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Determination of testosterone/epitestosterone concentration ratio in human urine by capillary electrophoresis

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ABSTRACT

A novel method for determining the testosterone/epitestosterone concentration ratio in human urine was established by capillary electrophoresis with diode-array detector. The urine samples were firstly purified by the solid extraction. The optimal experimental conditions were: running buffer pH = 4.74, 15.0 mmol L^{-1} HAc-NaAc, separation voltage 25 kV, temperature 25 °C, sample injection pressure 3.43 × 10^3 Pa, and duration 10 s. The testosterone and epitestosterone linear range were determined as 8.0–960.0 ng mL^{-1}, respectively. The testosterone and epitestosterone detection limits were determined as 4.6 and 4.5 ng mL^{-1}, respectively. The relative standard deviation was less than 0.36%.

1. Introduction

Testosterone is a male sex hormone, which can increase bone density and prevent osteoporosis. It can enhance the vitality of the human bodies, and increase the strengths of the muscles. It can help maintain the heart healthy, strengthen memory and relieve anxiety [1]. For women's health, testosterone is also indispensable and necessary. The lower testosterone will induce depression and cause osteoporosis and fat increase. It is also doping of bad athletes in the International Olympic Committee (IOC) the provisions. Therefore, a faster and reliable testosterone detection method is needed, which is of great importance.

The detection methods for testosterone include high performance liquid chromatography (HPLC) [2], high performance liquid chromatography–tandem mass spectrometry (LC-MS) [3–11], gas chromatography–tandem mass spectrometry (GC–MS) [12,13], electrochemical method [14–16], molecularly imprinted plasmon resonance method [17] and cloning the gene regulatory method [18]. Compared with LC-MS and GC–MS, sample pretreatment of HPLC is rather simple, and the testing cost is relatively low [3–5]. However, the determination of testosterone in steroidal drugs by capillary electrophoresis (CE) has not been reported. Testosterone is an endogenous hormone. Its concentration has great individual differences. Thus, the absolute quantitative standard can not be regarded as a positive criterion. The testosterone/epitestosterone concentration ratio is used as an evaluation index. Epitestosterone is the stereoisomer of testosterone. In normal urine, the testosterone/epitestosterone concentration ratio is about 1/1. For those who have taken testosterone, the testosterone/epitestosterone concentration ratio will significantly increase. In accordance with the standards of the International Olympic Committee Medical Commission (IOCMC), testosterone/epitestosterone (concentration ratio) > 6/1 in the urine is considered as testosterone positive. The structural formula of testosterone, epitestosterone and metandienone is shown in Fig. 1.

Herein, we used CE to systematically analyze the urine samples of those taking testosterone. A novel accurate testing method for determining testosterone/epitestosterone concentration ratio in urine was established.

2. Experimental

2.1. Materials

The materials were obtained from commercial suppliers and were utilized without the further purification. Tris (hydroxymethyl) aminomethane, potassium dihydrogen phosphate, phosphoric acid, acetic acid, sodium acetate, methanol, and hydrochloric acid were purchased from Shanghai Chemical Reagent Ltd., Co. Testosterone, epitestosterone, β-glucuronidase and metandienone were from Aladdin. All reagents were at least analytical grade. Deionized water was obtained by passing distilled water through a Mingche Water Purification System (Merk Millipore Lab Equipment (Shanghai) Ltd., Co., China). Deionized water was used throughout this work.

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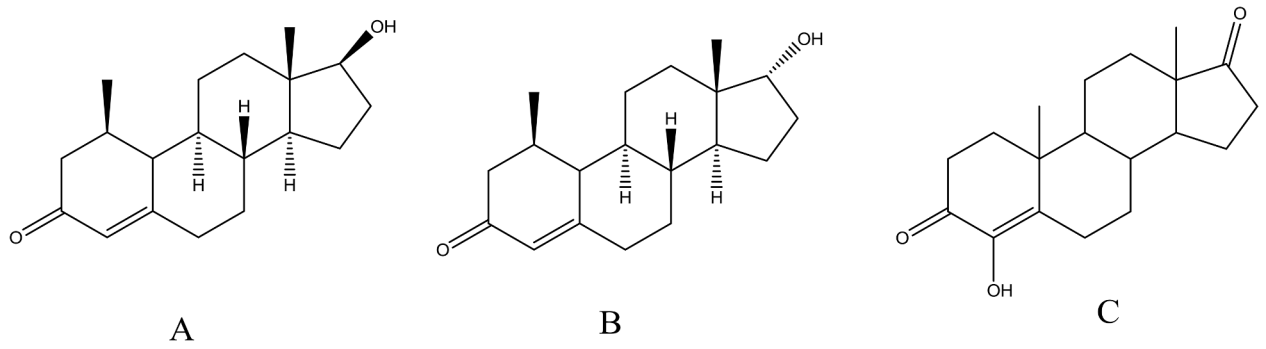

Fig. 1. The structural formula of testosterone (A), epitestosterone (B) and metandienone (C).

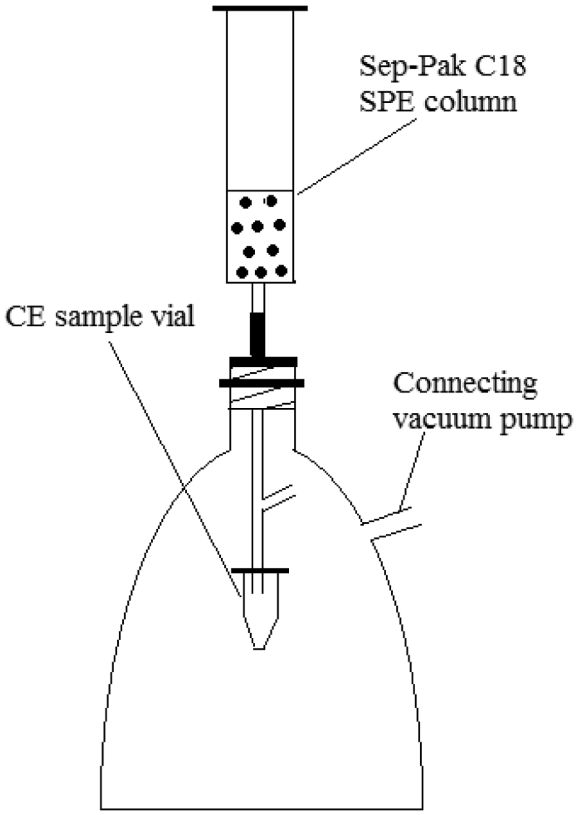

Fig. 2. Extraction set-up diagram of Sep-Pak C18 SPE column.

2.2. Instruments

Separations were performed on a P/ACE MDQ apparatus (Beckman Instruments Inc., Fullerton, CA, USA) equipped with the better diode-array detector. The capillary (Hebei Yongnian Ruifeng chromatographic components Co., China) of 75 μm i.d. (375 μm o.d.) and 57 cm total length (50 cm to the detector) were also used. Samples were injected in the hydrodynamic mode by overpressure (3.43×10^3 Pa). System Gold software was used for the data acquisition. Sep-Pak C18 SPE column was from Cherish Technology Ltd., Co. Agilent 5975 GC–MS was from Afilent Technologied Inc., USA. Lambda 950 UV/Vis/NIR Spectrophotometer was from PerkinElme, USA. All experiments were carried out at 25 °C by using a liquid thermostated capillary cartridge except for the temperature experiment. Sartorius Basic pH Meter PB-10 was from Shanghai Renhe Scientific Instrument Co., China.

2.3. Urine hydrolysis and preprocessing

Urine sample (5.0 ml) was mixed with 1.0 ml pH 5.15, 1.0 mol L^{-1} HAc-NaAc buffer, 10,000 units β-glucuronidase and 200 μL metandienone internal standard solution (concentration: 1.0 ng/ml methanol solution), separately. After well mixing, the mixture was incubated at constant temperature water bath (37 °C) for 20 h. Then, it was centrifuged for 15 min (4000 RPM). Under the condition of the negative pressure, the supernatant was passed through Sep-Pak C18 SPE column (particle diameter 10–30 μm) (Fig. 2). The column was washed with 4.0 ml high purity deionized water, 1.0 ml tris (hydroxymethyl) aminomethane (Tris) buffer (pH 8.25, 50.0 mmol L^{-1}), 4.0 ml of high purity deionized water, 1.0 ml of NaAc-HAc buffer (pH3.91, 50.0 mmol L^{-1}), 4.0 ml of high purity deionized water, and 1.0 ml of 30% methanol aqueous solution in turn. Finally, it was eluted with 1.0 ml 100% methanol. The elution solution was collected by clean CE sample vial (1.0 ml), and blow dried by air under 50 °C. The extractive was dissolved with 200 μL of 30% methanol solution and this solution was used directly as CE analysis sample.

3. Results and discussion

The UV spectrum of testosterone, epitestosterone and metandienone.

The UV spectrum of testosterone, epitestosterone and metandienone is shown in Fig. 3. Each of the testosterone, epitestosterone and metandienone had a maximum absorption peak at 242.0, 242.0 and 245.0 nm, respectively. Therefore, 242.0 nm wavelength was chosen in diode-array detector.

3.1. The separation voltage selection

Because the time of migration in CE is influenced by the separation voltage, the impacts of testosterone separation voltage on migration time in CE was examined in the range of 10 to 30 kV. The analysis showed that with the increase of the separation voltage, the migration time of testosterone was shortened and the resolution decreased as the enhancement of separation voltage. Under this condition, the separation voltage of 25 kV was selected.

3.2. The selection of buffer

The electrophoretic results of testosterone in HAc-NaAc buffer (pH 4.76, 15.0 mmol L^{-1}), Na_2HPO_4-KH_2PO_4 buffer and Tris buffer (pH 8.25, 15.0 mol L^{-1}) are shown Fig. 4. It can be seen that the signal

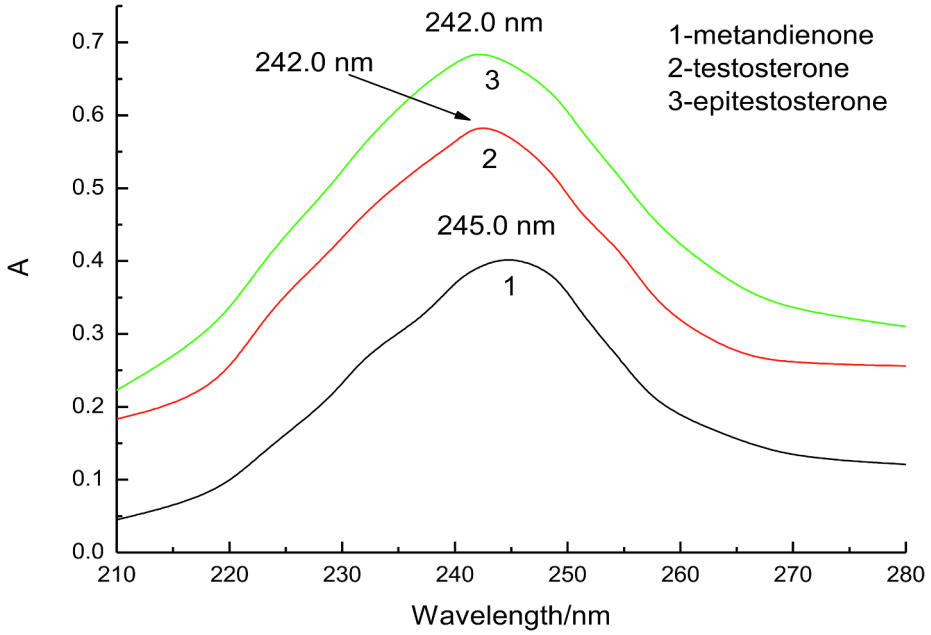

Fig. 3. UV spectrum of metandienone, testosterone and epitestosterone.

intensity and resolution were poor in Na_2HPO_4-KH_2PO_4 and Tris buffer, but very excellent in HAc-NaAc buffer. Thus, the HAc-NaAc buffer was used for the following experiments.

Separation conditions: separation voltage 25 kV, separation temperature 25 °C, sample injection pressure 3.43 × 10^3 Pa, duration 10 s, detection wavelength 242.0 nm.

3.3. The selection of HAc-NaAc buffer pH value and concentration

The electrophoresis was investigated in the pH value range of 3.5–6.0 and is shown in Fig. 5A. It can be seen that the signal intensity increased rapidly as the pH value increased from 3.5 to 4.74 and then it decreased slowly when the pH value was above 4.74. Thus, pH value 4.74 was used. The signal intensity with different buffer concentrations (5.0–30.0 mmol L^{-1}) was analyzed (Fig. 5B). The results showed that the signal intensity decreased slowly when the buffer concentration increased from 5.0 mmol L^{-1} to 30.0 mol L^{-1}. Since the migration time decreased as the increase of buffer concentration, HAc-NaAc buffer concentration of 15.0 mmol L^{-1} was used to shorten the time of measurement.

Separation conditions: separation voltage 25 kV, separation temperature 25 °C, sample injection pressure 3.43 × 10^3 Pa, duration 10 s, detection wavelength 242.0 nm.

3.4. The effect of capillary electrophoresis temperature

Fig. 6 shows the influences of temperature on the CE. It can be seen that the migration time decreased as the temperature increased from 10 to 30 °C. Thus, the temperature of 25 °C was selected to shorten time of measurement and it had relatively higher signal intensity.

Separation conditions: separation voltage 25 kV, sample injection pressure 3.43 × 10^3 Pa, duration 10 s, detection wavelength 242.0 nm, pH 4.74.

3.5. Linear range, precision and detection limit

Under the optimal condition, testosterone, epitestosterone and metandienone could be separated well (Fig. 7A). Electrophoregram of before and after taking testosterone is shown (Fig. 7B and C).

The 80.0 mg standard sample of testosterone was put into 50 ml beaker. After it was dissolved with 30 ml methanol, it was quantitatively transferred to 100 ml volumetric flask and then diluted with methanol to scale mark. The above standard solution was diluted to 40 ng μL^{-1} testosterone standard solution with methanol. The 40 ng μL^{-1} testosterone standard solution of 5.0, 10.0, 20.0, 40.0, 60.0, and 80.0 μL was added to five centrifugal tubes (Each centrifugal tube contained 5 ml of blank urine), respectively, and then 200.0 μL internal standard solution (2.0 ng/mL metandienone methanol solution) were added into the each tube. After urine sample pretreatment, CE determined the peak area ratio (PAR) of testosterone/metandienone. In the optimal condition, testosterone concentration (C_T) had good linear relationship with PAR of testosterone and metandienone in 0.2–2.0 range. The linear regression equation was C_T (ng mL^{-1}) = 509.01PAR − 48.22, R = 0.99981 (Fig. 8A). After the 13-times determination of 80.0 ng mL^{-1} testosterone, the peak area ratio RSD was 0.36%. The detection limit (3σ) was 4.6 ng mL^{-1}.

The method for epitestosterone determination was the same as the method of testosterone determination. Epitestosterone concentration (C_E) had good linear relationship with PAR of epitestosterone and metandienone in 0.2–2.0 range. The linear regression equation was C_E (ng mL^{-1}) = 515.40PAR − 47.88, R = 0.99933 (Fig. 8B). After the 13-times determination of 80.0 ng mL^{-1} epitestosterone, the peak area ratio RSD was 0.38%. The detection limit (3σ) was 4.5 ng mL^{-1}.

3.6. Recovery experiment

The same urine sample was divided into three parts, among which two parts were added with an amount of testosterone standard solution, respectively. All of the three samples were measured and the results are

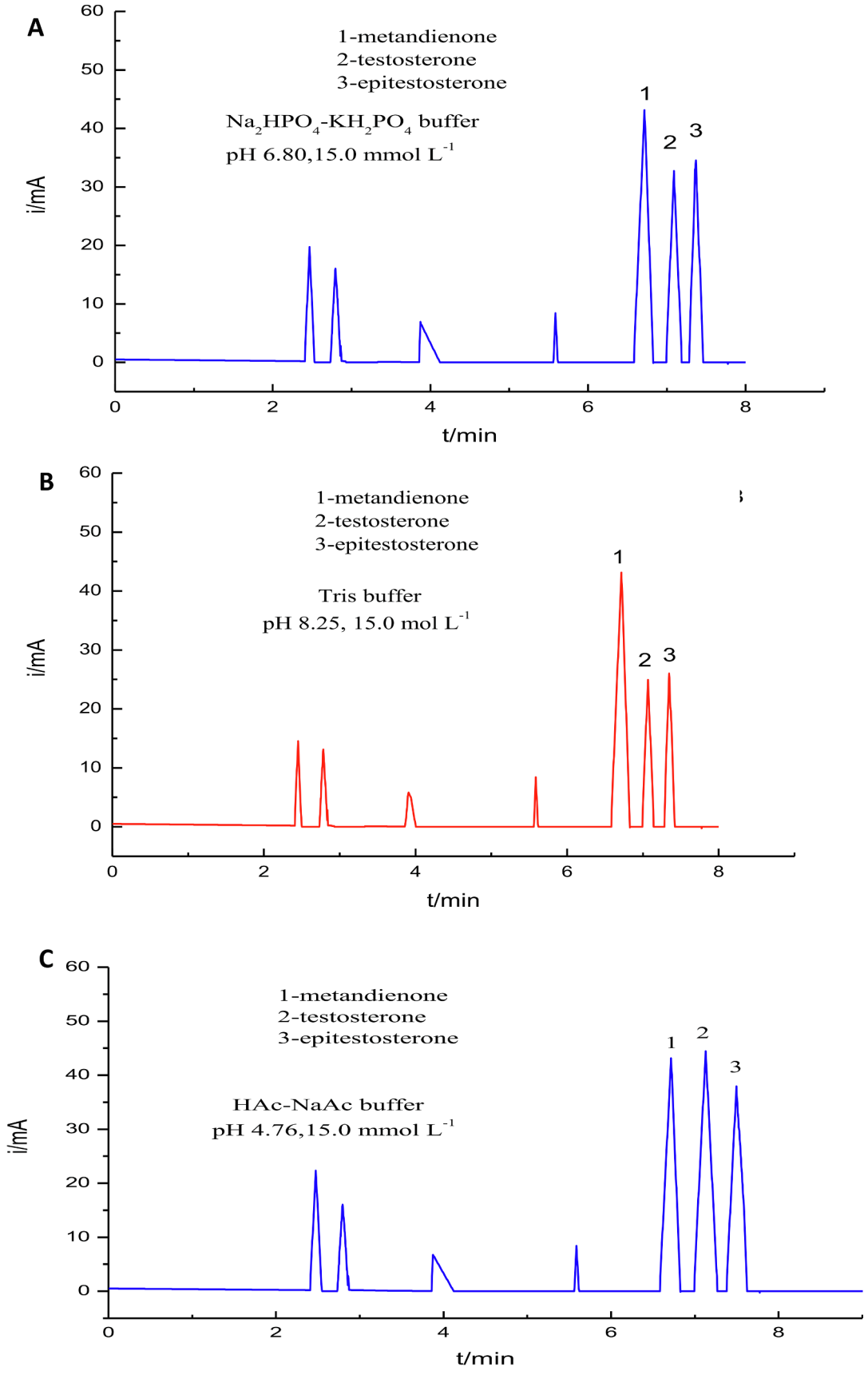

Fig. 4. The buffer effect on signal intensity and resolution.

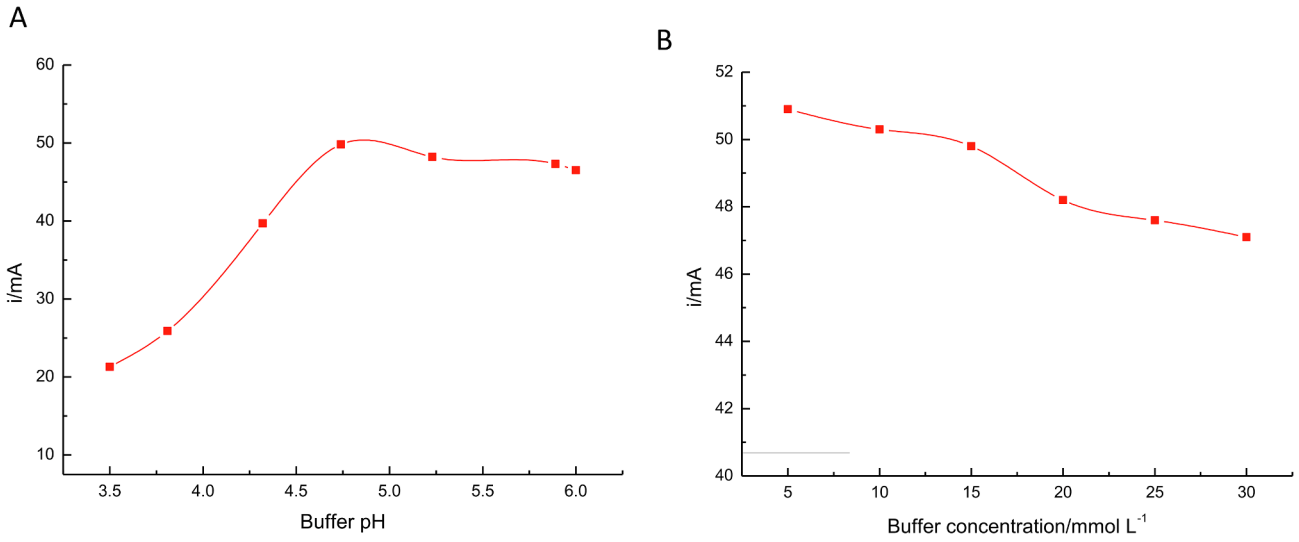

Fig. 5. The HAc-NaAc buffer pH value (A) and concentration (B) effect on signal intensity.

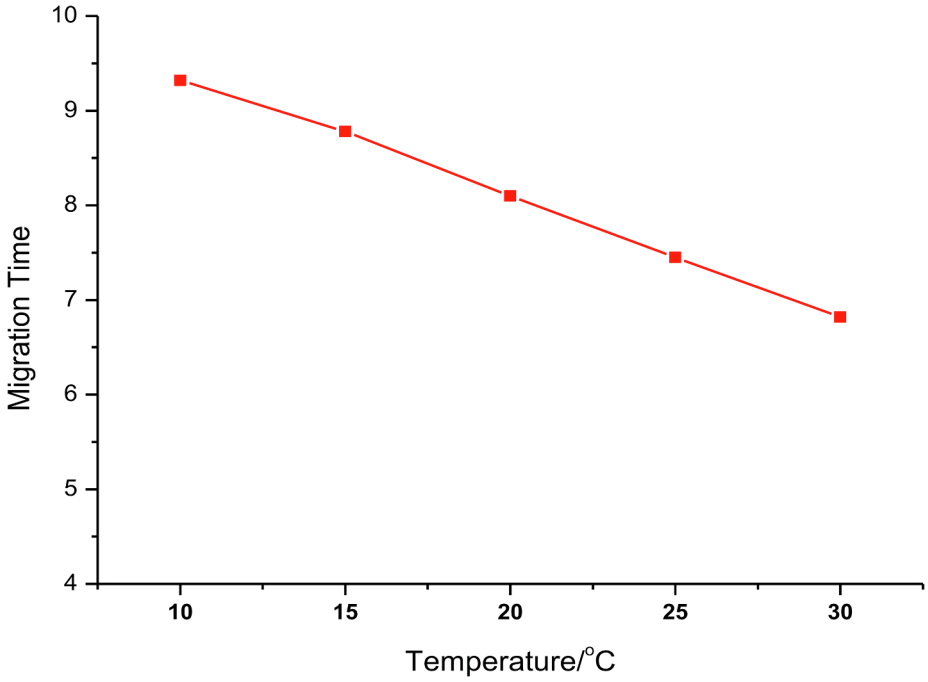

Fig. 6. The effect of temperature.

shown in Table 1. It can be seen that the recovery ratio of samples was 99.5%–101.1%.

The method for determining epitestosterone was the same as that for determining testosterone. As shown in Table 2, the recovery ratio of samples was 99.0%–100.5%.

Six healthy volunteers were randomized into two groups, with three volunteers in each group. One group took the testosterone, while the other group took placebo. After 36 h, urine sample was collected from each volunteer. All urine samples were analyzed by CE and GC–MS. The results showed that the results of CE were consistent with that of GC–MS. The volunteers taking the testosterone showed positive results, while those taking placebo showed negative results. The results of two methods are shown in Table 3.

4. Conclusions

We established a novel method for determining the testosterone/epitestosterone concentration ratio in the urine by capillary electrophoresis. The urine samples were firstly purified by Sep-Pak C18 SPE column. The optimal experimental conditions were running buffer pH = 4.74, 15.0 mmol L^{-1} HAc-NaAc, separation voltage 25 kV, temperature 25 °C, sample injection pressure 3.43 × 10^3 Pa and duration 10 s. The testosterone and epitestosterone linear range were determined as 8.0–960.0 ng mL^{-1}, and their detection limits were 4.6 and 4.5 ng mL^{-1}, respectively. The relative standard deviation was less than 0.36%. This method can directly determine testosterone/epitestosterone concentration ratio in human urine.

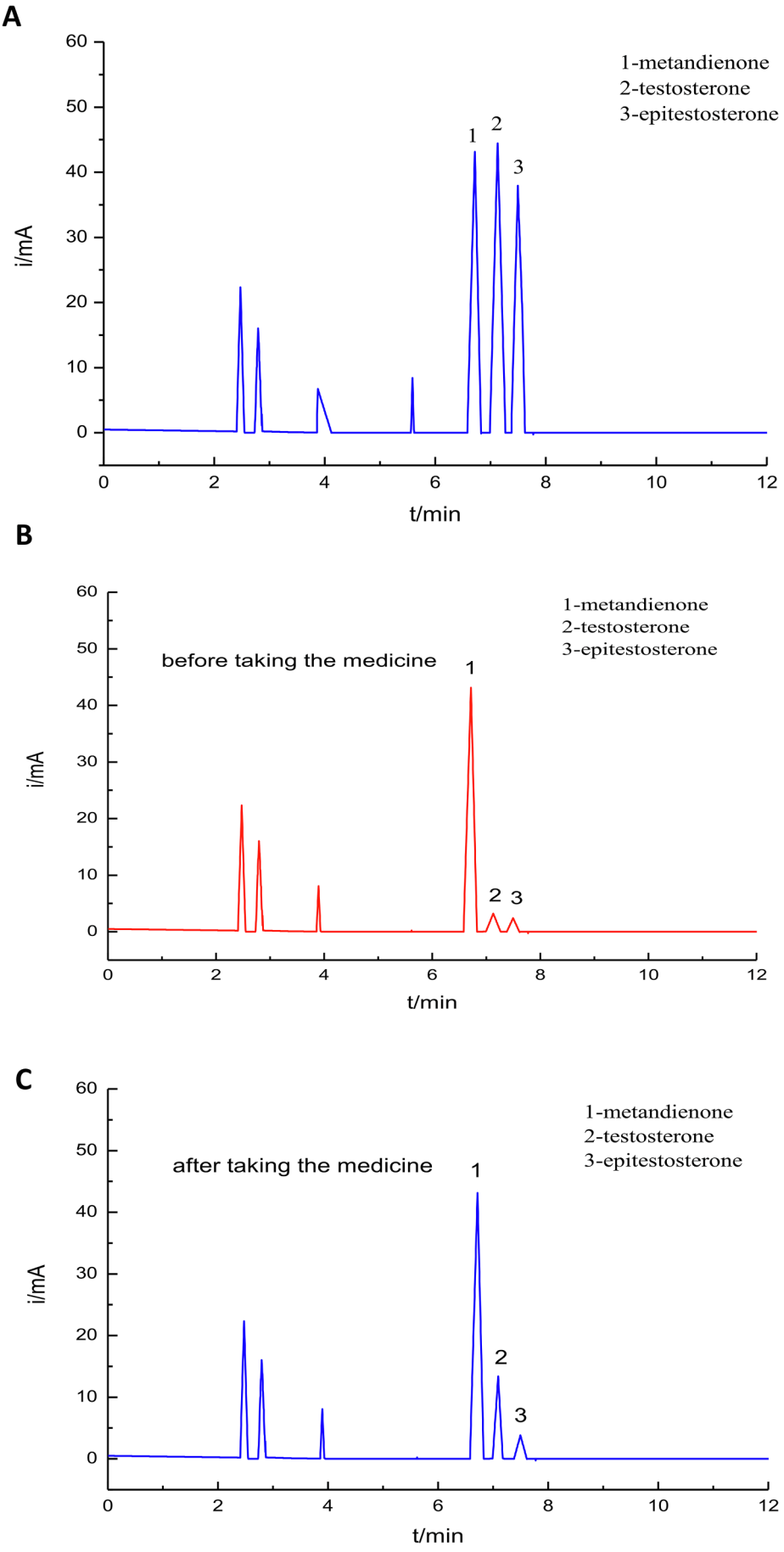

Fig. 7. Electrophoregram of testosterone, epitestosterone and metandienone separation.

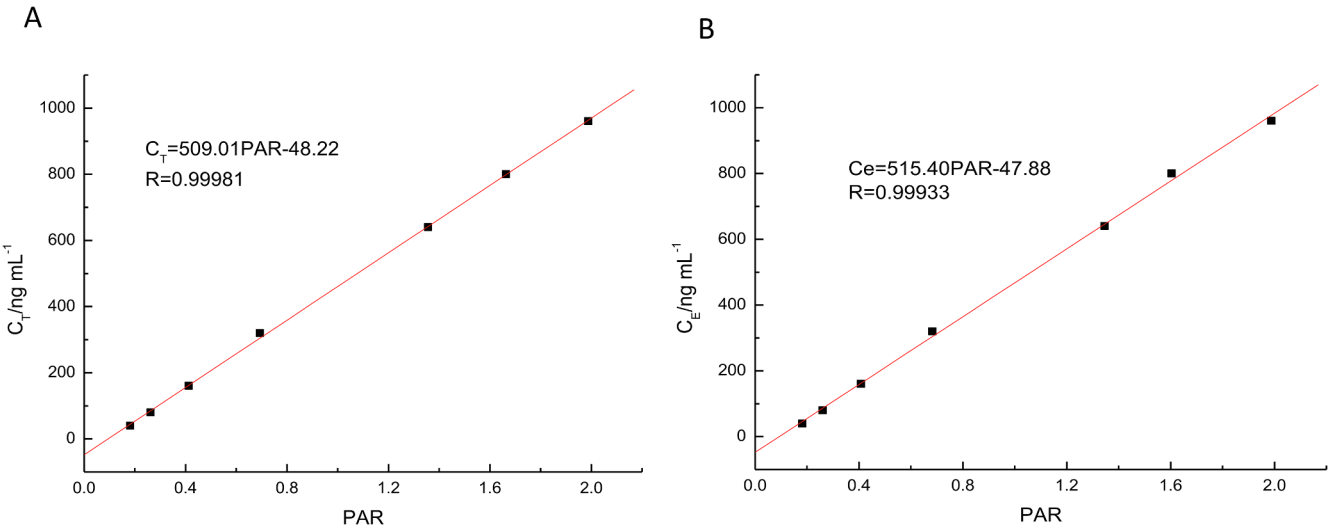

Fig. 8. Standard curve of testosterone (A) and epitestosterone (B).

Table 1
Determination results of testosterone (n = 5).

No.	Added testosterone concentration/ng mL^{-1}	Found testosterone concentration/ng mL^{-1}	Recovery Ratio/%	RSD/%
1	0	71.2	–	0.26
2	40.0	111.0	99.5	0.31
3	80.0	150.9	101.1	0.29

Table 2
Determination results of epitestosterone (n = 5).

No.	Added epitestosterone concentration/ng mL^{-1}	Found epitestosterone concentration/ng mL^{-1}	Recovery Ratio/%	RSD/%
1	0	70.4	–	0.28
2	40.0	110.0	99.0	0.36
3	80.0	150.8	100.5	0.31

Table 3
CE method was compared with the method of GC–MS (testosterone/epitestosterone).

No.	1	2	3	4	5	6
CE determination	1.46	19.80	22.36	23.57	4.23	3.25
GC–MS determination	1.49	20.56	22.68	23.89	4.53	3.42

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Author contribution statement: BD, YD and RS designed the study protocol. JZ, JW and LL contributed to the experiment of the study. BD wrote the manuscript, YD, BD and JZ revised the manuscript. All authors contributed to reviewing, revising the manuscript and agreed on the final draft.

Disclosure Statement

The authors declare that they have no conflicts of interest.

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