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Direct determination of diuretic drugs in urine by capillary zone electrophoresis using fluorescence detection

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Abstract

Four diuretic drugs banned in sport (amiloride, triamterene, bendroflumethiazide and bumetanide) have been separated by capillary zone electrophoresis (CZE) and detected using conventional fluorescence spectrometry. The effect of pH on electrophoretic parameters such as migration time, peak efficiency and peak height is discussed. Complete separation of the four drugs is achieved in less than 8 min at pH 8. No interference due to endogenous urine components is observed and thus direct urine analysis is feasible. Analytical figures of merit including precision and limits of detection are presented. Limits of detection range between 0.5 fmol for triamterene and 21.6 fmol for bumetanide.

Keywords: Amiloride; Triamterene; Bendroflumethiazide; Bumetanide

1. Introduction

Diuretics have important therapeutic applications. Primarily they are used to treat high blood pressure and problems in fluid retention. Many athletes use the drugs to decrease weight in sports such as wrestling, boxing, judo and weight lifting. In addition, diuretics are used illegally to evade detection of banned substances by reducing their concentration in urine. Diuretics cause weight loss by dehydrating fat that is 70% water. Diuretics may also cause an imbalance of the body's thermoregulatory system leading to exhaustion, irregular heartbeats, and ultimately stoppage of the heart or death [1]. For this reason the Medical Commission of the International Olympic Committee (IOCMC) added diuretics to the banned substance list in April 1986, and they are considered a doping class from the 1988 Olympic Games [2].

Many techniques have been developed for doping analysis, including radioimmunoassay (RIA), high-performance liquid chromatography (HPLC), polyacrylamide gel electrophoresis (PAGE), bioassay and enzyme-linked immunosorbent assay (ELISA) [3–17]. Most techniques have a range of limitations, including complex and time-consuming procedures, large analytical variability, low separation efficiency or the lack of sensitivity for some drugs. Consequently, there has been recently a growing interest in exploring capillary electrophoresis (CE) as an alternative method for drug analysis [18–21]. Capillary zone electrophoresis (CZE) has been evaluated for sport-oriented doping control [22–27]. Laser-induced fluorescence detection provides ultimate sensitivity while maintaining the extreme separation efficiency of CE [23]. Micellar liquid chromatography (MLC) and CZE have been evaluated as alternative techniques for doping control in sportsmen [28]. The complementary information provided by MLC and CZE has been demonstrated [28].

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In this paper, the development of a simple and rapid CZE method for the simultaneous analysis of four diuretic drugs (amiloride, triamterene, bendroflumethiazide and bumetanide) using conventional fluorescence detection is discussed. Migration times, limits of detection (LOD) and efficiency values for these drugs are presented. The suitability of CZE for separating these drugs and the feasibility of direct urine analysis are evaluated. A comparison of analytical figures of merit of various detection modes in the CZE of doping drugs is presented.

2. Experimental

2.1. Instrumentation

Experiments were performed using a laboratory-made capillary electrophoresis apparatus [23]. The fluorescence detector consisted of a xenon arc lamp (Carl Zeiss LX 501) and a glass filter (transmittance 270–380 nm) to set the excitation wavelength. The exciting beam was tightly focused onto a portion of the electrophoresis capillary with a plano-convex quartz lens (focal length 30 mm, f-number 1.2). Fluorescence emission was monitored by collecting the light from the capillary with a biconvex glass lens (focal length 80 mm, f-number 3.1) positioned at right angles to the excitation beam. A high-luminosity prism monochromator (Carl Zeiss, Model MQ3) was used for the selection of emission light. A photomultiplier tube connected to an amplifier (Carl Zeiss, Model PMQ3) was used to detect fluorescence at 433 nm.

2.2. Reagents and samples

Chemicals of analytical or research grade were used. Water was demineralized using a water purification system (Milli-Q, Millipore). Stock buffer solutions (ampoules containing concentrated buffer solutions) were purchased from Merck (Darmstadt, Germany). Citrate buffers were used for pH 5 and pH 6, phosphate buffer for pH 7, and borate buffers for pH 7–10. Working buffer solutions were prepared by diluting stock solutions with water to 500 ml at 20°C. Buffers at pH 6 and pH 9 were solutions ready for use. Working buffer solutions were diluted with water to obtain a constant current in the capillary of 42 μA. In all cases pH was kept unaltered. The drugs employed as reference compounds were obtained from Sigma (St. Louis, MO, USA). Stock solutions were prepared in methanol and diluted with appropriate buffer solutions to obtain working drug standards. Spiked urine samples were centrifuged at 1500 *g* for 10 min and injected directly into the capillary without further treatment.

2.3. Running conditions

For the laboratory-made instrument, a 100 cm (60 cm to the detector)×50 μm I.D. capillary column was used. Samples were injected electrokinetically at 10 kV for 10 s and then separated at 30 kV at room temperature. Under these conditions, ca. 6 nl of solution were injected into the capillary column. The capillary was rinsed with running buffer for 3 min prior to each run. The capillary was regenerated by flushing with 1 *M* NaOH (10 min), 0.1 *M* NaOH (10 min) and water (15 min). High-voltage power supply and fused-silica capillary tube were described previously [23].

3. Results and discussion

3.1. pH Influence

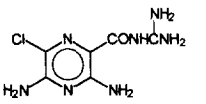
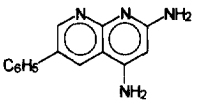
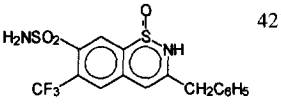
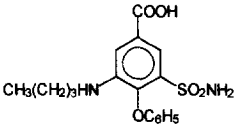
In capillary zone electrophoresis (CZE), the pH of the buffering medium affects the electrophoretic mobility, which in turn modifies the migration velocity, the time required for a solute to reach the detector, and the separation efficiency. Usually, variations in pH also affect detection sensitivity. At concentrations much lower than those of the buffer ions the migration velocity of an analyte (v_m) during electromigration is given by Eq. (1) [29]

$$v_m = k_a(I/A) \quad (1)$$

where I is the electrophoretic current and A is the cross-sectional area of the capillary and k_a is a constant determined by the buffer pH, dielectric constant, ionic strength, viscosity, size and shape of analyte as well as wall and analyte ζ potentials. Because v_m is linearly proportional to the electro-

phoretic current (I), strict control of electrophoretic current is necessary for reproducible migration results. For the drugs of interest, the effect of pH was studied at pH 5 and 6 with citrate buffer, at pH 7 with phosphate buffer and in the range pH 7–10 with borate buffer. All buffer solutions were diluted to obtain a constant current in the capillary of 42 μA, which remained within the range 42–45 μA during migration. Fig. 1 shows the effect of pH on the electrophoretic parameters. For the four drugs studied, a decrease in pH results in an increase in migration time (Fig. 1a). This observation agrees with the fact that the electro-osmotic mobility in capillaries has a sigmoid relationship with pH [30,31], increasing with pH. Regarding the migration order of drugs, this is related to the molecular structure and mass of the drugs (see Table 1). Diuretics containing amine groups (amiloride and triamterene, separated at acidic pH values as cations and at neutral or basic pH as neutral molecules) migrate faster in increasing order of molecular mass, while diuretics containing carboxylic and/or sulphonamide groups (bendroflumethiazide and bumetanide, separated at acidic pH as neutrals and at basic conditions as anionic molecules), migrate slower and in decreasing order of molecular mass. Fig. 1b shows the effect of pH on peak efficiency.

Table 1
Structure and molecular mass of diuretics of interest

Name	Structure	Molecular mass (g mol^{-1})
Amiloride		229.6
Triamterene		253.3
Bendroflumethiazide		421.4
Bumetanide		364.4

Efficiency in theoretical plates (N) was calculated using Eq. (2) [32,33]

$$N = 8 \ln 2(t_m / W_t)^2 \quad (2)$$

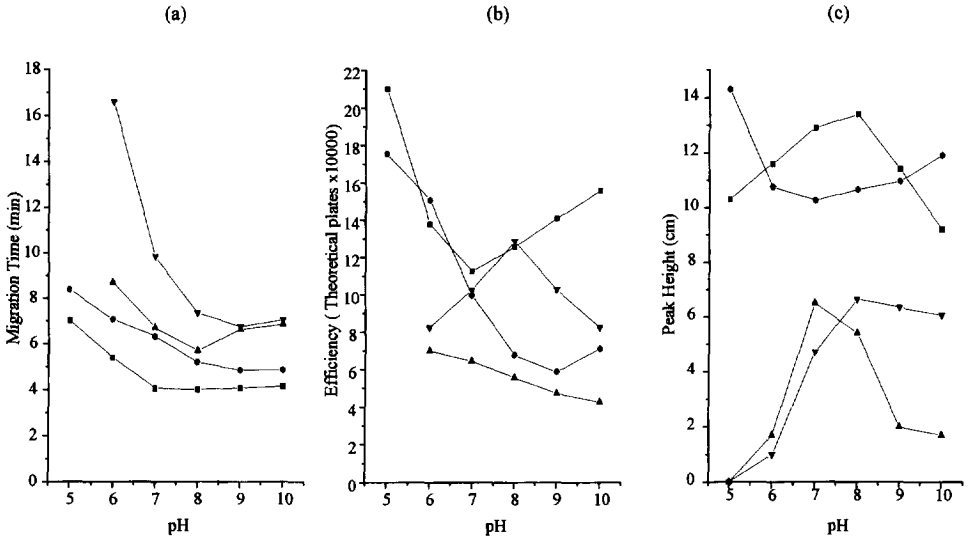


Fig. 1. Effect of pH on the electrophoretic parameters of diuretic drugs. (■) 5×10^{-5} M Amiloride, (●) 1×10^{-5} M triamterene, (▲) 2×10^{-5} M bendroflumethiazide and (▼) 5×10^{-5} M bumetanide. Running conditions in buffer (pH 8) at 30 kV and 42 μA.

where t_m is the migration time and W_t is the full width of the peak at half-maximum, expressed in terms of time. Fig. 1c shows the effect of pH on peak height. The best compromise between separation and detection sensitivity occurs at pH 8. This pH value was used for further studies.

3.2. Separation of drugs

Fig. 2 shows the electrophoregram of a mixture of amiloride (6×10^{-5} *M*), triamterene (2×10^{-6} *M*), bendroflumethiazide (2×10^{-5} *M*) and bumetanide (6×10^{-5} *M*). Fluorescence detection at 430 nm was used. The electrophoregram in Fig. 2a corresponds to the drug standards dissolved in running buffer, while that in Fig. 2b corresponds to the drugs dissolved in urine, both at identical drug concentrations. Separation is complete in less than 8 min in both cases. The only significant difference is that the peak of amiloride is broadened when dissolved in urine (Fig. 2b). Although this result is to be considered for quantitative purposes, migration time remains unchanged and separation is still satisfactory. Fig. 2c shows the electrophoregram of the urine blank. No interferences of endogenous urine components are observed, thus direct urine analysis is feasible without significant sample treatment. Quantitative data are reasonably precise (Table 2). Relative standard deviation values are better than 1% in most cases for migration time and peak width. Peak height R.S.D. values are within 2.5–8%. It is of interest that, in spite of the large differences in chemical structures of the drugs examined, separation is achieved in a short time, which is compatible with most practical analytical situations.

Table 2
Precision of the electrophoretic parameters of diuretics banned in sport for $n=8$

Drug	R.S.D. (%)		
	Migration time	Peak width	Peak height
Amiloride	0.45	0.26	7.89
Triamterene	0.60	0.22	2.53
Bendroflumethiazide	0.96	0.26	3.42
Bumetanide	1.64	0.03	3.11

3.3. Analytical figures of merit (AFOM)

Table 3 summarizes the AFOM based on peak height measurements for the diuretics of interest dissolved in urine. The maximum excitation and fluorescence wavelengths for the drugs are also listed. Linear response of peak height with concentration is observed for at least three concentration decades, with correlation coefficients better than 0.999. Limits of detection (LOD) calculated for the absolute amount detected are within the femtomole range, while LODs calculated for the sample injected range between 3.6×10^{-6} *M* (1.31 ppm) for bumetanide and 9×10^{-8} *M* (0.02 ppm) for triamterene. These values compare well with those obtained using laser-induced fluorescence detection [4] or UV detection at 254 nm [5]. It is difficult to assess

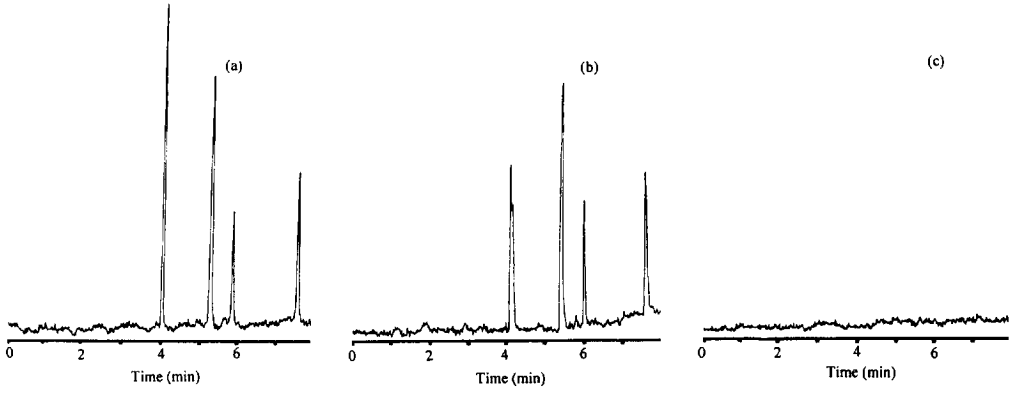


Fig. 2. Separation of diuretics by CZE. (a) Electrophoregram of a mixture of 6×10^{-5} *M* amiloride (t_m 4.0 min), 2×10^{-6} *M* triamterene (t_m 5.2 min), 2×10^{-5} *M* bendroflumethiazide (t_m 5.7 min) and 6×10^{-5} *M* bumetanide *M* (t_m 7.3 min). (b) Electrophoregram corresponding to an urine sample spiked with drugs at the same concentrations (drugs eluted in the same order). (c) Electrophoregram of an urine blank. Running conditions as in Fig. 1.

Table 3
Spectral characteristics and analytical figures of merit for the CZE method for various diuretics banned in sport

Drug	λ_{exc} (nm)	λ_{emi} (nm)	r	Limits of detection		Literature values M ($\times 10^{-7}$)[a,c]
				mol ($\times 10^{-15}$)[a,b]	M ($\times 10^{-7}$)[a,c]	
Amiloride	382	416	0.9993	17.0	29.7	–
Triamterene	370	434	0.9990	0.5	0.9	0.7[d], 2.4[e]
Bendroflumethiazide	272	381	0.9994	3.8	6.3	40[d]
Bumetanide	350	428	0.9990	21.6	36.0	12[e]

[a] For a signal-to-noise ratio of 2.
[b] Absolute amount detected.
[c] Concentration detectable.
[d] Ref. [5].
[e] Ref. [4].

the level of these drugs in urine of a doped athlete. Usually, the dose taken for doping purposes is well above that used for therapeutic uses. The limits of detection calculated in the low ppm range should be sufficient in both cases.

4. Conclusion

Capillary zone electrophoresis (CZE) using classical fluorescence detection is a useful analytical technique for separation and detection of traces of diuretic drugs in urine samples. The absence of column filling compared to liquid chromatography (LC) and the peak-free baseline provided by urine using fluorescence detection makes sample cleanup unnecessary. Solute separation in CZE is based on differences in electrophoretic mobility, rather than on different interactions with a stationary phase as in LC. Thus CZE could be used to conform the practice recommended by the IOCMC of using alternate analytical principles for screening and confirmatory analyses in doping control.

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