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Highly sensitive transient isotachophoresis sample stacking coupling with capillary electrophoresis-amperometric detection for analysis of doping substances

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ABSTRACT

A simple and effective method of capillary electrophoresis-amperometric detection (CE–AD) coupled with transient isotachophoresis (tITP) was developed for the trace determination of doping substances. Compared with the conventional capillary electrophoresis method, the maximum enhancement factor in terms of peak heights was up to 5500-fold when the tITP technique was adopted. Under the optimum conditions, the detection limit (S/N = 3) for methylephedrine (MDP), celiprolol (CEL), sotalol (SOT) and indapamide (IDP) were 4.2×10^{-14}, 6.3×10^{-13}, 5.8×10^{-14} and 9.5×10^{-13} mol L^{-1}, respectively. The RSDs of four analytes were 1.0–2.3% for migration time and 2.6–3.8% for peak current, respectively. The proposed method was successfully applied to determine the contents of SOT and IDP in real urine sample, and the excretion curve of IDP within 48 h was also investigated. The recoveries of the four doping in urine ranged from 90.0 to 102%.

1. Introduction

Doping control has been a hot topic in analysis research. The World Anti-Doping Agency (WADA) has established a list of prohibited substances including stimulants, β-block and diuretics. Methylephedrine (MDP) is a stimulant, celiprolol (CEL) and sotalol (SOT) are beta-blocking agents, and indapamide (IDP) is a diuretic. Various methods have been developed to control illegal use of the four doping including gas chromatography–mass spectrometry (GC–MS) [1,2], liquid chromatography–mass spectrometry (LC–MS) [3,4] and high performance liquid chromatography (HPLC) [5–7]. Though GC–MS is confirmed as the official method, considering different physical and chemical properties of various kinds of doping, many time-consuming and complicated derivatization steps are usually needed before analysis. Furthermore, LC–MS and HPLC method requires further purification and pre-concentration procedure due to the complex matrix and low content of targets. Besides, the expensive MS instrument needs much cost. Therefore, it is necessary to develop a simple and low-cost method for simultaneous determination of doping substances.

Capillary electrophoresis (CE) is a mature technique for separation and it has several advantages over other separation techniques, such as high efficiency, low consumption of sample, especially no consumption of organic solvents. Therefore, it has become a useful analytical technique for drug analysis [8,9] and doping [10,11]. The electrochemical detector, especially the amperometric detection (AD) is more sensitive than UV detector, while it is much cheaper and simpler than LIF and MS detectors. CE–AD provides excellent sensitivity and high selectivity towards electroactive substances, so it is preferred to be used in drugs analysis and in vivo biological analysis. Our group has also developed these methods for determination of doping substances in urine by CE–AD in the previous works [12,13]. However, due to the low injection volume (pL–nL), the sensitivity of the CE was greatly limited.

It is essential to develop some sample stacking method to meet the requirement of trace analysis. Some off-column sample stacking approaches, such as solid-phase extraction [14,15], solid-phase micro-extraction [16], and liquid-phase micro-extraction [17] have been proposed. However, these methods were obviously time-consuming and tedious. As a comparison, on-column sample stacking methods are more convenient and rapid. pH-mediated sample stacking [18], on-column transient isotachophoresis (tITP) [19,20], field-amplified [21,22] and large-volume sample stacking [23] have been applied to the pre-concentration of the polar analytes, which improve the analytical sensitivity.

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As the determination of the doping was usually carried out in the urine samples, high ionic strength in sample is inevitable. In on-column stacking methods, tITP mode is especially suitable for high-salt sample. tITP can be favorably used as a pre-concentration technique for diluted samples in CE, especially in those where ionic matrix constituents do not allow an efficient sample stacking due to little conductivity differences between sample zone and background electrolyte. In tITP mode, the sample solution is introduced between the leading electrolyte (LE) solution and the terminating electrolyte (TE) solution with higher and lower electrophoretic mobilities, respectively, than the sample compounds. When the voltage is applied, a potential gradient is established between the electrolyte and sample zones, and the field strength was inversely proportional to the mobility of the ions in every capillary zone. At equilibrium, every analyte moves as a discontinuous band according to its respective mobility, with high mobility ions migrating prior to low mobility ions. During the moving process, these stacking of sample zones always migrate with the identical velocity of leading ion [24]. In this way, high concentration with narrow sample zone can be obtained and sample pre-concentration would be realized.

The aim of this work was to develop a simple and high sensitive method for the simultaneous determination of four doping in urine sample, and the on-line sample stacking technique tITP coupled with CE–AD was studied. By using the developed method, high sensitivity (with detection limit of 10^{-14} mol L^{-1} level) of these target compounds namely MDP, CEL, SOT and IDP were obtained. The proposed method has been applied to determine these doping in human urine samples without any other facilities. It implied that the tITP–CE–AD method had great potentiality in doping control.

2. Experimental

2.1. Apparatus

In this study, a home-made CE–AD system was established. A ±30 kV high-voltage dc power supply (Shanghai Institute of Nuclear Research, Shanghai, China) provided the separation voltage between the two ends of capillary. An uncoated fused-silica capillary (70 cm length × 25 μm i.d. × 360 μm o.d., Yongnian Optical Fiber Factory, Hebei, China) was used in this study. A three-electrode electrochemical cell including a 300 μm diameter carbon disc working electrode, a platinum auxiliary electrode and an Ag/AgCl (saturated KCl) electrode as reference electrode, was connected to a BAS LC-4C amperometric detector (Bioanalytical Systems Inc., West Lafayette, IN, USA). The data were recorded by the TL9902 analytical system of chromatogram (Beijing Teleh Electronic Tech Co., Ltd.).

2.2. Chemicals

MDP, CEL, SOT and IDP were purchased from the Chinese Institute of Biological Products Control (Beijing, China). Indapamide tablets were obtained from Tianjin Lisheng Pharmaceutical Co., Ltd. Sotalol tablets were purchased from Lunan Beite Pharmaceutical Co., Ltd. All the reagents used were of analytical grade and bought from local commercial sources.

2.3. Preparation of standard solution and buffer solutions

Stock solution of MDP, CEL, SOT and IDP with the concentration of 1.0 mmol L^{-1} were all prepared in 3/7 (v/v) methanol-buffer solution then diluted with running buffer, respectively. 200.0 mmol L^{-1} H_3BO_3–$Na_2B_4O_7$ buffer solution (BB) was firstly prepared with 50.0 mmol L^{-1} $Na_2B_4O_7$ and 200.0 mmol L^{-1} H_3BO_3 solutions. The pH value of BB was adjusted with 200.0 mmol L^{-1} sodium hydroxide and hydrochloric acid solutions. Then the 200.0 mmol L^{-1} BB were diluted with fresh deionized water to the desired concentration. The running buffer used for electrophoresis in this experiment was 25.0 mmol L^{-1} BB (pH 8.8).

All experiment solutions were prepared with deionized water and passed through polypropylene filter (0.22 μm) prior to use.

2.4. Preparation of human urine sample

Ten healthy male volunteers aged from 24 to 28 were divided into two groups, and five volunteers for each. One group took a single dose of SOT tablets (80 mg) and a single IDP tablets (2.5 mg) at the same time. The other group took a single dose of IDP tablets (2.5 mg). All administrations abided by the principle of Public Health Bureau of China. The blank urine was collected before administration of drugs. The urine samples were collected at regular intervals and then stored in the refrigerator at −4 °C. Before analysis, the sample was thawed at room temperature and centrifuged for 15 min at 4000 rpm in order to remove precipitated proteins and other particulate substances.

2.5. Capillary precondition

New capillaries were pre-treated using 0.1 mol L^{-1} sodium hydroxide solution for 4 h, then rinsed with 0.1 mol L^{-1} HCl and deionized water for 10 min, respectively. Between each run, the capillary was rinsed with 0.1 mol L^{-1} sodium hydroxide solution, deionized water and running buffer for 5 min in sequence.

3. Results and discussion

3.1. Establishment of CE–AD conditions for four doping

As amperometric detection (AD) method was adopted in this study, the potential of the working electrode (ranging from 800 to 1100 mV), which greatly affects the peak current responses, was tested. And 1000 mV potential was applied to keep high sensitivity, good stability and high reproducibility.

The separation of these four compounds was a challenging task. The factors including the composition of the running buffer solution and separation voltage were considered as important parameters in this CE separation. The composition of running buffer was firstly studied, commonly used buffer such as BB solution and Na_2HPO_4–NaH_2PO_4 (PBS) solution were tested. The experiments showed that better resolution and sensitivity were obtained when the BB solution was used as the running solution. After further investigation, the optimum separation conditions were as follows: 25 mmol L^{-1} BB buffer with pH 8.8 was chosen as running buffer and 15 kV as separation voltage. Under the optimum CE–AD conditions, the electropherogram for the four doping was shown in Fig. 1.

3.2. Selection of LE and TE system

Compared with CE–UV, CE–AD is more sensitive and selective, but it still cannot solve the problem of trace analysis well. Indeed, the target compounds existed in urine were low to sub-μmol L^{-1} level. In addition, body fluids, especially plasma or urine contain high concentrations of protein and salt. Therefore, extensive sample pretreatment was usually essential for the quantification of these doping in body fluids. To improve the sensitivity, on-line pre-concentration procedure was firstly considered rather than complicated off-line sample treatment [25].

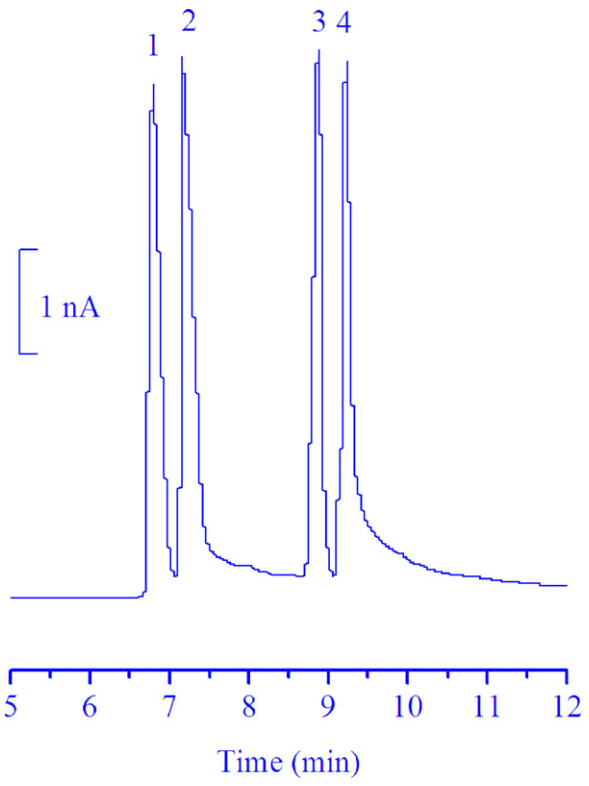

Fig. 1. The electropherogram under the optimum separation conditions. (1) MDP (1.0×10^{-4} mol L^{-1}); (2) CEL (3.0×10^{-4} mol L^{-1}); (3) SOT (1.5×10^{-4} mol L^{-1}); (4) IDP (5.0×10^{-4} mol L^{-1}). Fused-silica capillary: 70 cm × 25 μm; injection: 15 kV × 10 s; separation voltage: 15 kV; buffer solution: 25.0 mmol L^{-1} BB (pH 8.8); working potential: 1000 mV; the working electrode: 0.3 mm carbon disk electrode; the auxiliary electrode: platinum wire; the reference electrode: Ag/AgCl; temperature: 20 ± 0.5 °C.

As an effective on-line sample stacking technique, tITP is performed with a discontinuous buffer solution, and it is suitable for the analysis of high ion strength samples. In tITP mode, the sample zone is introduced between the background electrolyte of higher (leading electrolyte, LE) and lower (terminating electrolyte, TE) electrophoretic mobilities. Considering the following parameters including the type, concentration and injection time of LE and TE play crucial roles in the performance of tITP system [26], their influence were studied carefully.

Several recommended electrolyte systems [27], NaCl, KCl, HCl, and Tris as LE while β-alanine, histidine, glutamic acid, ethanolamine and glycine as TE were investigated in detail, respectively. The experimental results indicated that the signal-to-noise ratio of each analytes was drastically increased in the presence of HCl–Tris as LE and glycine as TE system in comparison with those of other electrolytes.

The amount of leading ion plays an important role in the ITP system performance. Therefore, the concentration and the injection amount of the LE were investigated. The effects of the LE concentration on the sensitivity were studied by use of 5.0, 10.0, 15.0, 20.0 and 25.0 mmol L^{-1} LE solutions, respectively. The results from Fig. 2(a) indicated that the highest peak current responses of four analytes were achieved when 10.0 mmol L^{-1} of LE was employed.

Additionally, the optimization objective of the loaded amount of LE solution (10.0 mmol L^{-1}) is to obtain the highest enrichment factors with good resolutions. As increasing the LE injection time when the injection voltage was 15 kV, the peak current response of four analytes was shown in Fig. 2(b). When 20 s was employed for the loading of LE, good resolution and sensitivity was obtained. Thus in the experiment the injected amount of the LE (10.0 mmol L^{-1}) was 15 kV × 20 s.

The glycine was chosen as the TE, and special care was taken to study the effect of the TE. At a fixed injection time of glycine, the concentration of TE in the range of 5.0–25.0 mmol L^{-1} was tested. As shown in Fig. 3, when 15.0 mmo/L glycine was used as TE, the highest peak currents were exhibited for all four analytes with good resolution. Furthermore, the injection amount of the TE was also studied. It was found that when the injection amount of TE was 15 kV × 20 s, the maximum responses of peak current could be obtained.

3.3. Optimization of the sample amounts for ITP

Two factors would affect the enhancement of analyte in CE stacking. One is the narrowing of analyte bands in the column. As the peak width of analyte is condensed, the peak height is dramatically increased, which results in a high signal-to-noise ratio and low detection limit. The other is the amount of sample loaded into the column. Because the width of the peak is significantly reduced by the stacking procedure, much larger sample volumes may be injected without losing separation efficiency. These two factors would give a greater mass of analyte in capillary and therefore a greater response at the detector can be obtained [28].

Compared with field-amplified sample stacking method, much larger volume of sample can be injected into the capillary in tITP

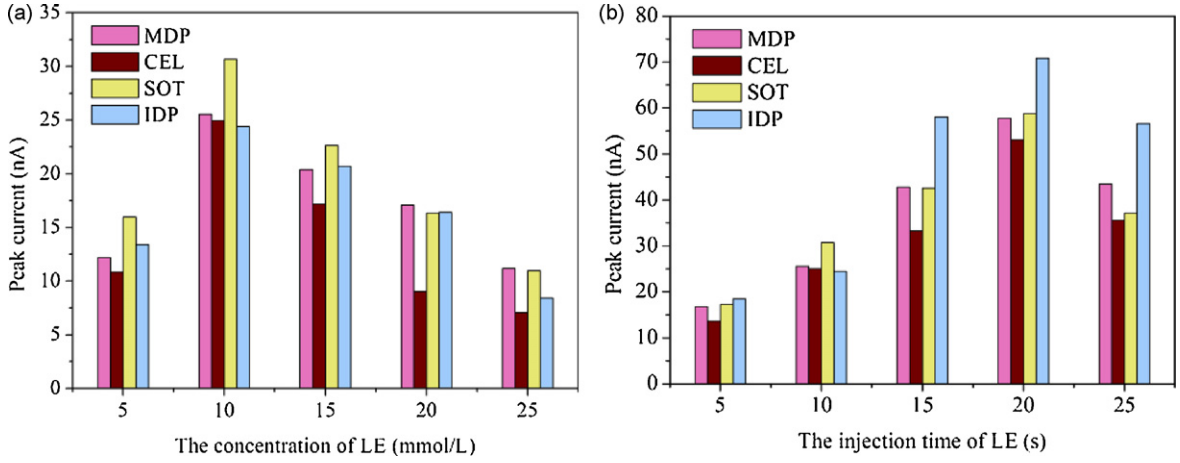

Fig. 2. (a) Effects of the concentration of LE on peak current. (b) Effects of time injection of LE on peak current. MDP (2.0×10^{-5} mol L^{-1}); CEL (2.6×10^{-5} mol L^{-1}); SOT (4.0×10^{-5} mol L^{-1}); IDP (3.8×10^{-5} mol L^{-1}). Other conditions were the same as Fig. 1.

Table 1
The stacking efficiencies of MDP, CEL, SOT and IDP.[a].

Analyte	Concentration without tITP (C_1, μmol L^{-1})	Peak currents without tITP (I_1, nA)	Concentration after tITP (C_2, nmol L^{-1})	Peak currents after tITP (I_2, nA)	Stacking efficiency (fold)[b]
MDP	0.12	1.11	0.11	5.60	5504
CEL	1.5	1.14	1.4	5.74	5394
SOT	0.12	1.15	0.11	5.45	5170
IDP	1.3	1.15	1.2	5.43	5115

[a] LE: 15 kV × 20 s, 10.0 mmol L^{-1} HCl–Tris; TE: 15 kV × 20 s, 15.0 mmol L^{-1} glycine; electrokinetic injection 15 kV × 45 s, other conditions were as Fig. 1.
[b] Stacking efficiency = $(C_1/C_2) \times (I_2/I_1)$.

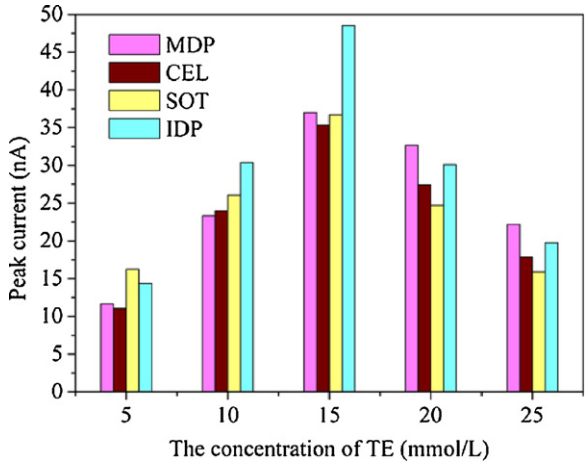

Fig. 3. Effects of concentration of TE on peak current. MDP (1.0×10^{-6} mol L^{-1}); CEL (1.3×10^{-6} mol L^{-1}); SAL (2.0×10^{-6} mol L^{-1}); FMT (1.9×10^{-6} mol L^{-1}). LE: 15 kV × 20 s, 10.0 mmol L^{-1} HCl–Tris; other conditions were the same as Fig. 1

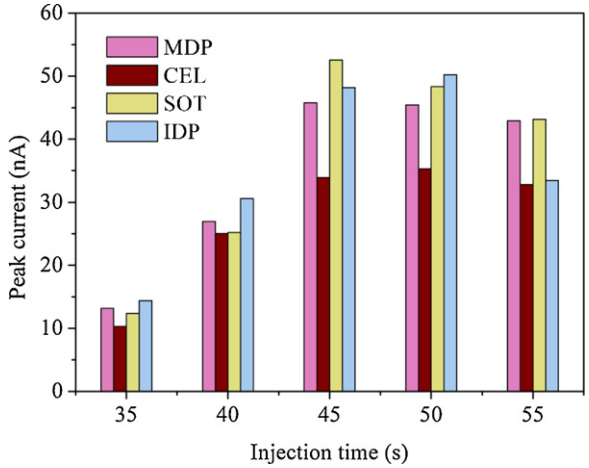

Fig. 4. Effects of time injection of samples on peak current. MDP (5.0×10^{-8} mol L^{-1}), CEL (6.5×10^{-8} mol L^{-1}), SOT (10.0×10^{-8} mol L^{-1}), IDP (9.5×10^{-8} mol L^{-1}). LE: 15 kV × 20 s, 10.0 mmol L^{-1} HCl–Tris; TE: 15.0 mmol L^{-1} glycine; other conditions were the same as Fig. 1

method because there is no restriction in low sample conductivity [29]. The injection time was tested from 35 to 55 s at 15 kV. As shown in Fig. 4, the peak current responses of four analytes increased with the extension of the sample injection time in the range of 35–45 s. However, peak width was broadened when injection time was greater than 45 s and then current response fell down. Therefore, 45 s was selected as sampling time with satisfactory results in this experiment.

The tITP pre-concentration were carried out by combining the optimum values of each parameter discussed above. The results suggested that the peak widths for doping in tITP sample stacking mode were much narrower than those in the conventional injection mode, and the sensitivity was thus drastically improved after stacking. The stacking efficiency (peak height enhancement factors) could be calculated by multiplying the peak height ratios with the concentration dilution factors [30], and the results were shown in Table 1. Compared with the conventional injection, the tITP provided a stacking efficiency of 5504-, 5394-, 5170-, and 5115-fold in standard solutions for MDP, CEL, SOT and IDP, respectively. It was shown that this method was very sensitive.

3.4. Method validation

For validation of the tITP method, linearity, sensitivity, accuracy and precision were evaluated as follows. A series of standard solutions of four doping diluted in the blank urine sample with different concentrations were analyzed under the optimum conditions, and each concentration was measured by three replicate injections. The results were listed in Table 2. The linearity was good, and the correlation coefficients for the four analytes was better than 0.9991. The detection limits (S/N = 3) were 4.2×10^{-14}, 6.3×10^{-13}, 5.8×10^{-14}, and 9.5×10^{-13} mol L^{-1} for MDP, CEL, SOT and IDP, respectively. Among these, the lowest detection limit of compound MDP was 42 fmol L^{-1} level. It has reported by World Anti-Doping Agency (WADA) that the LODs of MDP, CEL, SOT and IDP were about 5.6×10^{-6}, 2.6×10^{-6}, 3.7×10^{-6}, and 2.7×10^{-6} mol L^{-1}, respectively [31]. As for the tITP pre-concentration mode, the LODs had reduced about 10^7 times comparing with those of WADA. Above results showed that this method was very helpful to improve the sensitivity.

To testify the precision of the CE–Titp–AD method, the relative standard deviation (RSD) of both the peak current and migration time were studied and the experimental results were shown in

Table 2
Regression equation, linear ranges and detection limits of MDP, CEL, SOT and IDP.[a].

Compound	Regression equation $y = ax + b$[b]	R	Linear ranges (pmol L^{-1})	Detection limits[c] (pmol L^{-1})
MDP	$y = 0.050x + 0.0795$	0.9992	0.11–189	0.042
CEL	$y = 0.0040x + 0.0998$	0.9995	1.7–1590	0.63
SOT	$y = 0.049x + 0.0721$	0.9991	0.16–170	0.058
IDP	$y = 0.0045x + 0.0573$	0.9994	3.7–3200	0.95

[a] All conditions were the same as Table 1.
[b] Where the y and x are the peak current (nA) and the concentration of four analytes (pmol L^{-1}), respectively.
[c] (S/N = 3) the detection limits are estimated on the basis of a signal-to-noise ratio of 3.

Table 3
Intra-day ($n = 5$) and inter-day ($n = 15$)[a] precision for four doping with the same standard solution.[b].

Compound	Frequency	t (min) (mean ± SD)	RSD (%)	Peak current (nA) (mean ± SD)	RSD (%)
MDP	Intra-day	6.34 ± 0.11	1.74	6.13 ± 0.21	3.42
	Inter-day	6.45 ± 0.15	2.32	5.98 ± 0.18	3.01
CEL	Intra-day	6.68 ± 0.12	1.80	6.82 ± 0.25	3.66
	Inter-day	6.75 ± 0.14	2.07	6.60 ± 0.20	3.03
SOT	Intra-day	8.28 ± 0.08	0.97	6.76 ± 0.23	3.40
	Inter-day	8.41 ± 0.17	2.02	6.71 ± 0.18	2.68
IDP	Intra-day	9.04 ± 0.13	1.44	6.38 ± 0.24	3.76
	Inter-day	9.17 ± 0.16	1.74	6.27 ± 0.18	2.87

[a] Mean values of analytes performed on three different days.
[b] All conditions were the same as Table 1.

Table 3. Mean values were calculated by five consecutive injections of the standard solution performed each day and over 3 days under the same conditions. RSDs for inter-day and intra-day were less than 3.03 and 3.76%, respectively, which demonstrated that this method was of good reproducibility.

3.5. Urine sample analysis

IDP is a kind of diuretics, it can be used illegally in sport competition to reduce the concentration of the other banned substances in urine, and sometimes it was taken together with other kinds of doping. In this work, oral doses of IDP (2.5 mg) and SOT (80 mg) were simultaneously taken by healthy volunteers, and the corresponding urine samples were studied. The preparation of human urine sample was described in Section 2.4.

Prior to analysis, the urine samples were thawed at room temperature and centrifuged just with simple filtration, and then injected into the capillary for determination by conventional injection mode. The same urine sample was diluted 10-fold with running buffer solution (25.0 mmol L^{-1} BB, pH 8.8) and then was analyzed by the tITP sample stacking mode. Fig. 5 illustrates the electropherograms of the blank urine sample (a) and the real urine sample (b) of volunteers taking corresponding doses of SOT and IDP after 2 h. In Fig. 5(B), in order to identify the peaks of SOT and IDP, the standard solutions were added to the urine sample and then it was found that the peak current responses were obviously increased accordingly. The figure indicated that there was neither SOT nor IDP in the blank urine sample. The results shown in Fig. 5(A) (without pre-concentration) suggested that high concentration level of SOT (about 6.0×10^{-5} mol L^{-1}) can be detected in the conventional injection mode, but IDP in low concentration cannot be detected. In addition, SOT suffered from rather poor peak shape at its fairly high concentrations in the true urine matrix. However, Fig. 5(B) illustrated that both SOT and IDP could be successfully detected in real urine samples without the interference of endogenetic ground substances and interfering compounds in the tITP stacking mode, and good peak shapes of two compounds can be also obtained simultaneously. The content was so low (the concentration of IDP in urine is no more than nmol L^{-1} level) that it was difficult to be detected directly without pre-concentration in body fluids. It is indicated that the ITP sample stacking was excellent.

Furthermore, the excretion curve of the IDP was investigated in order to monitor the metabolism behaviors of doping. Another healthy volunteer was given with oral doses of IDP (2.5 mg). Urine samples were collected before administration (blank sample) and up to 48 h (three times of half-life) after administration at different collection periods. The IDP urine samples were diluted 10-fold with running buffer solution (25.0 mmol L^{-1} BB, pH 8.8). Fig. 6 illus-

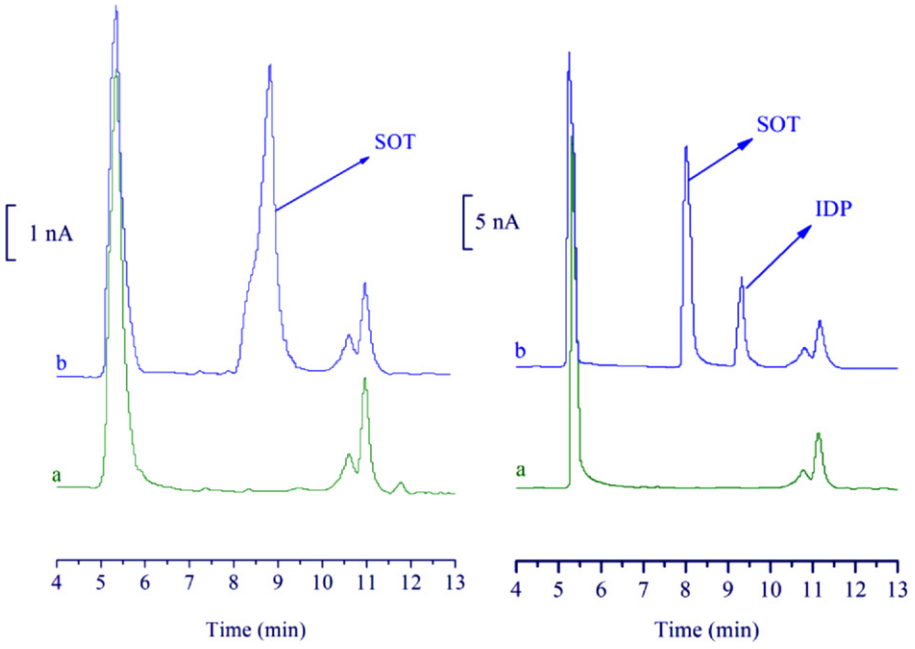

Fig. 5. The comparison electropherogram of blank urine (a) and the doping in urine sample (b) between without concentration (A) and after concentration (B). All conditions were the same as Table 1.

Table 4
Recoveries of four doping in the urine samples ($n = 5$).[a].

Doping	Content in urine sample ($\times 10^{-10}$ mol L^{-1})	Added ($\times 10^{-10}$ mol L^{-1})	Found ($\times 10^{-10}$ mol L^{-1})	Recovery (%)	RSD (%)
MDP	0	0.50	0.46	92.0	4.31
		1.00	0.93	93.0	3.84
CEL	0	5.00	4.57	91.4	4.62
		10.0	9.88	98.8	3.57
SOT	0.50	0.50	0.95	95.0	3.92
		1.00	1.41	94.0	3.21
IDP	1.00	5.00	5.51	91.8	4.43
		10.0	11.2	102	3.01

[a] All conditions were the same as Table 1.

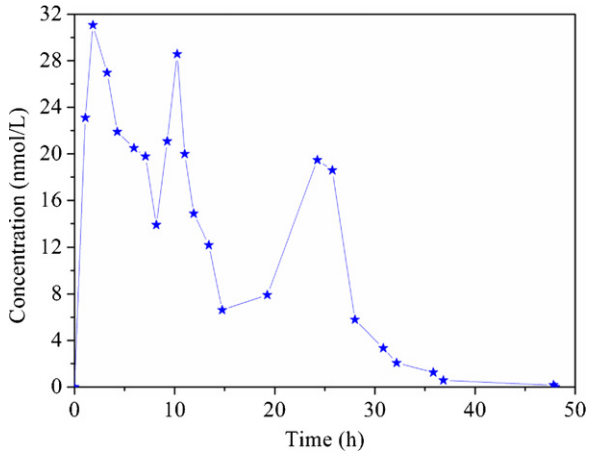

Fig. 6. Study of the urinary excretion of IDP after oral administration by healthy volunteer. All conditions were the same as Table 1.

trated the varied concentration of IDP with tITP–CE–AD method during the different excretion time. Seen from the excretion curve, two crest values and "saddle" shape were observed. This may be due to the hepatoenteral circulation of IDP. That is, the drugs were excreted from the bile, and then reabsorbed in the intestinal, so the excretion curve appeared more than one peak value. The experiment results showed that the total unchangeable percentages of IDP in all urine samples were 7.4%, which were calculated according to the regression equations. These results were in agreement well with the literature [32], which was ~7.0% of IDP excreted unchangeably in human urine.

At last, recovery experiments were used to validate the accuracy of the ITP sample stacking method. The recovery experiments for four targets were carried out by adding known concentration of mixture standards solution into the diluted urine samples which were collected after administration SOT tablet and IDP tablet. And then the samples were detected under the optimum preconcentration conditions. Table 4 showed the analytical results of the urine sample and the recoveries with this method. The average recoveries ranged between 90.0 and 102%, and the RSDs were less than 4.6% ($n = 5$). The results demonstrated that tITP–CE–AD approach for doping detection was reliable.

4. Conclusions

A simple, sensitive tITP–CE–AD method for simultaneous analysis of four doping (MDP, CEL, SOT and IDP) has been firstly developed in this paper. Excellent on-line sample preconcentration effect was achieved by selective discontinuous electrolyte system. The present research exhibited several advantages compared with former reported work. Firstly, by this tITP on line stacking technique, highest sensitivity and enhancement factor was gained, where the detection limit was lower than 4.2×10^{-14} mol L^{-1} and enhancement factor was higher than 5500-fold in terms of peak heights. In addition, compared with CE–AD, tITP–CE–AD method appear to much higher sensitivity and resolution in the determination of doping substances in human urine samples. Finally, the excretion curve of IDP was obtained to demonstrate the practicability of this method. Although this work was focused on only four analytes in urine, undoubtedly, the tITP–CE–AD system showed very promising potentialities in the identification and quantitation of trace analytes presented in biological matrices.

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