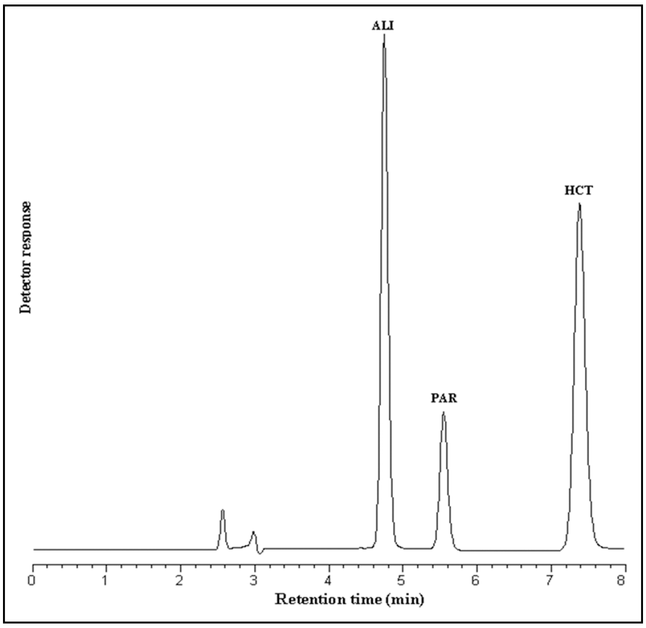

Fig. 2: Typical chromatogram of aliskiren (ALI; 60 μg/mL), paracetamol (PAR; 10 μg/mL) and hydrochlorothiazide (HCT; 10 μg/mL) Chromatographic conditions: Promosil C-18(250 mm × 4.6 mm, 3 μm) column; mobile phase: methanol-50 mM phosphate buffer (containing 6 mM tetrabutyl ammonium bromide IP reagent)- water 25:50:25 (v/v/v), pH 7.2; flow rate 1.0 mL/min; column temperature of 30 °C; detection: 210 nm.

The retention of both drugs increased with increasing TBAB ion-pairing reagent concentration from 0.5 to 5 mM, but no noticeable further increase was observed when the concentration of TBAB was increased to 8 mM. Peak shape was also affected by concentration of ion-pairing reagent, peaks were broad and tailing, especially for ALI in mobile phase containing 8 mM TBAB. The basis for RP-HPIPC separation is still controversial. Two different processes are possible. In process 1, the retention of the studied drugs can be explained by the formation of "ion-pairs" between the positively charged ion-pairing reagent and negatively charged drugs. These ion-pairs mask the charges on them, which in turn facilitates their partitioning on the hydrophobic column. In process 2, the adsorption of the positively charged ion-pairing reagent onto the C18 column, renders it into an ion-exchange column. In this study, 3 mM TBAB was chosen for more symmetrical peaks and more sensitivity.

Two different organic solvents, methanol and acetonitrile were used. It was found that methanol enhanced ion pair solubility with more symmetrical peaks and short analysis time. The effect of methanol content was evaluated at constant ion-pairing reagent concentration (3 mM) and pH (7.2). The retention time of HCT decreased linearly with increasing methanol concentration with less pronounced effect on ALI. When methanol content was increased from 15 to 35%, the retention time of HCT decreased approximately from 17.5 min to 3.5 min, which affect on the resolution of the two drugs. Using 25% of methanol in the mobile phase for this method resulted in excellent resolution within reasonable retention times.

The temperature of the column was varied from 25 °C to 40 °C (with 5 °C interval). Similarly the effect of flow rate was examined at 0.8, 1.0 and 1.2 mL/min. It was found that, a column temperature of 30 °C and a flow rate of 1 mL/min were the optimum conditions for the separation of ALI and HCT.

Based on these experiments, a mobile phase comprising 25% methanol, buffer concentration of 25 mM containing 3 mM TBAB at pH 7.2 with a column temperature of 30 °C was selected as optimum conditions for the separation of ALI and HCT (Fig. 2).

Table 1: Results of linearity, intermediate precision and system suitability determinations

Parameter	ALI	HCT
Concentration range (μg/mL)	0.250–60	0.1–10
Regression equation	Y = 0.029x + 0.008	Y = 0.361x-0.013
Coefficient of determination (R^2)	0.999	0.999
Standard error of estimate ($S_{y/x}$)	0.008	0.043
Intermediate precision (R.S.D., n = 30)	0.5	0.6
Retention time (min)	4.8	7.4
Tailing factor	1.035	1.090
Theoretical plates	10381	12562

Where, y: mean (n = 3) peak area ratio; x: concentration (μg /mL); R.S.D.: relative standard deviation.

2.2. *Method validation*

The proposed method was validated according to the ICH guidelines [ICH 2005] for its specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy, selectivity and robustness. Characteristics parameters of system suitability are given in Table 1.

2.2.1. *Specificity*

The specificity of the developed method was determined by examining the presence of possible interference from excipients or sample matrix. The chromatogram overlay of the spiked mixture of the two drug products and the blank showed that the proposed method is specific for both ALI and HCT as there was no interference observed. Absence of interference was verified for all forms. Two chromatographic columns of the same characteristics but with a different production batch number demonstrated comparable chromatographic profiles.

2.2.2. *Limit of detection (LOD) and limit of quantification (LOQ)*

The LOD and LOQ for ALI and HCT were determined based on signal-to-noise ratio of 3 and 10, respectively [USP 2000]. The baseline noise was measured in a blank experiment in the region of retention time of ALI and HCT using chromatographic software. It was found that for ALI, the LOD and LOQ values were 0.075 μg/mL and 0.198 μg/mL (RSD = 0.6%), respectively and for HCT, the LOD and LOQ values were 0.04 μg/mL and 0.062 μg/mL (RSD = 0.7%), respectively. The sensitivity of the measurement can be easily increased by using a larger sample loop as needed.

2.2.3. *Linearity*

Under the above described experimental conditions, a linear relationship was established by plotting the peak area ratio of each drug to the internal standard against the drug concentration (μg/mL). The points in the residual plots were randomly distributed around the horizontal axis. The random dispersion of the residuals suggests that, the linear model gives a good fit of the data. The coefficients of determination of the regression lines, the standard error of estimate and the linear regression equations are shown in Table 1.

2.2.4. *Precision and accuracy*

The precision of the method was evaluated as repeatability and intermediate precision (Ermer et al. 2005). Repeatability was examined by three fold analyses of two preparations of 150 μg/mL of ALI and 25 μg/mL of HCT in one day. The RSD on the peak areas of these six determinations was not more than 0.4%. Intermediate precision was also determined for five consecutive days. The RSD on the peak areas (n = 30, 5 days) was not more than 1.0% (Table 1) suggesting that the proposed method is suitable for simultaneous analysis of ALI and HCT in combined dosage forms. In addition, the between days (intermediate) precision suggests that the developed method gave repeatable results for five consecutive days. Accuracy of the method was determined as percent recovery of a known added amount of analyte to the sample. The proposed method was found to give a mean recovery of 101.1% for ALI and 100.9% for HCT in the examined dosage form. So, the developed method gave satisfactory recoveries for both ALI and HCT.

2.2.5. *Selectivity*

To investigate the selectivity of the method, solutions of several drugs that are sometimes co-administered with ALI and HCT were prepared. None of the following drugs were found to interfere with analysis: ramipril, lisinopril, metoprolol, valsartan, atenolol, indipamide and lacidipine.

2.2.6. *Robustness*

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in the method parameters and provides an indication of its reliability during normal usage. When the method is robust, these little changes do not have a significant influence on the results (Vander Heyden et al. 2001). To evaluate the robustness of the method, experimental factors that might cause variability in the method responses were examined. Four factors (the amount of methanol (MeOH) in the mobile phase, the pH of the buffer, the temperature and the amount of TBAB in the mobile phase) were investigated.

A two-level full factorial experimental design runs and three replicates of the central point needed 19 runs to complete a whole factorial design. The design was generated by using Design-Expert 8.0 software (Stat-Ease Inc. Minneapolis, MN, USA). For this test, one lower value and one higher value of the factors were used. The different values used for each factor in the design are given in Table 2. The response evaluated were resolution of

Table 2: Chromatographic parameters settings applied in the robustness investigation, corresponding to low (−), central (0) and high (+) levels

Parameter	Low value (−)	Central value (0)	High value (+)
TBAB (mM)	2.5	3	3.5
MeOH (%)	23	25	27
pH	6.9	7.2	7.5
Temperature	28	30	32

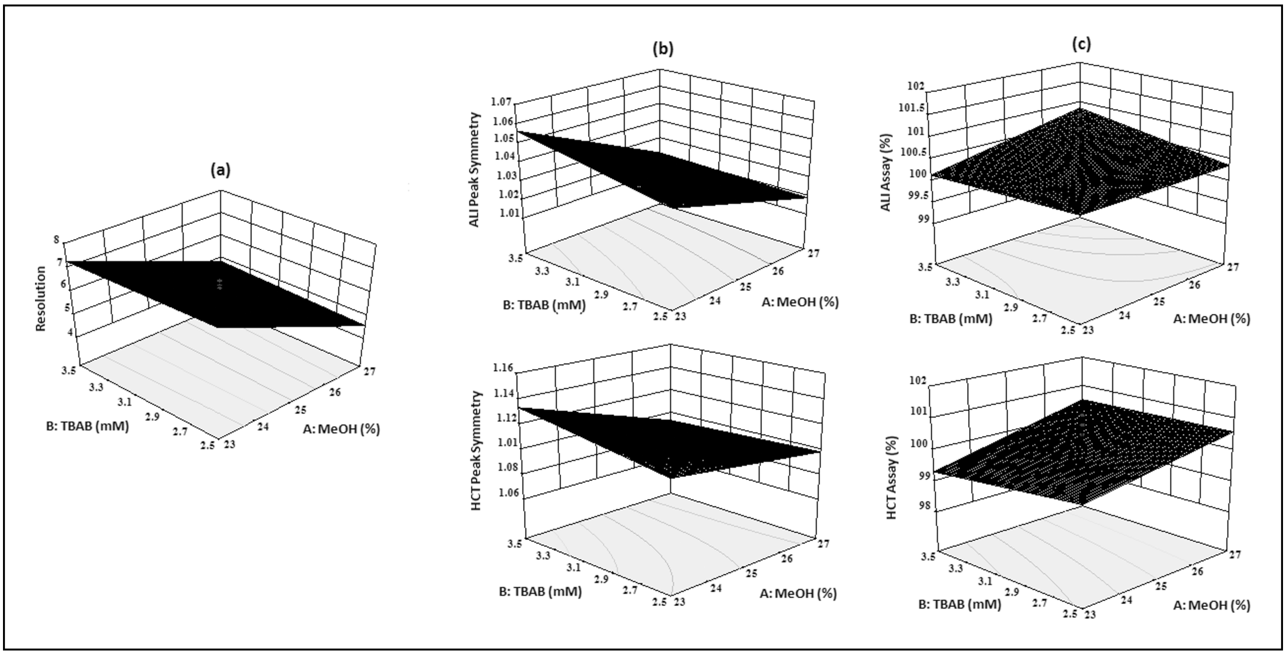

Fig. 3: Three dimentional response surface plots showing the influence of tetrabutylammonium bromide (TBAB) and methanol (MeOH) on **(a)** the resolution of ALI-HCT; **(b, c)** the peak symmetry and the assay (%) of both drugs, respectively. The other parameters are kept at their central points.

ALI-HCT, peak symmetry and the assay (%) of both drugs. The effects of the examined factors were estimated by ANOVA. Figure 3 illustrates some of 3-dimensional plots, which show the variation of the responses as a function of the two most influencing parameters while the other parameters were kept constant at the central value. Methanol and TBAB variations somewhat affect the responses, in the range examined. From the results it was concluded that in all cases, there was no significant factor affecting robustness ($P > 0.05$).

2.3. Method application

2.3.1. Dosage form analysis

The proposed method was applied for the determination of ALI and HCT in two different tablet products (Rasilez-HCT tablets). The results obtained are accurate and precise as indicated by the excellent percentage recovery and S.D. < 2 (Table 3). Common excipients in tablets, such as talc, lactose, starch, polyvinyl pyrrolidone, microcrystalline cellulose, aluminium chloride and magnesium stearate did not interfere with the assay. Statistical analysis of the results obtained by this method and by those given by the reference HPLC method (Sangoi et al. 2011a) was performed using the Student's t-test and the Variance ratio F-test. The calculated values did not exceed the theoretical ones, indicating no significance difference between the compared methods regarding accuracy and precision, respectively (Table 3).

2.3.2. Application to spiked human urine

According to the literature, ALI is rapidly absorbed, resulting in an oral bioavailability of about 3% (Waldmeier et al. 2007; Cromer et al. 2008). ALS excretion is almost completely via the biliary–fecal route (91%), with less than 4.5% excreted in the urine. Unchanged ALI accounts for approximately 80% of the drug in the plasma following oral administration, indicating low exposure to metabolites (Waldmeier et al. 2007). HCT is not metabolized to a relevant degree and the bulk of the dose (95% of dose) is excreted unchanged in urine (Ernst et al. 2007).

The urine calibration curves were linear with the following equations: ALI, $y = 0.293x + 0.004$ ($r^2 = 0.998$); and HCT, $y = 1.954x – 0.025$ ($r^2 = 0.999$). The results of analysis of ALI and HCT in urine samples are provided in Table 4. The accuracy of the proposed method was assessed by investigating the recovery of each of the studied drugs at three concentration levels covering the specified range. The tabulated recoveries indicate good accuracy. Fig. 4 represents a typical chromatogram of the three drugs in spiked human urine sample.

2.4. Conclusion

The proposed method is specific, sensitive, precise, accurate and easy to apply for the simultaneous separation and determination of ALI and HCT in tablets, as apparent from the validation data. It can, therefore, be easily and conveniently adopted for routine quality-control analysis of ALI and HCT in pharmaceutical formulations in which economy and time are essential. Moreover, it can be applied to the determination of both drugs in spiked human urine with good results.

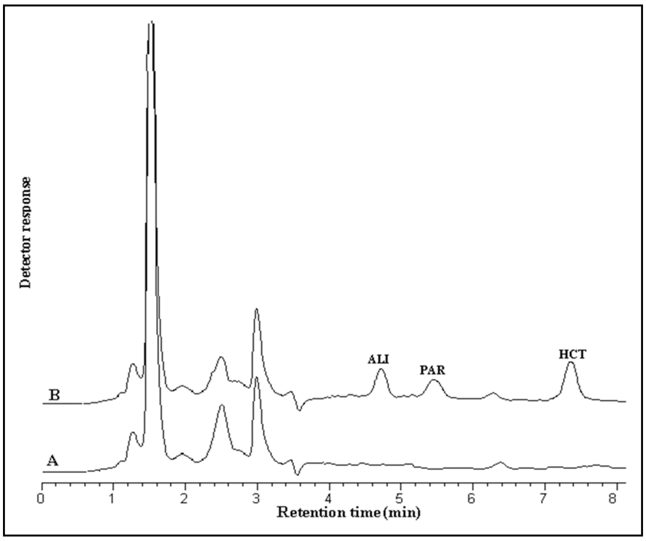

Fig. 4: HPLC chromatograms of (A): urine blank; (B) ALI (1 μg/mL), PAR (3 μg/mL) and HCT (1 μg/mL) in spiked human urine.

Table 3: **Results obtained for ALI and HCT determination in rasilez-HCT tablets**

Labeled amount	Mean found	Recovery* ± S.D. (%)	Reference method
300 mg ALI	303.3	101.11 ± 0.25 (t = 1.09, F = 2.82)	101.42 ± 0.42
150 mg ALI	151.81	101.06 ± 0.36 (t = 1.86, F = 1.34)	100.55 ± 0.31
25 mg HCT	25.2	100.93 ± 0.30 (t = 1.48, F = 1.13)	101.30 ± 0.32

Theoretical values of t and F at P = 0.05 are 2.78 and 19.00, respectively.
* Mean recovery of three separate determinations.

Table 4: **Recovery and precision of ALI and HCT from spiked human urine**

Compound	Concentration added (μg/mL)	Recovery ± S.D. (%) (n = 3)	Intra-day R.S.D. (%) (n = 5)	Inter-day* R.S.D. (%) (n = 5)
Aliskiren	0.5	98.5 ± 0.45	0.43	
	1.0	98.6 ± 0.31	0.40	0.59
	2.0	99.2 ± 0.32	0.35	
Hydrochlorothiazide	0.5	97.9 ± 0.34	0.51	
	1.0	98.1 ± 0.32	0.45	0.61
	2.0	98.3 ± 0.36	0.33	

R.S.D: relative standard deviation.
*Three successive days.

3. Experimental

3.1. Chemicals and reagents

Aliskiren hemifumarate reference substance was kindly provided by Novartis (Basel, Switzerland). Hydrochlorothiazide reference substance was obtained from Chemipharm Pharmaceutical Industries (6th October City, Egypt). Paracetamol (PAR) as internal standard was obtained from Sigma Chemicals. Two tablet formulations containing 300 mg of ALI and 25 mg of HCT/tablet (Rasilez HCT 300/25) or 150 mg of ALI and 25 mg of HCT/tablet (Rasilez HCT 150/25) were obtained from commercial sources. Tetrabutylammonium bromide, methanol and acetonitrile (HPLC grade) were purchased from Sigma-Aldrich (Germany). Orthophosphoric acid (85% w/v) was obtained from Riedel-deHaën (Sleeze, Germany). Sodium hydroxide and sodium dihydrogen phosphate were obtained from Adwic Co. (Cairo, Egypt). Through the whole work, ultrapure deionized (DI) water was used from an Easypure RODI water system (Barnstead Int.).

3.2. Apparatus

LC analyses were performed on a Shimadzu (Japan) HPLC system consisting of a CMB-20 Alite system controller, a pump (LC-20 AD), a column oven (CTO20AC) and UV/VIS detector (SPD-20A). This equipment has a degasser system (DGU 20 A). For data processing and acquisition, LC solution software version 1.3 from Shimadzu was used. An ultrasonicator from Merck L-7612 and a pH Meter from Hanna (USA) were used.

3.3. Chromatographic conditions

Chromatographic separations were achieved on a Promosil C-18 (250 mm × 4.6 mm, 3 μm) column (Agela Technologies, USA) thermostatted at 30 °C. A security guard holder (23.9 mm × 0.5 mm i.d.) was used to protect the analytical column. The mobile phase consisted of methanol: phosphate buffer pH 7.2 : water (25:50:25 v/v/v). The phosphate buffer of pH 7.2 was prepared by dissolving 6.89 g of sodium dihydrogen phosphate monohydrate and 1.93 gm of tetrabutylammonium bromide in 800 mL of purified water, and adjusting the pH to 7.2 with 2 M NaOH before bringing up to 1000 ml with purified water. Thus buffer solution contains 50 mM sodium dihydrogen phosphate monohydrate and 6 mM tertrabutylammonium bromide at pH 7.2. The mobile phase was prepared daily and was filtered (Millipore, 0.45 μm) under vacuum. The injection volume was set at 20 μL and the UV detection was made at 210 nm. The total run time was 8 min.

3.4. Determination of ALI and HCT

3.4.1. Preparation of standard solutions

A stock solution of ALI was prepared by dissolving 30.85 mg of aliskiren hemifumarate (equivalent to 25 mg of ALI base) in 25.0 mL of methanol. A similar stock reference solutions of HCT and PAR (25 mg/25 mL) were made. These stock solutions were further diluted with the same solvent and then with the mobile phase as appropriate to obtain the working standard solutions. The peak area ratio was plotted versus the concentration of the drugs (μg/mL) to get the calibration graph. Alternatively the corresponding regression equations were derived. The stock solutions were found to be stable at least for two weeks when kept in the refrigerator.

3.4.2. Preparation of tablet solutions

Ten tablets from each formulation were weighed and pulverized well. Appropriate amounts were transferred into individual 100 mL volumetric flasks, sonicated with 80 mL methanol for 10 min and then the solution was completed to volume with the same solvent. This solution was filtered through a 0.2 μm nylon filter (Whatman, Dassel, Germany) and aliquot of this solution was diluted with the mobile phase as appropriate. The nominal contents of the tablets were calculated using either the calibration graph or the corresponding regression equation.

3.4.3. Preparation of spiked human urine

Urine samples (0.5 mL) were diluted with 250 μL water and spiked with different concentration of ALI and HCT standard solutions to give a final concentration range of 0.2–3 μg/mL at constant concentration of PAR (3 μg/mL). After vortex mixing for 30 s, 1.5 mL of acetonitrile was added and the mixture was then centrifuged for 15 min at 4000 rpm. The resulting supernatant filtered through a 0.45 μm syringe filter, then evaporated to dryness under nitrogen at 40 °C. The residual mass was reconstituted with 0.25 mL of mobile phase, vortex-mixed for 30 s, transferred to 1.5 mL Eppendorf tubes and centrifuged at 4000 rpm for 5 min to precipitate any particulate matter. A blank experiment was carried out simultaneously. The peak area ratio was plotted *versus* the concentration of the drugs (μg/mL) to get the calibration graph. Alternatively, the regression equations were derived.

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