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Short communication

Simultaneous determination of aminomethylbenzoic acid, cefminox sodium and etamsylate in human urine by capillary electrophoresis

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ABSTRACT

A sensitive and selective capillary electrophoresis method is developed, for the first time, for effective separation and simultaneous determination of aminomethylbezoic acid (PAMBA), cefminox sodium (CMNX) and etamsylate (ETM). The electrophoresis conditions were investigated and optimized. A 25 mM phosphate solution (pH 8.5) was used as a buffer and the peak area was determined with UV detection at 216 nm wavelength under 18 kV separation voltage. Under optimal conditions, the three drugs can be separated effectively. Good linearity was achieved in 3.13–150 μg/mL for PAMBA, 6.25–150 μg/mL for CMNX and 3.13–150 μg/mL for ETM, with the correlation coefficients of >0.999. The limit of detection (LOD) for PAMBA, CMNX and ETM was 1.04, 2.08 and 1.04 μg/mL, respectively. Their recoveries in human urine were in the range from 90.2% to 101% with the RSD (n = 5) of 0.7–3.1%. The proposed method is simple, rapid and accurate, and provides the sensitivity and linearity necessary for analysis of the test drugs in human urine at clinically relevant concentrations.

1. Introduction

Etamsylate (diethyl ammonium 2,5-dihydroxy benzene sulphonic acid, ETM) is a systemic, nonthrombogenic haemostatic agent that has been used in the treatment of capillary hemorrhage, hematemesis, hemopthysis, malena, hematuria, epistaxis and menorrhagia as well as post-partum hemorrhage [1]. Aminomethylbenzoic acid (PAMBA) is a kind of antihemorrhagic agent, which can inhibit the activation of lactase and clinically be used for the treatment of alimentary tracthaemorrhage, obstetrics haemorrhage, and common capillary haemorrhage [2]. Cefminox sodium (CMNX) is a new semisynthetic cephalosporin and has better curative effect for the control of pneumonia, faucitis and urinary tract infection than cefazolin sodium and cefmetazole sodium. CMNX was frequently combined with ETM and PAMBA, for clinical application [3]. The structures of the three drugs are shown in Fig. 1.

ETM in pharmaceuticals has been determined by a variety of analytical techniques including spectrometry [4,5], electrochemistry [6,7] and chemiluminescence (CL) [8,9] as well as liquid chromatography (LC) [10,11]. The determination of ETM in biological fluids has been reported employing spectrophotometry [12]. The above-mentioned techniques, of course, are sensitive enough but are only suited to ETM alone. The reported spectrophotometric methods have been applied for the assay of ETM and mefenamic acid [13,14], ETM and other three drugs [15] as well as ETM and captopril in pharmaceutical preparations and biological fluids [16]. Few works had been published for the determination of PAMBA. Wang et al. [17] reported the determination of PAMBA and aminophylline using flow injection CL method with poor selectivity. An ultraviolet spectrophotometric method was also used for the determination of PAMBA in injection with relatively narrow linear range and low sensitivity [18]. An effective method has been established for the determination of PAMBA in the injection, but a chemical derivative chromogenic reagent was required [19]. CMNX in pharmaceuticals has been determined by HPLC [20–23]. The ion-exchange, reversed-phase and ion-pair chromatographic methods have been developed for the determination of CMNX, cefoxitin and cefmetazole in spiked serum and urine [24]. However, in some cases, measurement conditions should be carefully controlled to achieve good selectivity for the reported methods.

To study the compatible stability of PAMBA and CMNX, each content of the two drugs after compatibility were determined by second order derivative UV spectrophotometry [25] and HPLC method [26], but these methods showed lower sensitivity and narrow linearity range. Since the maximal absorption peaks for PAMBA and ETM present serious germination, a multivariate calibration spectrophotometric method was developed for simultaneous determination of the two drugs in spiked rabbit serum, with higher sensitivity but narrow linearity range and trouble [27]. Capillary electrophoresis (CE) is an analytical tool that has shown great promise in replacing many conventional clinical laboratory methods, especially electrophoresis and HPLC. The main attrac-

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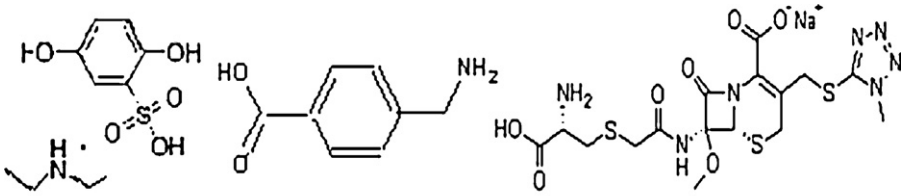

Fig. 1. Structure of the three drugs.

tion of CE was that it was fast, used small amounts of sample and reagents, and was extremely versatile, being able to separate large and small analytes [28]. A CE method with electrochemiluminescence detection was used for simultaneous determination of ETM, tramadol and lidocaine in human urine [29]. However, capillary electrophoresis for the determination of CMNX and PAMBA has not been found in literature. It is very important to find an effective separation and sensitive method to simultaneously determine ETM, CMNX and PAMBA in clinical medicine. In this work, an effective CE method was designed for separation and determination of the three drugs. The proposed method has successfully applied for simultaneous determination of the test drugs in urine samples.

2. Experimental

2.1. Instrumentation

All experiments were performed with an Agilent 3D CE system with air-cooling and a diode-array detector (Agilent, Waldbronn, Germany). Data were collected with the Agilent Chemstation version A.10.02 chromatographic data system. A 48.5 cm (40.0 cm to the detector) 75 μm i.d. uncoated fused silica capillary (Yongnian Optical Fabric Factory, Handan, China) was utilized. A centrifuge TGL-16M (Xiangyi Centrifuge Co., Hunan, China), ultrasonic cleaner (Ultrasonic Instrument Co., Kunshan, China) and PHS-3C pH meter (Shanghai Precision & Scientific Instrument Co., Shanghai, China) were used in sample treatment.

2.2. Material and reagents

Etamsylate, aminomethylbenzoic acid and cefminox sodium were purchased from the Jinyue Amino Acid Co. Ltd. (Tianjin, China), Dongting Pharmaceutical Co. Ltd. (Hunan, China) and North China Pharmaceutical Co. Ltd. (Shijiazhuang, China), respectively. All reagents were of analytical grade. Purified water was prepared by an XGJ-30 highly pure water machine (Yongcheng Purification Science & Technology Co. Ltd., Beijing, China).

A stock solution (2.0 mg/mL) of etamsylate, aminomethylbenzoic acid and cefminox sodium was prepared by dissolving an accurately weighed amount of 0.1 g in a 50-mL volumetric flask and adjusting to the volume with water, and stored in refrigerator at −4 °C The standard solutions were prepared by diluting the stock solution with water. A 25 mM phosphate buffer was prepared by dissolving an appropriate amount of Na_2HPO_4 in water, the pH value of which was adjusted with hydrochloric acid and sodium hydroxide. All standard solutions and buffer solutions were prepared weekly, and filtered through 0.45 μm cellulose acetate filters (Shanghai Xinya Purification Material Factory) prior to injection.

2.3. Sample treatment

The patient urine sample was provided by Hospital of Hebei University. A 4.0 mL of acetonitrile was added in 2 mL of urine sample to remove the protein. The mixture was shaken for 10 min, then centrifuged at 10,000 rpm for 10 min. The supernatant was filtered through 0.45 μm microporous membrane of mixed cellulose ester. The filtrate was applied for CE analysis and recovery test.

2.4. Electrophoresis conditions

Prior to its use every day, the capillary was consecutively flushed with 0.1 M NaOH for 10 min, distilled water for 10 min and the running buffer for 10 min. After each analysis run the capillary was flushed for 3 min with the running buffer to maintain the reproducibility of the analysis. Sample introduction was made at the positive side using 50 mbar pressure for 4 s. The high-voltage power supply was set at 18 kV. The 25 mM Na_2HPO_4 solution (pH 8.5) was used as a running buffer. Capillary temperature was kept at 25 °C. Considering the three compounds sensitivity comprehensively, direct UV detection was employed at a wavelength of 216 nm.

3. Results and discussion

3.1. Effect of running buffer

The running buffer is an important factor for the separation of the drugs. PAMBA, CMNX and ETM are acidic compounds. Use of an alkaline buffer solution is required. In our work, a Na_2HPO_4 buffer (pH adjusted with 0.1 M hydrochloric acid) was used for the separation of the three drugs. The effects of pH and concentration of the buffer on the resolution and sensitivity were investigated with 10 μg/mL PAMBA, 70 μg/mL CMNX and 50 μg/mL ETM.

The results showed that when pH < 5 PAMBA could not be baseline-separated, and the sensitivity was lower at pH lower than 7.0. In an acidic environment, these compounds were cationic due to the protonation of tertiary amino groups [30], and the dissociation degree of silanol groups was little, resulting in a low magnitude and small change of the electroosmotic flow [31]. Thus these compounds showed both small changes and low values of resolution at low pH values. With the increasing of pH, the electroosmosis increased due to the dissociation of the silanol groups, so the retention time for the three compounds became shorter. Otherwise, with the increasing of pH, the peak heights were increased giving a higher sensitivity. The pH value of running buffer influenced mainly their resolution and electroosmotic flow (EOF). A high pH value led to high degree of dissociation and high EOF [32]. Thus the resolution increased and reached gradually the maximum value. However, when the pH was greater than 9.5, ETM was not observed in the electropherigram. A high pH value of the running buffer resulted in a decrease of resolution due to the fact that the tertiary amino groups were deprotonated and did not associate with the capillary silanol groups. To obtain maximum sensitivity and resolution, an optimal pH level of 8.5 was ultimately selected for the CE conditions.

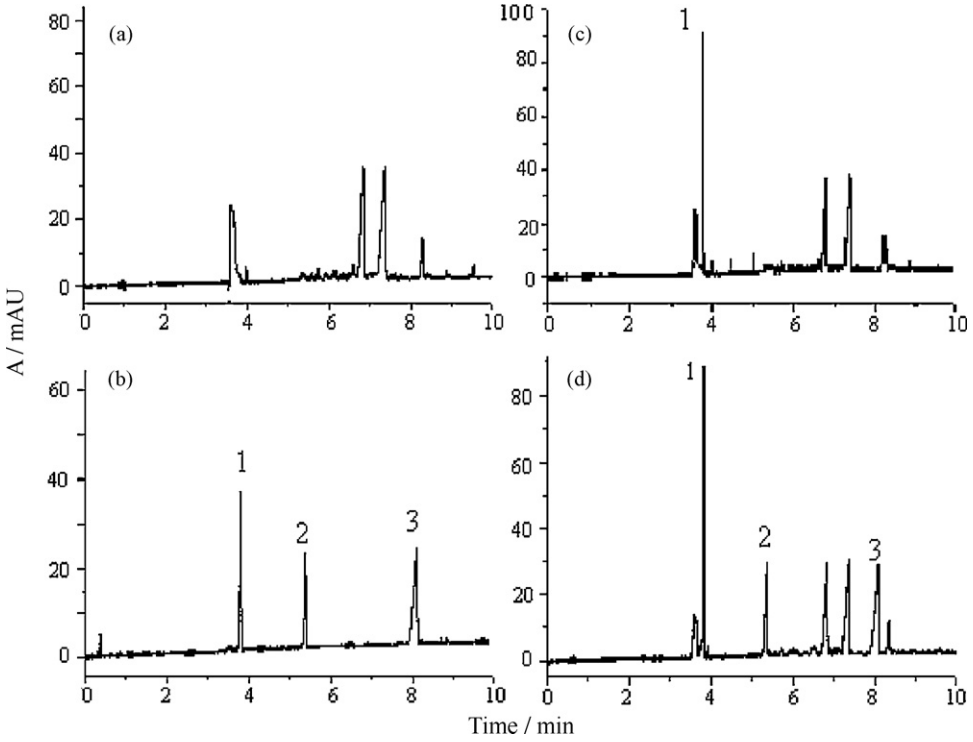

Fig. 2. Electrophotogram of the three drugs. (a) Blank urine sample; (b) standard solution: (1) PAMBA, 50 μg/mL; (2) CMNX, 50 μg/mL; (3) ETM, 100 μg/mL; (c) patient urine sample; (d) patient urine sample; spiked with 50 μg/mL CMNX and 100 μg/mL ETM; detection at a 216 nm and separation with a 25 mM Na_2HPO_4 buffer (pH 8.5) at 18 kV voltage

The effects of buffer concentration from 15 to 40 mM on the resolution were further tested at pH 8.5. The retention time and peak height of the three drugs increased with the increasing of buffer concentration from 10 to 25 mM, and decrease over 30 mM. The high concentration buffer with high viscosity made the thickness of electric double layer between the capillary walls and the buffer solution to be decreased. Otherwise, the EOF decreased with increasing buffer concentration, it is linked to both a decrease in counter-ion layer thickness as well as an increase in adsorption of counter-ion, which results in an increase in the coverage of sites on the silica surface [33]. These factors result in cataphoretic migration speed to be decreased.

It can be indicated that the migration time of the three analytes increased in order of PAMBA, CMNX and ETM, and the migration sequence could not be affected by pH and concentration of the buffer.

The effective separation of the peaks of the three drugs from the peaks of impurity was observed in the presence of 25 mM buffer. Therefore, to achieve higher resolution and without disturbances a 25 mM buffer (pH 8.5) was selected for the separation of the three drugs.

3.2. Separation voltage

The increase of separation voltage would increase the EOF and shorten the analytical time. However, the high voltage led to Joule heating and affected the separation of the analytes. The effect of voltages on the separation of the three drugs was investigated in the range of 15–22 kV with 25 mM phosphate buffer solution (pH 8.5). It was found that with increasing in separation voltage, EOF increased and retention time decreased. However, the higher voltage caused the decrease in peak areas of the analytes, and would cause higher current and lead to more Joule heating, which produces radial temperature gradient and decreases separation efficiency [34]. The peaks of the drugs could not be separated absolutely from the impurity when voltage was beyond 18 kV. Based on the experiments mentioned above, 18 kV voltage was selected for capillary electrophoresis in this work with higher sensitivity and shorter analytical time.

3.3. Analytical performance of the method

3.3.1. Specificity

Under the optimal conditions, specificity was determined for each analyte in the assay. The electrophotograms of the three analytes were obtained with high resolution, as shown in Fig. 2.

It is shown that the migration time of PAMBA CMNX and ETM in both standard solution and spiked blank urine was 3.8, 5.4 and 8.1 min, respectively. PAMBA, CMNX and ETM could be separated absolutely from the noise of the blank urine. Otherwise, for the blank urine samples at a spiked level 18.75, 75 and 112.5 μg/mL, the relative standard deviation (RSD) of migration times was determined for intra-day ($n = 5$) and inter-day ($n = 5$). The results are listed in Table 1.

The intra- and inter-day RSDs of the migration times were ≤2.1% and ≤2.8% for PAMBA, ≤2.8% and ≤3.4% for CMNX, and ≤2.5% and ≤2.8% for ETM, respectively. The good reproducibility and effective baseline separation were achieved.

3.3.2. Linearity

The linearity of the CE method was evaluated by analysis of seven concentration samples of the studied drugs. The calibration curves were constructed by least-squares linear regression. The equations of calibration curves obtained based on three parallel measurements are listed in Table 2. It can be seen that the linearity is satisfactory based on the criteria ($R^2 \geq 0.98$) described by Green [35].

Table 1
Intra-day and inter-day analytical precisions of three synthetic drugs.

Drug	Concentration (μg/mL)	Intra-day (n = 5, RSD%)		Inter-day (n = 5, RSD%)	
		Migration time	Peak area	Migration time	Peak area
PAMBA	18.75	1.0	1.7	2.2	3.2
	75	2.1	2.4	2.8	3.5
	112.5	0.8	1.6	1.7	2.4
CMNX	18.75	1.1	3.0	2.3	3.2
	75	2.8	2.9	3.0	3.6
	112.5	2.2	3.5	3.4	4.1
ETM	18.75	1.3	2.0	1.9	2.6
	75	1.7	3.9	2.8	4.7
	112.5	2.5	2.6	2.7	3.8

Table 2
Regression equations, linearity range and detection limit.

Analyte	Regression equation[a]	Correlation coefficient (R^2)	Linearity range (μg/mL)	LOD (μg/mL)
PAMBA	$A = 1.25678C + 1.19952$	0.9996	3.13–150	1.04
CMNX	$A = 0.34598C + 0.86131$	0.9982	6.25–150	2.08
ETM	$A = 0.90491C - 2.29866$	0.9984	3.13–150	1.04

[a] A: peak area; C: analyte concentration (μg/mL).

Table 3
Analysis of patient urine samples.

Analyte	Content (μg/mL)	Added (μg/mL)	Found (μg/mL)	Recovery (%)	RSD, n = 5 (%)
PAMBA	84.07	10	89.8	95.5	2.3
	84.07	35	107.0	89.9	2.7
	84.07	60	136.3	94.6	1.8
CMNX	–	10	9.86	98.6	1.0
	–	35	34.7	99.1	1.1
	–	60	56.8	97.4	0.8
ETM	–	10	9.02	90.2	1.1
	–	35	32.3	92.3	2.6
	–	60	59.3	98.8	3.2

3.3.3. Detection limit and precision

The limit of detection (LOD) was determined as the sample concentration that produces a peak with a height three times the level of the baseline noise. The LOD values for PAMBA, CMNX and ETM were 1.04, 2.08 and 1.04 μg/mL, respectively.

Based on the US FDA guidance [36], the intra-day and inter-day variabilities at three typical assay concentrations (18.75, 75 and 112.5 μg/mL) were evaluated for five replicates within 1 day and over 5 successive days. The results are given in Table 1. The intra- and inter-day RSD values of peak areas were ≤2.4% and ≤3.5% for PAMBA, ≤3.5% and ≤4.1% for CMNX, ≤3.9% and ≤4.7% for ETM, respectively. It is indicated that the precision of the method is satisfactory.

3.3.4. Stability

The stability of the three analytes in blank urine and running buffer within 24 h was investigated. Under the optimal conditions, they were determined at different time (0, 1, 2, 4, 8, 12, 20, and 24 h) respectively. No significant change was observed in this study. The RSD of peak area for PAMBA, CMNX and ETM were lower than 2.94, 3.09 and 3.58%, respectively. It was shown that the three drug analytes were stable in urine and running buffer in this time span.

3.4. Application

A normal dosage of PAMBA through intravenous injection could reach 0.3 g, and the mean serum concentration of PAMBA after a dose would be about 100 μg/mL [2]. After oral administration of 500 mg ETM, peak plasma concentration in the range of 10–20 μg/mL were achieved 2–4 h after dosing, and about 72% of the administered dose are excreted in the first 24 h-urine. A 69.17 μg/mL of CMNX in serum was obtained at 1 h after the administration of CMNX [37]. These drugs can be administered perorally or intravenously, and are excreted mainly into the urine.

The urine sample was collected from one patient dosed with PAMBA of 0.1 g within 1 h. The optimal conditions were applied to the separation and determination of PAMBA, CMNX and ETM in human urine. The urine sample was treated according to the procedure described in Section 2.3. The peaks were identified by comparing the migration time and spiking the standards to the real sample solution. The concentration in urine was 84.07 μg/mL. The electropherograms of blank urine sample, patient urine sample (within 1 h), standard solution as well as spiked patient urine sample were shown in Fig. 2. It was shown that the peaks in the blank urine did not interfere with the determination of the three analytes in real human urine sample.

The recovery test of the assays for PAMBA, CMNX and ETM in human urine was determined by adding known amounts (10, 35 and 60 μg/mL) of these drugs to the urine samples. The results are listed in Table 3. The data showed that the recoveries ranged from 90.2% to 98.6% with the RSD of 1.0–2.3% for PAMBA, and that from 89.9% to 99.1% with the RSD of 1.1–2.7% for CMNX, and that 94.6–98.8% with the RSD of 0.8–3.2% for ETM.

The results indicate that the proposed method provides the selectivity, sensitivity and linearity necessary for analysis of the test drugs in human urines at clinically relevant concentrations.

4. Conclusion

A new method for effective separation and simultaneous determination of PAMBA CMNX and ETM by capillary electrophoresis is proposed in the present study. Compared to the methods reported in literature for simultaneous determination of PAMBA and CMNX or ETM, the present method offers advantages of speediness, simplicity, sensitivity and accuracy, and is applicable to the determination of these drugs in clinical studies. The proposed method can provide basis for the clinical application and pharmacokinetics of the three drugs.

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