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Simultaneous determination of atenolol and amiloride in pharmaceutical preparations by capillary zone electrophoresis with capacitively coupled contactless conductivity detection

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ABSTRACT: Capillary zone electrophoresis coupled with a capacitively coupled contactless conductivity detector (CE-C^4D) has been employed for the determination of atenolol and amiloride in pharmaceutical formulations. Acetic acid (150 mM) was used as background electrolyte. The influence of several factors (detector excitation voltage and frequency, buffer concentration, applied voltage, capillary temperature and injection time) was studied. Non-UV-absorbing L-valine was used as internal standard; the analytes were all separated in less than 7 min. The separation was carried out in normal polarity mode at 28°C, 25 kV and using hydrodynamic injection (25 s). The separation was effected in an uncoated fused-silica capillary (75 μm, i.d. × 52 cm). The CE-C^4D method was validated with respect to linearity, limit of detection and quantification, accuracy, precision and selectivity. Calibration curves were linear over the range 5–250 μg/mL for the studied analytes. The relative standard deviations of intra- and inter-day migration times and corrected peak areas were less than 6.0%. The method showed good precision and accuracy and was successfully applied to the simultaneous determination of atenolol and amiloride in different pharmaceutical tablet formulations. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: capillary zone electrophoresis; contactless conductivity detector (CE-C^4D); atenolol; amiloride; drug analysis

Introduction

Hypertension is a disease that affects millions of people all over the world, causing devastating action over time. High blood pressure causes serious consequences such as cardiovascular accidents and kidney disease. The treatment to avoid hypertension targets a healthy lifestyle, even though 60% of people with hypertension need medical help.

A more effective treatment of hypertension is through the association of antihypertensive with diuretic drugs. The first is a pressure regulator, while the second keeps liquid retention under control. By combining these two pharmaceuticals with different action mechanisms, it is possible to use low doses and to minimize their side effects (Balesteros *et al*., 2007). Chlorthalidone (CD) is a diuretic with actions and indications similar to those of the thiazide diuretics. It is prescribed with atenolol (AT), which is a cardioselective β-blocker and amiloride hydrochloride (AM), which is a potassium-sparing diuretic.

Liquid chromatography with mass spectrometry (LC–MS) methods for the determination of β-blocker in biological specimens have been reported (Moraes *et al*., 2008; Johnson and Lewis 2006; Khuroo *et al*., 2008). Capillary zone electrophoresis (CZE) methods for the determination of AT in human urine and plasma (Maguregui *et al*., 1997; Arias *et al*., 2001) were developed. A CZE method for the determination of AT, and AM, hydrochlorthiazide, and bendroflumethiazide in pharmaceutical formulations and urine was reported (Maguregui *et al*., 1998). The simultaneous determination of AT and CD in formulations using CZE method was also reported (Al Azzam *et al*., 2009). Balesteros *et al*. (2007) developed a CZE method for the determination of CD and losartan in capsules. Reports for determining CD by gas chromatography (GC) (Magnar and Klas, 1974; Degen and Schweizer, 1977) or liquid chromatography (LC) for the simultaneous determination of both AT and CD (Dadgar and Kelly, 1988; Muirhead and Christie, 1987; Giachetti *et al*., 1997; Rapado-Martinez *et al*., 1997; El-Gindy *et al*., 2008; Sa'sa *et al*., 1988) can also be found, or for the simultaneous determination of β-blocker drugs (AT, pindolol, metoprolol and labetolol) using HPLC coupled with a capacitively coupled contactless conductivity detector (HPLC-C^4D) (Kubáň *et al*., 2006). A high-performance thin-layer chromatographic

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Abbreviations used: AM, amiloride hydrochloride; AT, atenolol; CT, chlorthalidone.

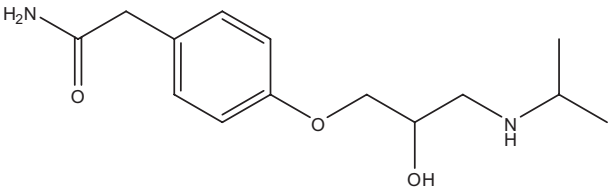
method for the determination of certain antihypertensive mixtures (Salem, 2004) was also conducted.

UV spectrophotometric methods for the determination of AT and metoprolol in pharmaceutical formulations (Bonazzi *et al*., 1996), and for simultaneous determination of AT and CD in pharmaceutical formulations (Wehner, 2000; Vetuschi and Ragno, 1990) have also been reported. In some of these works, partial least squares analysis of the UV spectral data for the determination of AT and CD in synthetic binary mixtures and pharmaceutical formulations (Ferraro *et al*., 2003; Mohamed and Salem 2005) or for the simultaneous determination of AT, AM and CD in pharmaceutical formulations (El-Gindy *et al*., 2005) or for determination of AM, AT, hydrochlorothiazide and timolol maleate in synthetic mixtures and pharmaceutical formulations (Ferraro *et al*., 2004) were conducted.

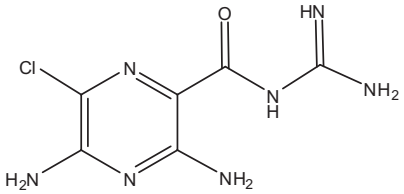
Capacitively coupled contactless conductivity detection (C^4D) is a conductometric measuring approach that is based on the conductivity differences between the sample zones and the background electrolyte (BGE). The technique is based on the application of an alternate voltage over a couple of cylindrical electrodes positioned outside the capillary and measure the current that flows through them is measured. This current is a function of the solution conductivity at the gap between the electrodes (Felix *et al*., 2006). Sensitive detection can be achieved using BGE with low conductivity (Yang and Qin, 2009). C^4D overcomes the disadvantage of short path length that is encountered by on-column optical detections.

Furthermore, it avoids the direct contact of working electrodes with BGE in the conventional conductivity detection (Šolínová and Kašička, 2006) and eliminates the laborious work of fabricating detection cells in potential gradient detection (Fan *et al*., 2007). It prevents the potential damage of the conductmetric detection cell, especially in cases where organic solvents are employed as additives. Additionally, the detection sensitivity of C^4D could be tuned by optimizing the oscillation voltage (Tanyanyiwa and Hauser, 2002). CE-C^4D has been applied in the determination of small inorganic and organic ions, peptides, proteins, oligo- and polynucleotides and nucleic acid fragments (Kubáň and Hauser, 2008, 2009). For reviews on capacitvely coupled contracless conductivity detection and their application in pharmaceutical and environmental analyses, see Quek *et al*. (2008) and Henchoz *et al*. (2007).

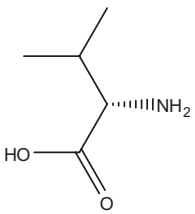
In this paper the determination of AT and AM in pharmaceutical tablet formulations using CZE-C^4D is presented. The method was further validated as per ICH-Q2A guidelines (International Conference on Harmonization, 1995), and was applied to the quality control of co-formulated preparations and AT in single active ingredient preparations. L-Valine (Fig. 1) was used as internal standard (IS).



Atenolol (pK_a 9.6)



Amiloride (pK_a 8.7)



L-Valine

Figure 1. The chemical structures of atenolol, amiloride and L-valine.

Experimental

Chemicals and Reagents

Analytical-grade methanol was purchased from Merck (Darmstadt, Germany). Deionized water was purified by a Milli-Q system (Millipore, Bedford, USA), and was used throughout for the preparation of solutions. Acetic acid, sodium hydroxide, L-valine and AM were purchased from Sigma-Aldrich (St Louis, MO, USA). Commercial tablet pharmaceutical preparations (claimed to contain 100, 25 and 5 mg of active ingredients—AT, CD and AM, respectively) were obtained from Alpha Chem Advanced Pharmaceutical Industries, Egypt, where the combination of the three drugs was available. However only tablet formulations with single or combinations of two active drugs are available in Malaysia and were obtained from local drug stores. Hypoten tablets (100 mg AT), AT and CD standards were kindly donated by HIKMA Pharmaceutical Company, Jordan.

CE Apparatus and Operating Conditions

Separations were conducted on a HP^{3D}CE CZE system (Agilent Technologies, Waldbronn, Germany). The CE unit was coupled to eDAQ detector (eDAQ, Denistone East, Australia). Data acquisition was performed with ChemStation Software (Agilent Technologies, Waldbronn, Germany) to control the CE and data handling, and PowerChrom software (eDAQ, Denistone East, Australia) was used for conductivity detector control and data acquisition. An uncoated fused-silica capillary 75 µm i.d × 52 cm, from Agilent Technologies was used. The new capillary was conditioned by flushing for 30 min with 1 M NaOH, 10 min with 0.1 M NaOH and 15 min with water. Between injections, it was preconditioned for 3 min with water, 3 min with 0.1 M NaOH and 3 min with the BGE prior to each subsequent run in order to maintain the reproducibility of the analysis. Samples and standards were injected hydrodynamically at 50 mbar for 25 s and the CE was operated under the following conditions: voltage, 25 kV (normal polarity); capillary temperature, 28°C; and BGE, 150 mM acetic acid. The final configuration of the C^4D detector was set at a fixed amplitude of 100 V, while the frequency was optimized from 50 to 500 kHz. At the end of the day, a final 5 min washing with water was performed. All standards, sample solutions, the running buffer and NaOH solution were filtered through 0.2 µm regenerated cellulose membrane filter using Agilent solvent filtration kit.

Preparation of Standard Solutions

Stock solutions of AT and AM (500 μg/mL each) were prepared by adding 2 mL methanol, then topped up with water to the desired concentrations. L-Valine stock solution (1000 μg/mL) was prepared in water and used throughout as internal standard. The stock solutions were used to prepare calibration standards. Working solutions for AT and AM were prepared by serially diluting the stock solution with water after spiking with the L-valine internal standard to a final concentration of (200 μg/mL). All solutions were stored refrigerated in the dark when not in use.

Preparation of Sample Solutions

Ten tablets from each sample were ground into fine powder in a mortar. The powder (equivalent to about 7.5 mg AT) were quantitatively transferred into 50 mL volumetric flasks and dissolved with 2 mL of methanol, sonicated for 5 min then another 20 mL water was added, sonicated for another 5 min, spiked with L-valine internal standard (final concentration 200 μg/mL) and finally topped up to the mark with water. The solution was filtered through a 0.2 μm regenerated cellulose membrane filter before being subjected to the CZE analysis.

Results and Discussion

For C^4D in CZE it is necessary to use a BGE solution of low conductivity in order to obtain good sensitivity and to minimize the electrophoretic current. The electrophoresis current influences the detection stability due to the effect of Joule heating (Pormsila *et al*., 2009).

BGE Selection

The selection of the BGE was based on conductivity detection of the β-blocker drugs selected for the study. In C^4D, the response arises from the difference in conductivity between the analytes and BGE co-ions (Nussbaumer *et al*., 2009). For obtaining the highest signal-to-noise ratio, the largest possible difference of the conductance of the analytes and electrolyte is required. The detection of AT and AM was performed in a BGE of 150 mM acetic acid. This simple acidic buffer solution was chosen as it yields high sensitivity for the studied drugs, which are characterized by dissociation constants, pK_a, of about 9. The BGE used had to have a pH value of <3 to render a significant fraction of the drugs used in this study in cationic form, which is a prerequisite for their electrophoretic separation and detection by conductivity measurement. Therefore, 150 mM acetic acid was chosen as BGE for the next measurements.

Under these conditions, CD was not detected since it is an amide and does not form ions in the presence of acidic pH. In contrast to amines, amides are completely nonbasic. Amides do not form ions when treated with aqueous acids, and they are poor nucleophiles. There are two reasons for the difference in basicity between amines and amides. First, the ground state of an amide is stabilized by delocalization of the nitrogen lone-pair electrons through orbital overlap with the carbonyl group. Second, a protonated amide is higher in energy than a protonated amine because the electron-withdrawing carbonyl group inductively destabilizes the neighboring positive charge. Both factors increased stability of amide vs an amine and decreased stability of a protonated amide vs a protonated amine led to substational difference in $\Delta G°$ (McMurry, 1996).

Influence of the Buffer Concentration

The effect of buffer concentration on the sensitivity was investigated by varying the concentration of acetic acid from 50 to 500 mM. Better sensitivity could be achieved at a BGE concentration of 150 mM. Therefore, 150 mM acetic acid was selected and, under these conditions, the generated current was still acceptable (~ 25 μA).

Influence of Detector Excitation Voltage and Frequency

C^4D detector frequencies from 50 to 550 kHz were tested at a fixed excitation voltage of 100 V in order to find the maximal sensitivity. The highest peak intensity was obtained at 350 kHz (figure not shown). The decreasing signal beyond 350 kHz may be due to the higher stray capacitance between the electrodes at increasing excitation frequency (Yang and Qin 2009); therefore, an excitation signal of 100 V × 350 kHz was chosen for further studies.

The influence of voltage (10–30 kV) on the migration time was evaluated under the optimized BGE conditions. When the applied voltage was increased, peak broadening and decrease in both migration time and resolution were observed. This is probably due to the fact that, as the applied voltage increases, the increasing Joule heat leads to a reduction in migration time due to the increase in the apparent mobility with increasing voltage. Therefore, 25 kV was chosen.

The influence of capillary temperature (19–30°C) was evaluated under the chosen BGE conditions. When the temperature is increased, migration time decreases. It is imperative to control Joule heating since this parameter is directly linked to the analyte mobility and stability, as well as system reproducibility. Decreasing viscosity with temperature is responsible for the nonlinearity of the dependence of velocity on temperature, while increase in the diffusion coefficient of analyte is responsible for the poorer than expected performance at high temperatures. An increase in temperature may also cause an increase in the number of ions in solution due to dissociation of molecules, thus increasing its conductivity (http://www.camlab.co.uk//sitefiles/Tech_papers/TempCondMeas.pdf). Therefore, 28°C was chosen as the working temperature for the analysis.

Optimization of sample injection time (5–20 s) at 50 mbar was conducted to achieve a lower detection limit without affecting the quality of the peak shape and reproducibility, migration time, and resolution. An injection time of 25 s offered best results and was selected for the rest of the studies.

From the above experiments, the adopted conditions for AT and AM were: 150 mM acetic acid as BGE; injection time, 25 s; applied voltage, 25 kV; capillary temperature, 28°C; and detector excitation signal 100 V × 350 kHz. A typical eletrophoregram obtained under the adopted conditions is shown in Fig. 2. The suitability of L-valine as internal standard is obvious as it is well resolved from the analyte peaks. All components including the internal standard migrated in less than 7 min.

Validation Procedure

Calibration curves, limits of detection and quantitation. Working solutions containing standard compounds were all prepared as described to construct the calibration curves. Each calibration curve contained seven different concentrations (5–250 μg/mL) and was performed in triplicate. Calibration

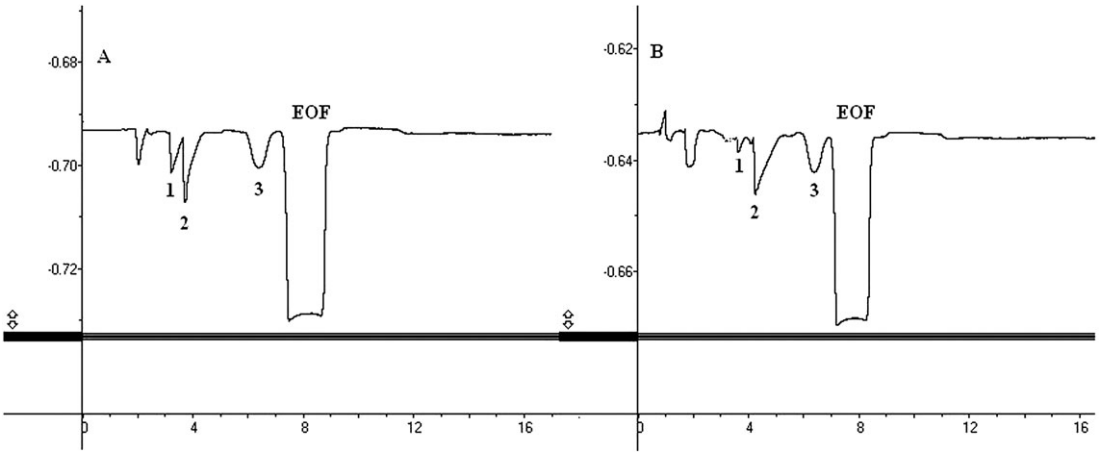


Figure 2. Typical electropherograms obtained when operated under the adopted conditions. (A) 150 μg/mL standard, (B) Teklo tablet. 1, Amiloride; 2, atenolol; and 3, internal standard (L-valine). Conditions: 150 mM acetic acid; voltage, 25 kV; temperature, 28°C; injection time, 25 s; and detector excitation signal, 100 V × 350 kHz.

curves with regression equations for AT and AM were $y = 0.00761x + 0.04274$ and $y = 0.00480x - 0.00195$, respectively, which were obtained by plotting the corrected peak area (y) as a function of analyte concentration (x) in μg/mL. The limits of detection (LOD) for AT and AM were 1.14 and 2.38 μg/mL, respectively, while the limits of quantitation (LOQ) were 3.45 and 7.24 μg/mL, respectively. LOD was calculated as the amount of the injected sample to yield a signal-to-noise ratio of 3, and the LOQ was taken as the amount of the injected sample to give a signal-to-noise ratio of 10. All the standards showed good linearity ($r^2 > 0.999$) over a relatively wide concentration range (5–250 μg/mL). As expected, the sensitivity of the proposed CE method is slightly inferior compared with the reported HPLC-UV method (El-Gindy *et al*., 2005; LODs for AT, AM and CD were 0.003, 0.004 and 0.002 μg/mL, respectively) or to the HPLC-C^4D (Kubáň *et al*., 2006; LOD for AT was 0.5 μM). However the analysis time of the proposed CE is slightly faster (<7 min compared with ~8 min in the HPLC report). Although the sensitivity is inferior compared with the reported methods, nevertheless it posses adequate sensitivity for the analysis of these active ingredients in pharmaceutical formulations.

Precision. Intra- and inter-day variations were used to determine the precision of the developed method by analyzing three concentrations (5, 50 and 250 μg/mL) of standard solutions. The intra-day variation was determined by analyzing the nine replicates on the same day while inter-day variation was conducted over six consecutive days. Intra-day precision for AM and AT migration times, expressed as the percent relative standard deviation, RSD, were 0.52–2.10 and 1.81–2.14%, respectively, while for the corrected peak areas ranged from 2.72–5.60 and 3.24–4.38%, respectively, (Table 1). Inter-day precision for AM and AT migration times was 1.88–4.09 and 1.65–2.85%, respectively, while for the corrected peak areas it was 4.32–5.77 and 5.40–5.78%, respectively, (Table 1), indicating the good precision of the newly developed method.

Accuracy. The accuracy of the method was determined by performing recovery tests. An appropriate amount of Teklo tablet powder was weighed and spiked with known amount of the standard compounds and each sample was analyzed in triplicate.

Table 1. Intra and inter-day precision for the determination of atenolol (AT) and amiloride (AM)

Analyte (μg/mL)	RSD (%)			
	Migration time		Corrected peak areas	
	AT	AM	AT	AM
Intra-day precision (n = 9)				
5	1.81	0.52	4.37	3.97
50	1.91	1.94	4.38	2.72
250	2.14	2.10	3.24	5.60
Inter-day precision (n = 54)				
5	1.65	1.88	5.78	5.71
50	2.85	3.07	5.40	4.77
250	2.36	4.09	5.61	4.32

n = number of determinations (three preparations for each concentration).

Table 2. Accuracy results for the determination atenolol (AT) and amiloride (AM) spiked to tablet

Amount (μg/mL)	(%) Recovery ± SD	
	AT	AM
10	96.41 ± 3.85	98.73 ± 2.73
50	95.97 ± 1.36	97.15 ± 3.41
150	99.64 ± 2.32	94.96 ± 2.21

Accuracy values ranged from 96.41 to 99.64% (for AT) and from 94.96 to 98.73% (for AM) (Table 2). The good accuracy values obtained indicate the potential of this method for the determination of both analytes in pharmaceutical formulations.

Analysis of Pharmaceutical Formulations

The developed method has been successfully applied for the simultaneous determination of AT and AM in different tablet formulations. Good agreement between the proposed method and

Table 3. Assay results of atenolol and amiloride in different pharmaceutical formulations

Trade name	Manufacturer	Generic name	Label claim (AT[b]/CD[c]/AM[d]) mg	Agreement (%) (%) ± SD	CD (%)	AM (%) ± SD
Teklo	Acapi	AT + CD + AM	100/25/5	95.21 ± 0.48	N/D[a]	99.01 ± 2.05
Tenedone	Sigma	AT + CD	50/25	97.70 ± 2.58	N/D	N/D
Tenoret 50	Astrazeneca	AT + CD	50/12.5	96.52 ± 0.46	N/D	N/D
Tenoretic	Astrazeneca	AT + CD	100/25	97.54 ± 1.24	N/D	N/D
Noten	α-Alphapharm	AT	50	97.21 ± 2.07	N/D	N/D
Apo-atenol	Apotex	AT	50	94.54 ± 0.36	N/D	N/D
Ternolol	Hovid	AT	50	95.83 ± 0.66	N/D	N/D
Ternolol	Hovid	AT	100	96.6 ± 1.22	N/D	N/D
Normaten	Mims	AT	100	95.6 ± 1.07	N/D	N/D
Hypoten	Hikma	AT	100	97.9 ± 1.49	N/D	N/D

[a] N/D, not detected. [b] AT, atenolol. [c] CD, chlorthalidone. [d] AM, amiloride.

the manufacturer's claimed values was found for all samples (Table 3). Figure 2(B) shows a typical electropherogram of the pharmaceutical formulations.

Conclusions

The suitability of CE-C^{4}D as an alternative method for the determination of AT and AM in pharmaceutical formulations has been demonstrated. Under the adopted conditions, baseline separation of AT and AM and the internal standard were obtained in less than 7 min. Good analytical performance with regards to linearity, reproducibility, and accuracy was achieved. All the validated data obtained are in compliance with the ICH-Q2A guidelines (International Conference on Harmonization, 1995). When compared with the HPLC-UV method (El-Gindy *et al*., 2005), the proposed method exhibits less sensitivity, but nevertheless provides slightly faster analysis time (<7 min compared with ~8 min by HPLC method). The inherent advantages of the C^{4}D detector over the UV detector (e.g. low cost, simplicity and greater robustness) have been highlighted (Pormsila *et al*., 2009). Furthermore, another advantage of this detector is that it can be placed any where along the length of the capillary since it is not necessary to remove the polyimide coated in order to perform the detection (Chen *et al*., 2009).

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