Research Article

Biomedical Chromatography

Received 15 October 2009, Accepted 8 December 2009 Published online in Wiley InterScience: 12 January 2010

(www.interscience.wiley.com) DOI 10.1002/bmc.1395

Simultaneous determination of atenolol, chlorthalidone and amiloride in pharmaceutical preparations by capillary zone electrophoresis with ultraviolet detection

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ABSTRACT: Capillary zone electrophoresis methods for the simultaneous determination of the β-blocker drugs, atenolol, chlorthalidone and amiloride, in pharmaceutical formulations have been developed. The influences of several factors (buffer pH, concentration, applied voltage, capillary temperature and injection time) were studied. Using phenobarbital as internal standard, the analytes were all separated in less than 4 min. The separation was carried out in normal polarity mode at 25°C, 25 kV and using hydrodynamic injection (10 s). The separation was effected in an uncoated fused-silica capillary (75 μm i.d. × 52 cm) and a background electrolyte of 25 mm H_3PO_4 adjusted with 1 m NaOH solution (pH 9.0) and detection at 198 nm. The method was validated with respect to linearity, limit of detection and quantification, accuracy, precision and selectivity. Calibration curves were linear over the range 1–250 μg/mL for atenolol and chlorthalidone and from 2.5–250 μg/mL for amiloride. 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, chlorthalidone and amiloride in various pharmaceutical tablets formulations. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: capillary zone electrophoresis; atenolol; chlorthalidone; amiloride; drug analysis.

Introduction

Hypertension is one of the most serious health problems faced by modern society; the disease is capable of silently and progressively affecting different organs until damage is evident and irreversible, causing diminished quality of life. Hypertension therapy can be carried out using a single drug and, in those cases which are not suited to single drug therapy, the addition of a second drug is recommended. These therapies demand strict quality control measures, necessitating techniques for the determination of each of the active ingredients without interference of the others (Ferraro *et al*., 2003).

Chlorthalidone (CD) is a sulfonamide diuretic that has a very long duration of action. It is often used to treat many hypertension cases. Atenolol (AT) is a β-adrenergic receptor-blocking agent usually used for management of hypertension. Amiloride hydrochloride (AM) is a potassium-conserving drug with weak diuretic hypertensive activity. They are useful alone in the management of mild hypertension by reducing the blood volume or in combination with other classes of hypertensive agent for more severe cases. Mixtures of CD with AT and AM are examples of such combinations which are still in common use (Mohamed and Salem, 2005).

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) has been reported. Liquid chromatography mass spectrometric (LC-MS) methods for the determination of several β-blockers in biological specimens have been dicussed (Moraes *et al*., 2008; Johnson and Lewis, 2006; Khuroo *et al*., 2006). Capillary zone electrophoresis (CZE) methods for the determination of AT in human urine and plasma (Maguregui *et al*., 1997; Arias *et al*., 2001) or for the determination of AT, AM, hydrochlorthiazide and bendroflumethiazide in pharmaceutical formulations and urine have also been reported (Maguregui *et al*., 1998). Moreover, a CZE method for the simultaneous determination of AT and CD in formulations was recently 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) can also be found. A high-performance thin-layer chromatographic method for the determination of certain antihypertensive mixtures (Salem 2004) was also reported.

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

UV spectrophotometric methods for the determination of AT and metoprolol in pharmaceutical formulations (Bonazzi *et al*., 1996), and for the 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) was conducted.

To the best of our knowledge, a CZE method for the simultaneous determination of AT, CD and AM in co-formulated preparations has not been reported. This communication describes, for the first time, a simple, sensitive CZE method for the simultaneous determination of the three active ingredients, namely AT, AM and CD. The method has been further validated as per ICH-Q2A guidelines (International Conference on Harmonization, 1995), and applied to the determination of the active ingredients in pharmaceutical tablet formulations. Phenobarbital (Fig. 1) was used as internal standard (IS).

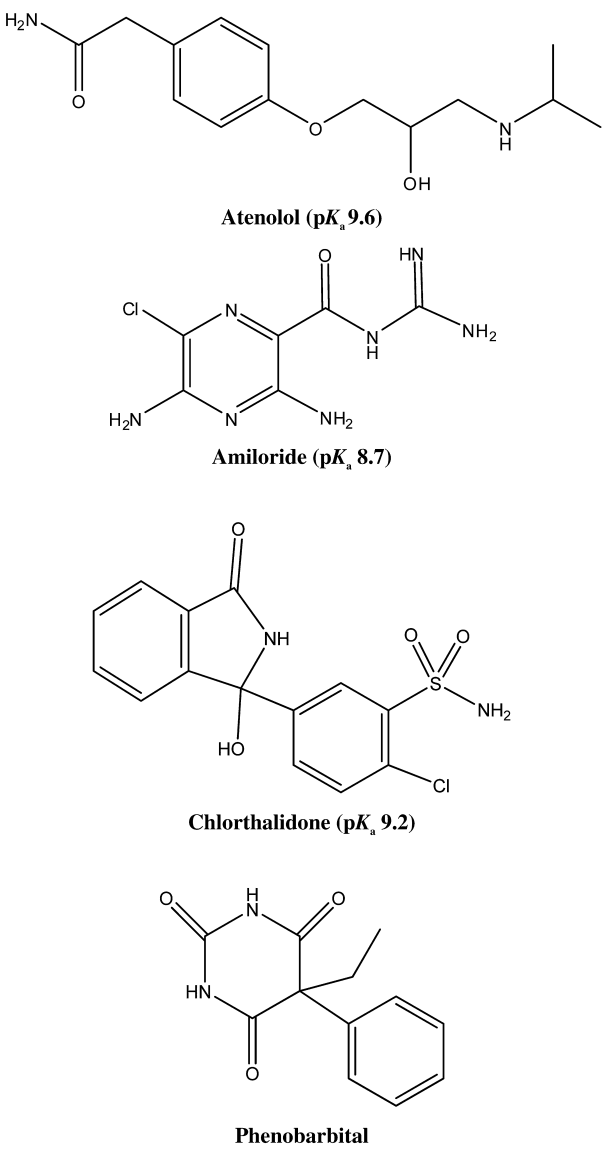

Figure 1. The chemical structures of atenolol, amiloride, chlorthalidone and phenobarbital.

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. *Ortho*-phosphoric acid (85%), sodium hydroxide and AM were purchased from Sigma-Aldrich (St Louis, MO, USA). Commercial tablet pharmaceutical preparations (claimed to contain 100, 25 and 5 mg active ingredients; AT, CD and AM, respectively) were obtained from Alpha Chem Advanced Pharmaceutical Industries, Egypt. 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, CD and phenobarbital standards were kindly donated by Hikma Pharmaceutical Company, Jordan.

CZE Apparatus and Operating Conditions

Separations were conducted on an HP^{3D}CE CZE system (Agilent Technologies, Waldbronn, Germany). The unit was equipped with photodiode array detector. Uncoated fused-silica capillary 75 μm i.d × 52 cm, (detection length, 8.5 cm from the outlet end of the capillary) from Agilent Technologies was used. Data acquisition was performed with ChemStation Software. 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 0.1 m NaOH and 4 min with the background electrolyte (BGE) prior to each subsequent run. Samples and standards were injected hydrodynamically at 50 mbar for 10 s and the CE was operated under the following conditions: voltage, 25 kV (normal polarity); capillary temperature, 25°C; detector wavelength, 198 nm; BGE, 25 mm H_3PO_4 adjusted with 1 m NaOH solution to pH 9.0. At the end of the day, a final 5 min washing with water was performed. All standards, sample solutions and the BGE and NaOH solutions were filtered through 0.2 μm regenerated cellulose membrane filter using an Agilent solvent filtration kit.

Preparation of Standard Solutions

Stock solutions of AT, CD, AM and phenobarbital (500 μg/mL each) were prepared by adding 2 mL methanol, then topped up with water to the desired concentrations. The stock solutions were used to prepare calibration standards. Working solutions for AT, CD and AM were prepared by serially diluting the stock solution with water after spiking with the internal standard (phenobarbital) to a final concentration of 20 μg/mL. All solutions were stored refrigerated in the dark when not in use.

Preparation of Sample

Ten tablets from each sample were ground into fine powder in a mortar. The powder (equivalent to about 7.5 mg AT) was 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 phenobarbital internal standard (final concentration 20 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.

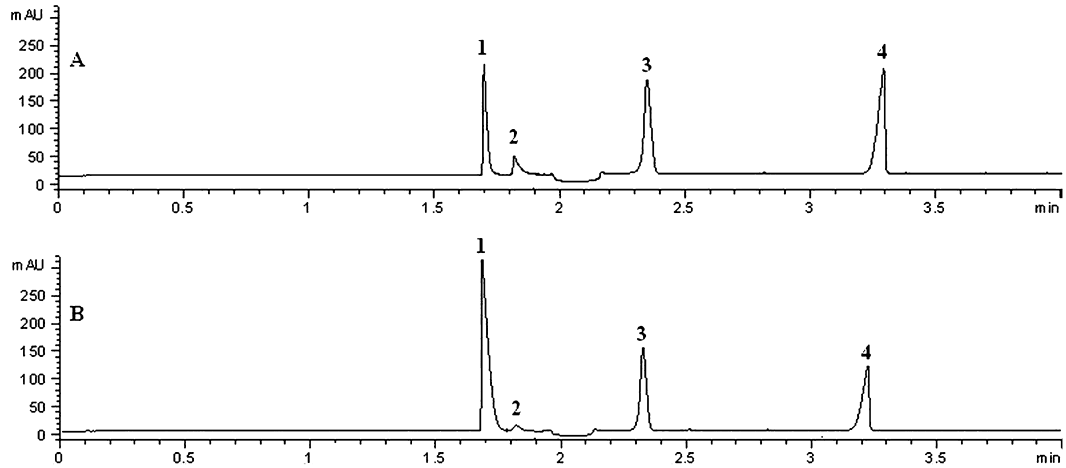

Figure 2. Typical electropherograms obtained when operated under the adopted conditions. (A) 100 µg/mL standard, (B) Teklo tablet. **1**, Atenolol; **2**, amiloride; **3**, chlorthalidone; and 4, internal standard (phenobarbital). Conditions: 25 mm H_3PO_4 adjusted with 1 m NaOH, pH 9.0; voltage, 25 kV; temperature, 25°C; and injection time, 10 s.

Results and Discussion

Optimization of Electrophoretic Conditions

pH is an important parameter to optimize as it has a direct effect on the ionization of the silanol group of the capillary wall, which in turn affects the magnitude of the electroosmatic flow (EOF). Also it determines the extent of the ionization of the analyte. Under basic pH conditions, AT ($pK_a = 9.6$) exists predominantly in the negatively charged form and therefore migrates toward the anode. Moreover, since the pK_a values of the analytes under study are around 9, they were almost completely de-protonated under the BGE pH used. To study the effect of pH on the peak shape and migration time, a constant concentration of 25 mm H_3PO_4 adjusted with 1 m NaOH solution as BGE was investigated over the pH range 7.5–10.0. In general, the migration time decreased as pH was increased. The best results were achieved at pH 9.0; good peak shapes were obtained compared with when other pH values were used. Therefore, BGE with pH 9.0 was chosen for the subsequent measurements.

The effect of buffer concentration was studied by varying both the concentration of H_3PO_4 from 25 to 100 mm at a constant pH of 9.0. It is known that buffer concentration affects the magnitude of the EOF: a higher buffer concentration provides a lower EOF and vice versa. Using a high concentration of buffer will result in more Joule heating. If excessive Joule heat cannot be efficiently dissipated, peak efficiency will decrease and the migration time will increase. The migration time increases with increasing buffer concentration. When 25 mm H_3PO_4 buffer (adjusted with 1 m NaOH) was used, good peak shape was obtained. Under these conditions less Joule heat was generated. Based on the migration time and the current generated, 25 mm H_3PO_4 buffer was chosen.

In order to determine the optimal voltage to be applied, the influence of voltage (10–30 kV) on the migration time was investigated under the optimized BGE conditions. Voltage greater than 25 kV induced an increase on the current intensity; thus 25 kV was selected as the optimum.

Capillary temperature can influence several parameters, such as sample stability. The migration velocity of analyte and the number of theoretical plates are expected to be proportional to the temperature. Ohmic heating of the capillary causes distortions, and the migration velocity increases more rapidly while the plate count increases less rapidly. These distortions are serious for large bore capillary and higher concentration of buffer. 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 upon temperature, while increase in the diffusion coefficient of analyte is responsible for the poorer than expected performance at high temperatures. Therefore, 25°C was chosen as the working temperature for the analysis.

Optimization of sample injection time (5–20 s) at 50 mbar was conducted in order to achieve a lower detection limit without affecting the quality of the peak shape and reproducibility, migration time and resolution. An injection time of 10 s was chosen as it offered the best results and therefore was selected for the rest of the studies.

From the above experiments, the adopted conditions for the simultaneous analysis of AT, CD and AM were decided: 25 mm H_3PO_4 adjusted with 1 m NaOH to pH 9.0 as BGE; injection time, 10 s; applied voltage, 25 kV; capillary temperature, 25°C; and detection wavelength, 198 nm. A typical eletrophoregram obtained under the adopted conditions is shown in Fig. 2. The suitability of phenobarbital as internal standard is evident as it is well resolved from the analyte peaks. All components migrated in less than 4 min.

Validation Procedure

Calibration curves, limits of detection and quantitation. Working solutions containing all the three standard compounds were prepared as described above to construct the calibration curves. Each calibration curve contained nine different concentrations (1–250 µg/mL) for AT and CD and seven different concentrations (2.5–250 µg/mL) for AM and each concentration was performed in triplicate. Calibration curves with regression equations for AT, CD and AM were $y = 0.03947x + 0.01623$,

$y = 0.04802x - 0.01534$ and $y = 0.01084x - 0.01131$, 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, CD and AM were 0.78, 0.47 and 1.63 μg/mL respectively, while the limits of quantitation (LOQ) were 2.36, 1.43 and 4.97 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 studied. The sensitivity of the proposed CZE method is slightly lower compared with the reported HPLC-UV method (El-Gindy *et al*., 2005; the LOD for AT, CD and AM were 0.003, 0.002 and 0.004 μg/mL, respectively). However the analysis time of the proposed CE is faster (<4 min compared with ~8 min in the HPLC report).

Precision. Intra- and inter-day variations were used to determine the precision of the developed method by analyzing three concentrations (2.5, 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 precisions for migration times and corrected peak areas, expressed as the percentage relative standard deviation, RSD, were 0.11–0.22 and 0.32–4.03%, respectively (Table 1), while inter-day precisions were 1.41–4.11 and 2.67–5.55% for migration times and corrected peak areas, respectively, indicating the good precision of the 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. Accuracy values were 98.32–101.82% (for AT), 100.26–104.08% (for AM) and 102.12–105.11% (for CD) (Table 2).

Analysis of Pharmaceutical Formulations

The developed method has been successfully applied for the simultaneous determination of AT, CD and AM in one co-formulated tablet, AT and CD in three co-formulated tablets and six AT tablets. Good agreement between the proposed method and the manufacturer's claimed values were found for all samples (Table 3). Figure 2(B) shows a typical electropherogram of the pharmaceutical formulations.

Conclusions

A CZE method for the simultaneous determination of AT, AM and CD was successfully developed. Under the adopted conditions, baseline separation of AT, AM and CD and the internal standard was obtained in less than 4 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 method (El-Gindy *et al*., 2005), the proposed method, as expected,

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

Analyte (μg/mL)	RSD (%)					
	Migration time			Corrected peak areas		
	AT	AM	CD	AT	AM	CD
Intra-day precision (n = 9)						
2.5	0.11	0.22	0.14	3.29	2.96	4.03
50	0.13	0.22	0.17	1.32	1.08	0.86
250	0.13	0.14	0.22	0.53	2.16	0.32
Inter-day precision (n = 54)						
2.5	4.11	2.66	2.74	3.22	4.67	5.55
50	1.79	3.00	2.74	3.60	4.86	4.75
250	1.41	1.95	2.14	2.62	5.33	2.76

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

Table 2. Accuracy results for the determination atenolol (AT), amiloride (AM) and chlorthalidone (CD) spiked in tablets

Amount (μg/mL)	(%) Recovery ± SD		
	AT	AM	CD
2.5	98.32 ± 3.43	104.08 ± 3.65	105.11 ± 2.97
50	99.21 ± 1.10	103.32 ± 0.86	102.42 ± 1.38
150	101.82 ± 0.45	100.26 ± 1.21	102.12 ± 0.93

Table 3. Assay results of atenolol (AT), amiloride (AM) and chlorthalidone (CD) in different pharmaceutical formulations

Trade name	Manufacturer	Generic name	Label claim (AT/CD/AM) (mg)	Agreement (%) AT (%) ± SD	CD (%) ± SD	AM (%) ± SD
Teklo	Acapi	AT+ CD + AM	100/25/5	102.21 ± 0.48	100.10 ± 0.69	98.14 ± 1.18
Tenedone	Sigma	AT + CD	50/25	100.23 ± 1.04	96.63 ± 0.89	N/D[a]
Tenoret 50	Astrazeneca	AT + CD	50/12.5	102.42 ± 0.58	98.43 ± 0.78	N/D
Tenoretic	Astrazeneca	AT + CD	100/25	103.20 ± 0.87	98.11 ± 0.38	N/D
Noten	α-Alphapharm	AT	50	98.83 ± 0.92	N/D	N/D
Apo-atenol	Apotex	AT	50	97.65 ± 0.79	N/D	N/D
Ternolol	Hovid	AT	50	99.64 ± 0.56	N/D	N/D
Ternolol	Hovid	AT	100	101.21 ± 0.46	N/D	N/D
Normaten	Mims	AT	100	104.22 ± 0.66	N/D	N/D
Hypoten	Hikma	AT	100	101.04 ± 0.96	N/D	N/D

[a] N/D, not detected.

exhibits less sensitivity due to the shorter path length of the flow cell, but nevertheless provides faster analysis time (<4 min compared with ~8 min by HPLC). Higher separation efficiency and minimization of use of solvents are other inherent features of the CZE methods. The proposed method is therefore recommended to be adopted as a quality control protocol in pharmaceutical industries.

Acknowledgements

This work was supported financially by a Universiti Sains Malaysia Research University and Postgraduate Research Grant Scheme (USM-RU-1001/PKIMIA/841008).

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