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Extraction of testosterone and epitestosterone in human urine using aqueous two-phase systems of ionic liquid and salt

Chiyang He, Shehong Li, Huwei Liu, Kean Li, Feng Liu *

The Key Laboratory of Bioorganic Chemistry and Molecular Engineering, Ministry of Education, College of Chemistry, Peking University, Chengfu Road, Beijing 100871, China

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Abstract

Based on aqueous two-phase systems (ATPS) consisting of 1-butyl-3-methylimidazolium chloride, a hydrophilic ionic liquid (IL), and K_2HPO_4, a new and simple extraction technique, coupled with a reversed-phase high performance liquid chromatography (RP-HPLC), was developed for the simultaneous concentration and analysis of testosterone (T) and epitestosterone (ET) in human urine. Under the optimal conditions, the extraction efficiencies for both analytes were 80–90% in a one-step extraction. The method required only 3.0 mL of urine and a single hydrolysis/deproteinization/extraction step followed by direct injection of the IL-rich upper phase into HPLC system for analysis. The method has been satisfactorily applied to the analysis of T and ET in human urine with detection limits of 1 ng/mL and linear ranges of 10–500 ng/mL for both compounds. Compared with conventional liquid–liquid extraction or solid phase extraction, this new method is much "greener" due to no use of volatile organic solvent and low consumption of IL. The proposed extraction technique opens up new possibilities in the separation of other drugs.

Keywords: Extraction; Ionic liquid; Aqueous two-phase systems; Testosterone; Epitestosterone; Human urine

1. Introduction

The use of anabolic androgenic steroids (AAS) to improve athletic performance is one of the widest problems in human sports despite the fact that it was banned in 1976. Testosterone, one of the endogenous AAS, was forbidden in sports by the International Olympic Committee (IOC) since 1983. This ban was expressed as the testosterone (T)/epitestosterone (ET) ratio (T/ET > 4 in the 2005 Prohibited List published by the World Anti-Doping Agency). If the T/ET ratio greater than four to one in the urine is reported, further investigation will be obligatory in order to determine whether the ratio is due to physiological or pathological condition.

Presently, there are several different testing methods for testosterone and epitestosterone, but the pretreatment processes are time-consuming and somewhat harmful to environment. In gas chromatography–mass spectrometry (GC–MS) method, hydrolysis, liquid–liquid extraction (LLE) or solid-phase extraction (SPE), and derivatization should be carried out prior to analysis of compounds, in which volatile organic solvents (VOCs) are used for concentration of analytes and usually evaporated to dryness [1]. The methods also suffer from the disadvantages of instability of derivatives and their thermal decomposition during analysis, resulting in that the reproducibility is not always sufficient [2,3]. High-performance liquid chromatography (HPLC) is another method of choice [4,5]. However, it can neither avoid the use and evaporation of VOCs during concentration of compounds by LLE or SPE and the relatively tedious pretreatment. Moreover, emulsion formation is another main drawback of LLE. Therefore, development of simple and "green" pretreatment methods is of great interest.

Aqueous two-phase system (ATPS) might be an alternative for extraction of T and ET from human urine samples.

* Corresponding author. Tel.: +86 10 62761187; fax: +86 10 62751708.
E-mail address: liufeng@pku.edu.cn (F. Liu).

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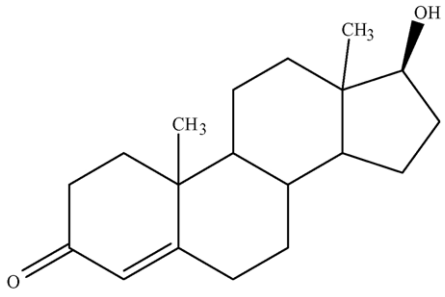
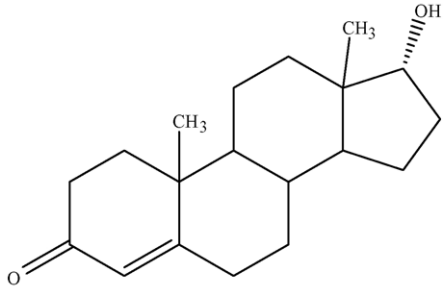
ATPS is usually composed of two or more polymers [6], a polymer and a salt [7], or two surfactants (including quaternary ammonium surfactants and anionic surfactants) [8,9]. Compared with conventional organic-solvent extraction or SPE, ATPS is considered to be environmentally friendly due to no use of VOC in the whole process. Its applications have been well documented [10–12]. However, most of phase-forming polymers and surfactants have high viscosity, form an opaque solution, and sometimes interfere with the analysis of analytes.

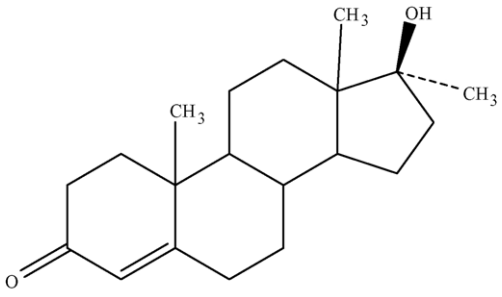
Recently, ionic liquids (ILs) have been gaining great exposure for potential use as green solvents and possible replacements for traditional VOCs. ILs are sometimes called molten salts with melting point below 100 °C and consist entirely of ionic species. Most of the ILs consists of a nitrogen-containing organic cation (such as imidazolium or pyridinium) and a large organic or inorganic anion, but they



Testosterone, T



Epitestosterone, ET



Methyltestosterone, MT

Fig. 1. Chemical structures of steroids.

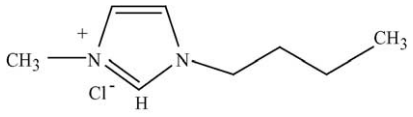


Fig. 2. Chemical structure of [C_4mim]Cl.

are very different from traditional quaternary ammonium salts due to their unique characters. Besides having many properties of conventional organic solvents, such as excellent solvation qualities, a variable viscosity range and a wide temperature range, ILs are nonvolatile and exhibit excellent chemical and thermal stabilities [13,14]. Furthermore, at least a million binary ionic liquids and 10^{18} ternary ionic liquids exist potentially [15], which enables the ILs to be effectively designed for various applications. ILs have been primarily explored for applications in synthesis [16], electrochemistry [17], catalysis [13,18], chromatographic separation [19,20], extraction processes [21–27], and mass spectrometry analysis [28]. More recently, a new type of ATPS consisting of IL and salts was reported for recycle, metathesis and study of the distribution ratios of short chain alcohols [29]. However, to our knowledge, no ATPS of IL and salt has been previously used for biochemicals or drugs separation.

The aim of this study was to develop a simple and "green" extraction method based on the ATPS of IL and salt as a new pretreatment strategy for the analysis of T and ET (Fig. 1) in human urine by HPLC. We selected 1-butyl-3-methylimidazolium chloride ([C_4mim]Cl, Fig. 2), a hydrophilic IL, as the phase-forming IL, and studied the phase behaviors of ATPS including phase diagrams and effect of salts. After optimizing the extraction conditions involving the type and amount of salts, the concentration of analytes, and temperature, the method was successfully applied to the analysis of T and ET in human urine samples.

2. Experimental

2.1. Materials and reagents

1-Butyl-3-methylimidazolium chloride ([C_4mim]Cl, ≥95%) was obtained from Fluka (Switzerland). Testosterone (T, >99%), methyltestosterone (MT, >98%), and epitestosterone (ET) were purchased from Acros (NJ, USA), Tokyo Kasei Kogyo (Tokyo, Japan) and Sigma (St. Louis, MO, USA), respectively. β-Glucuronidase from *Escherichia coli* was acquired from Sigma and stored at −20 °C. Sep-Pak C_{18} cartridges (500 mg/3 mL) were obtained from Waters (USA). Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany) and methanol (HPLC grade) was bought from Tianjin Shield (Tianjin, China). All other chemicals were of analytical grade.

Stock solutions of T, ET and MT were each prepared by dissolving in acetonitrile at the concentration of 1.0 mg/mL and stored at 4 °C, while working standard solutions were pre-

pared at 100 μg/mL by diluting the stock solution with acetonitrile. IL solution was prepared by dissolving [C_4mim]Cl in deionized water at the concentration of 0.4 g/mL. The solution of β-glucuronidase from *E. coli* was prepared at 50 mg/mL in 0.2 M phosphate buffer (pH 6.9) and stored at −20 °C.

2.2. Preparation of aqueous two-phase systems

To a 10 mL graduated tube, 3.0 mL water (which included 333.3 ng/mL T, MT or ET when the distribution behaviors of steroid were studied), 0.2 g IL (in order to economize IL and gain a higher enrichment factor) and a suitable amount of salt were added. After the mixture was vortexed and stood for 10 min, the two-phase volumes were read, and consequently the phase volumes ratio (V_u/V_l) (where V_u and V_l are the volumes of the upper and lower phases, respectively) and enrichment factor (F) (defined as $F = V_{H_2O}/V_u$, where V_{H_2O} is equal to 3.0 mL) were calculated.

The distribution of steroids is described by the distribution ratio D (given as $D = c_u/c_l$, where c_u and c_l are the concentrations of the steroids in the upper and lower phases, respectively) and extraction efficiency (E, %) (defined by $E = V_u c_u/m_s$, where m_s is the amount of steroids added).

2.3. Preparation of SFUS or urine sample

Steroids-free urine samples (SFUS) were prepared following the procedure described by Gonzalo-Lumbreras et al. [4], checked for endogenous steroids with negative result by HPLC coupled with the following ATPS extraction procedure, and then used as matrix for steroid spikes.

To a 10 mL centrifuge tube containing 3.0 mL human urine or SFUS, 10 μL of 100 μg/mL MT as internal standard (I.S.), 0.1 mL solution of β-glucuronidase from *E. coli*, 0.1 mL of 0.2 M sodium phosphate buffer at pH 6.9 were added. The mixture was heated at 55 °C for 1 h. After hydrolysis, 0.25 mL of 0.5 g/mL trichloroacetic acid solution (TCA) as deproteinizing agent was added. The contents were then mixed thoroughly and centrifuged at 6000 rpm for 10 min to remove the precipitant, and then 0.2 g IL and 3.4 g K_2HPO_4 were added to the supernatant. After the mixture was vortexed and centrifuged to facilitate the phase separation, the upper phase was directly injected into the HPLC system for determination of T and ET.

2.4. HPLC analysis of steroids

Chromatographic analyses were performed on an Agilent 1100 HPLC system including a quaternary pump and a variable wavelength UV detector (Agilent, USA). The instrument control and data processing were carried out by an Agilent ChemStation software.

In the studies of the distribution behaviors of steroids, a μBondapak^{TM} C_{18} (10 μm, 300 mm × 3.9 mm i.d.) column from Waters was employed for determination of steroids in the upper phase with external standard method. The mobile phase consisted of acetonitrile, methanol and 40 mM Tris–HCl buffer (pH 6.9) with a volume ratio of 25/15/60 at a flow-rate of 1.0 mL/min. The injection volume was 20 μL. The UV detection wavelength was 245 nm.

For the determination of T and ET in urine (or spiked SFUS), with MT as internal standard (I.S.), an Agilent Zorbax SB-C_{18} (5 μm, 250 mm × 4.6 mm i.d.) analytical column and an Agilent Zorbax SB-C_{18} guard-column (5 μm, 12.5 mm × 4.6 mm i.d.) were used. The eluent was acetonitrile/H_2O (39/61, v/v) at a flow-rate of 1.0 mL/min. The injection volume was 100 μL, and the column effluent was monitored at 245 nm.

3. Results and discussion

3.1. ATPS studies

3.1.1. Effect of the property of salt on phases separation

A series of salts were tested for the formation of ATPS with [C_4mim]Cl. Results show that ATPS can be formed by adding appropriate amount of K_2HPO_4, K_3PO_4, K_2CO_3, KOH, Na_2HPO_4 or NaOH to aqueous [C_4mim]Cl, while KH_2PO_4, K_2SO_4, $(NH_4)_2SO_4$, KCl or NaCl cannot cause phase separation. According to Gutowski and Weingärtner [29,30], this phenomenon is probably a solvophobic one. The kosmotropic ions, e.g. HPO_4^{2-}, SO_4^{2-}, OH^-, CO_3^{2-} and PO_4^{3-} [29,31–33], which exhibit stronger interaction with water molecule than that between water molecules, are beneficial to the ATPS formation [29]. However, the chaotropic ions, e.g. Cl^-, NH_4^+, K^+, $H_2PO_4^-$ [31–33], have the opposite effect because of their weaker interactions with water. Although SO_4^{2-} is almost the same kosmotropic as HPO_4^{2-} [34], the solubility of K_2SO_4 (11 g/100 mL H_2O) and $(NH_4)_2SO_4$ (43.5 g/100 mL H_2O) are much less than that of K_2HPO_4 (150 g/100 mL H_2O) [35]. As a result, even in the saturated solutions of K_2SO_4 and $(NH_4)_2SO_4$ the concentration of SO_4^{2-} is not high enough for the formation of ATPSs. Among the salts which can cause phase separation, four types were chosen to determine the phase diagrams of IL-salt–water systems by cloud point method at 25 °C (Fig. 3). As shown in Fig. 3, the abilities of the salts studied for phase separation were in an order of $K_2HPO_4 \approx K_2CO_3 \approx K_3PO_4 >$ KOH.

3.1.2. Effect of the amount of salt on phase separation

The effect of the amount of four salts on the phase behavior of ATPS was also investigated. When 2.7–4.0 g K_2HPO_4, 1.9–3.7 g K_2CO_3, 2.9–3.6 g K_3PO_4 and 3.8–5.4 g KOH were added into 3.0 mL water containing 0.2 g IL for the phase separation, the enrichment factors (F) were 9–10, 13–15, 10–14 and 15–17, correspondingly, which almost kept constant in each type of ATPSs (Fig. 4). On the other hand, with

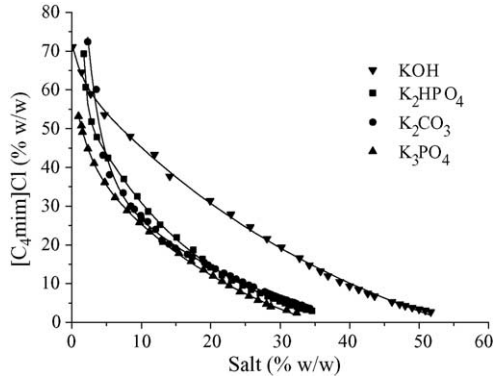


Fig. 3. Phase diagrams for IL/salt systems.

the increase of salt mass, the phase volumes ratios (V_u/V_l) increased at the beginning, and decreased after the amount of salts reached certain values. It could be interpreted by the fact that, with the increasing amount of salts, the upper-phase volume increases greatly at first, while varies little after the salt mass surpasses certain value, meanwhile, the volume of lower phase increases gradually. Two distinct phases can be formed after the mixture is thoroughly votexed and set for 5 min, and two-phase volumes keep constant within at least 12 h. Furthermore, no emulsion formation in the whole extraction process was observed.

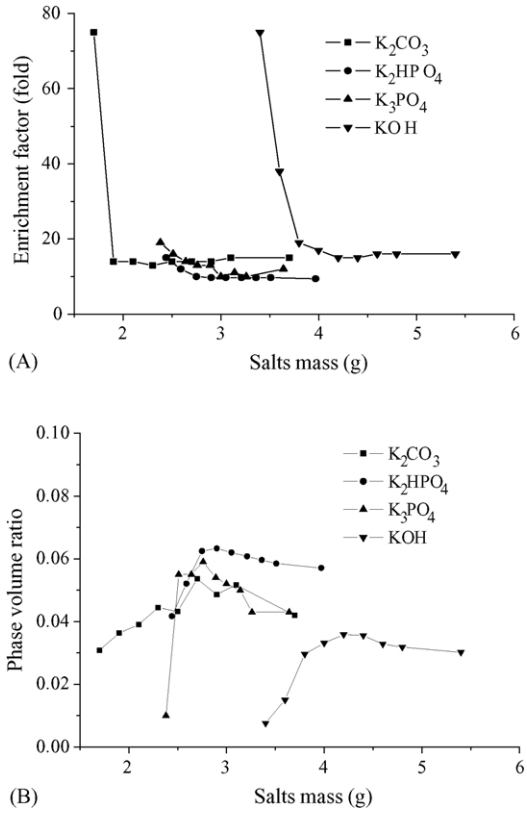


Fig. 4. Effects of salts on: (A) the enrichment factor; (B) phase volumes ratio. 3.0 mL H_2O and 0.2 g IL were added for the ATPS formation.

3.2. Steroids distribution

3.2.1. Effect of salt on the distribution behaviors of steroids

In various IL/salt systems tested, the distribution behaviors of steroids are very different. When 2.7–3.7 g K_2HPO_4 was used for phase separation, the extraction efficiencies (E) of steroids almost kept constant with the value of 85–90%, 81–87% and 81–85% for T, MT and ET, respectively, while their distribution ratios (D) increased with the increase of salt mass (Fig. 5). In IL/K_2CO_3 system, with the amount of K_2CO_3 increased from 1.7 to 3.3 g, the values of E decreased obviously from 44%, 41% and 43% to 0%, 0.3% and 0.3% for T, MT and ET, respectively, and the D decreased similarly (Fig. 5). However, when 2.2–3.3 g K_3PO_4 or 3.6–5.2 g KOH was employed in ATPS, the concentration of T, MT or ET in the IL-rich upper phase was negligible. The results show that strongly basic condition is detrimental to the extraction of steroids. Therefore, 3.4 g K_2HPO_4 was used as the phase separation salt in this study.

Due to the hydrophobicity of steroids, the hydrophobic interactions between the steroids and the IL-rich upper phase in ATPS probably are the main driving force for their extraction, which is similar to the traditional ATPS [7].

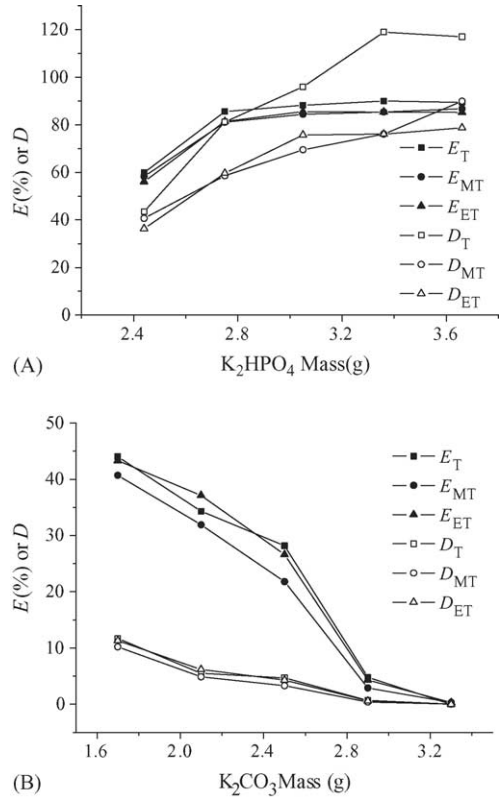


Fig. 5. Effect of salts on the distribution behaviors of steroids: (A) K_2HPO_4; (B) K_2CO_3. E_T, E_{MT}, E_{ET}, D_T, D_{MT}, and D_{ET} are the extraction efficiencies and distribution ratios of T, MT and ET, respectively. The tested concentrations of steroids were all 333.3 ng/mL. 3.0 mL H_2O and 0.2 g IL were added for the ATPS formation.

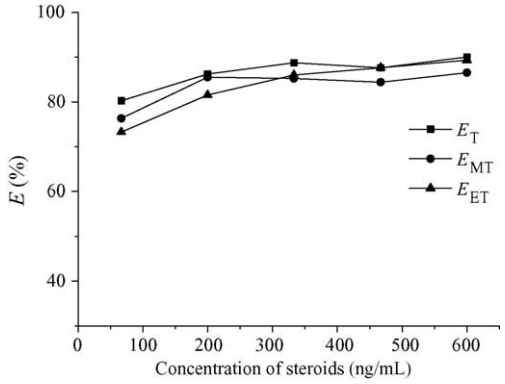


Fig. 6. Effect of steroid concentration on the extraction efficiency. 3.0 mL H_2O, 0.2 g IL and 3.4 g K_2HPO_4 were added for the ATPS formation.

3.2.2. Effect of steroid concentration on the extraction efficiency

To further confirm the possibility of applying the ATPS studied above to the quantitative extraction of T, MT and ET, the effect of steroid concentration on its distribution behavior was also studied (Fig. 6). Within 67–600 ng/mL, the extraction efficiencies were about 80–90%, 76–86% and 73–89% for T, MT and ET, respectively, which were high enough to quantitatively extract steroids from aqueous solution. Additionally, the values of E_T/E_{MT} (1.01–1.05) and E_{ET}/E_{MT} (0.95–1.04) almost kept constant. These results are crucial for the determination of T and ET by HPLC method with MT as internal standard.

3.2.3. Effect of temperature on the extraction efficiency of steroid

The effect of temperature on the extraction efficiencies of steroids were also investigated as shown in Fig. 7. Within 10–50 °C, the extraction efficiencies of T, MT or ET fell in a narrow range of 80–90% when 333.3 ng/mL of steroid was added, indicating that temperature had little influence on the distribution behavior of steroids. Thus, this method provides a relatively wide range of temperature for the study on the extraction behavior of analytes. The following studies were carried out at room temperature.

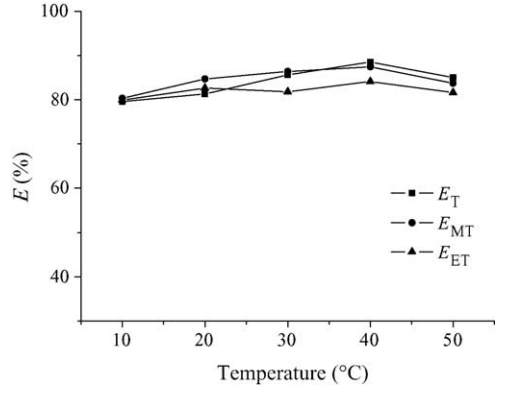


Fig. 7. Effect of temperature on the extraction efficiency of steroid. 3.0 mL H_2O, 0.2 g IL and 3.4 g K_2HPO_4 were used for the ATPS formation, and the tested concentrations of steroids were all 333.3 ng/mL.

3.3. Human urinary analysis

3.3.1. Effect of the deproteinizing agent on HPLC analysis

A comparison was carried out to demonstrate the effect of the deproteinizing agent (TCA) on the extraction efficiencies of steroids. When 0.25 mL of 0.5 g/mL TCA was added or not, the values of A_T/A_{MT} and A_{ET}/A_{MT} were 1.154 and 0.962, or 1.143 and 0.989, respectively. Herein A_T, A_{ET} and A_{MT} are the chromatographic peak areas of T, ET and MT, correspondingly. The concentrations of the tested steroids were all 333.3 ng/mL. The results show that TCA has little influence on the determination of T and ET with the HPLC method described above.

3.3.2. Calibration curves

Calibration curves were obtained by adding the standard testosterone and epitestosterone to SFUS at five concentrations in the range of 10–500 ng/mL, using 333.3 ng/mL methyltestosterone (MT) as internal standard (I.S.) under the same HPLC conditions for urinary sample. Quantification was based on peak-area ratios of compound to I.S. versus concentration of compound spiked. Linear regression equations and correlation coefficients (r) for T and ET are $A_T/A_{MT} = 0.00317\ c_T + 0.01095$ ($r = 0.9997$) and $A_{ET}/A_{MT} = 0.00307 c_{ET} - 0.01237$ ($r = 0.9999$), respectively, where c_T and c_{ET} are the concentrations of T and ET spiked, correspondingly, with the unit of ng/mL.

3.3.3. Method validation

The limits of detection for both compounds were 1 ng/mL based on S/N (signal-to-noise) = 3. The precision and accuracy were estimated using five different replicate samples containing 40.0, 200.0 and 400.0 ng/mL analytes, respectively. Standards of T and ET were added to SFUS, processed under the proposed extraction conditions, and analyzed using the HPLC method developed in this study. Obtained recoveries were 96–100% with a RSD of 2.0–6.8% and 98–104% with a RSD of 1.5–5.5% for T and ET, correspondingly (Table 1), showing that the present method has a satisfac-

Table 1
Analytical accuracy and reproducibility for T and ET in spiked SFUS[a]

Sample		Concentration added (ng/mL)	Concentration found (ng/mL)	Recovery (%)	RSD (%)
1	T	40.0	38.3	95.8	6.8
	ET	40.0	40.4	101.0	5.5
2	T	200.0	197.0	98.5	3.4
	ET	200.0	195.9	97.9	2.3
3	T	400.0	399.1	99.8	2.0
	ET	400.0	416.0	104.0	1.5

[a] Each value represents the mean for five different samples ($n = 5$).

Table 2
Determination and recovery data of testosterone and epitestosterone in human urinary samples

Sample		Steroids in urine found by present method[a] (ng/mL)	Steroids in urine found by SPE-HPLC[a,b] (ng/mL)	Steroids added (ng/mL)	Recovery[a] (%)
1	T	56.6 (3.9%)	62.7 (3.0%)	50.0	95.2 (6.5%)
	ET	37.5 (2.9%)	35.7 (2.7%)	50.0	102.0 (10.2%)
2	T	155.3 (2.3%)			
	ET	29.3 (8.6%)			
3	T	10.9 (5.2%)			
	ET	29.3 (6.4%)			

[a] Average of three determinations with respective RSD in the parentheses.
[b] SPE follows a procedure described by Gonzalo-Lumbreras et al. [4].

tory reproducibility and accuracy for the determination of the analytes in a wide range of concentrations.

3.3.4. Analysis of human urine samples

The proposed extraction method was used to separate and determine T and ET in several human urinary samples. After hydrolysis, T and ET in the urine samples were extracted and determined with the proposed HPLC method (Fig. 8 and Table 2). As shown in Table 2, the determined mean values of testosterone and epitestosterone in sample 1 correspond very well to the values that obtained by the SPE-HPLC [4] method and the recoveries of the method were 95.2% and 102.0%, respectively. Furthermore, the analytes in samples 2 and 3 were also determined by the proposed method with satisfactory reproducibility as given in Table 2, showing that the present method can be satisfactorily applied to the quantitative determination of testosterone and epitestosterone in human urine. To economize urine sample, the volume of urine used herein is 3.0 mL resulting 10-fold of enrichment factor for both analytes, which is high enough for their analysis. Furthermore, as confirmed by other experimental results, the enrichment factor can be further improved by increasing the volume of sample.

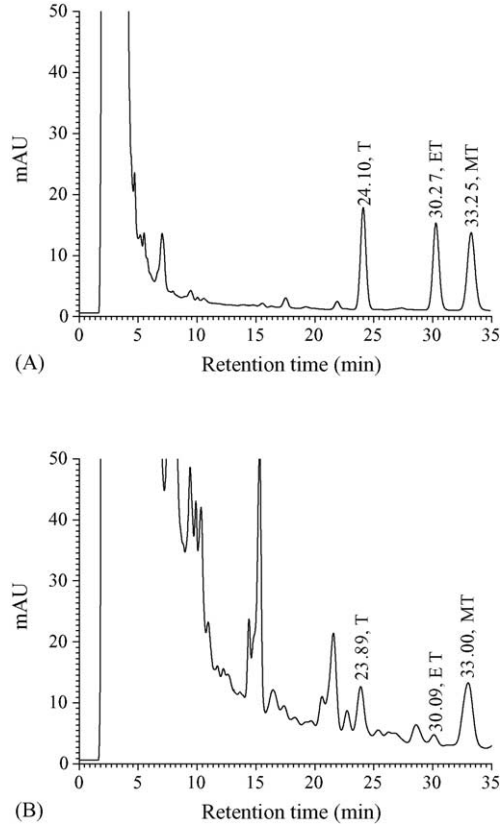


Fig. 8. HPLC chromatograms with UV detection of: (A) SFUS spiked with standard (300.0 ng/mL) and I.S. (333.3 ng/mL); (B) a human urine sample added with I.S. (333.3 ng/mL). Column: Zorbax SB-C_{18}. Mobile phase: 39/61% (v/v), acetonitrile–water. Flow rate: 1.0 mL/min. UV detection at 245 nm.

4. Conclusions

This study demonstrates that the ATPS consisting of $[C_4mim]Cl$ and K_2HPO_4 was an excellent strategy for the simultaneous extraction of testosterone and epitestosterone from human urine. Compared with previously reported extraction methods such as traditional LLE or SPE, the proposed one is “greener”, simpler and more effective due to no using of volatile organic solvent, low consumption of extraction solvent, no emulsion formation in the whole extraction process, high extraction efficiencies in a one-step extraction and direct injection of the upper IL-rich phase into HPLC system for the determination of the analytes. This novel extraction technique can be employed in combination with HPLC as a viable method for the quantitative determination of testosterone and epitestosterone in human urine. As a novel pretreatment method it would have great potential in coupling with other instruments such as FIA and CE, etc. Furthermore, the proposed method has also opened up new possibilities in the separation and concentration of other bioactive drugs.

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References

[1] M.H. Choi, J.Y. Kim, B.C. Chung, Analyst 124 (1999) 675.
[2] A. Leinonen, T. Kuuranne, R. Kostiainen, J. Mass Spectrom. 37 (2002) 693.
[3] D.H. Catlin, R.C. Kammerer, C.K. Hatton, M.H. Sekera, J.L. Merdink, Clin. Chem. 33 (1987) 319.
[4] R. Gonzalo-Lumbreras, D. Pimentel-Trapero, R. Izquierdo-Hornillos, J. Chromatogr. Sci. 41 (2003) 261.
[5] R. Draisci, L. Palleschi, E. Ferretti, L. Lucentini, P. Cammarata, J. Chromatogr. A 870 (2000) 511.
[6] P.-Å. Albertsson, Partition of Cell Particles and Macromolecules, third ed., Wiley, New York, 1986.
[7] L. Li, C.Y. He, S.H. Li, F. Liu, S. Su, X.X. Kong, N. Li, K.A. Li, Chin. J. Chem. 22 (2004) 1313.
[8] J.X. Xiao, U. Sivars, F. Tjerneld, J. Chromatogr. B 743 (2000) 327.
[9] H.Q. Yin, M. Mao, J.B. Huang, H.L. Fu, Langmiur 18 (2002) 9198.
[10] Y. Akama, A. Sali, Talanta 57 (2002) 681.
[11] H.D. Willauer, J.G. Huddleston, R.D. Rogers, Ind. Eng. Chem. Res. 41 (2002) 1892.
[12] I.H. Pan, H.H. Chiu, C.H. Lu, L.T. Lee, Y.K. Li, J. Chromatogr. A 977 (2002) 239.
[13] J. Dupont, R.F. de Souza, P.A.Z. Suarez, Chem. Rev. 102 (2002) 3667.
[14] J.L. Anderson, J. Ding, T. Welton, D.W. Armstrong, J. Am. Chem. Soc. 124 (2002) 14247.
[15] R.D. Rogers, K.R. Seddon, Science 302 (2003) 792.
[16] T. Fukushima, A. Kosaka, Y. Ishimura, T. Yamamoto, T. Takigawa, N. Ishii, T. Aida, Science 300 (2003) 2072.
[17] F. Zhao, X. Wu, M.K. Wang, Y. Liu, L.X. Gao, S.J. Dong, Anal. Chem. 76 (2004) 4960.
[18] T. Welton, Chem. Rev. 99 (1999) 2071.
[19] J. Ding, T. Welton, D.W. Armstrong, Anal. Chem. 76 (2004) 6819.
[20] M. Vaher, M. Koel, J Chromatogr. A 1068 (2005) 83.
[21] H.M. Luo, S. Dai, P.V. Bonnesen, Anal. Chem. 76 (2004) 2773.
[22] K. Shimojo, M. Goto, Anal. Chem. 76 (2004) 5039.
[23] H.M. Luo, S. Dai, P.V. Bonnesen, A.C. Buchanan, J.D. Holbrey, N.J. Bridges, R.D. Rogers, Anal. Chem. 76 (2004) 3078.
[24] S. Carda-Broch, A. Berthod, D.W. Armstrong, Anal. Bioanal. Chem. 375 (2003) 191.
[25] J. Liu, G. Jiang, Y. Chi, Y. Cai, Q. Zhou, J. Hu, Anal. Chem. 75 (2003) 5870.
[26] J. Liu, Y. Chi, G. Jiang, C. Tai, J. Peng, J. Hu, J. Chromatogr. A. 1026 (2004) 143.
[27] J. Liu, N. Li, G. Jiang, J. Liu, J.Å. Jönsson, M. Wen, J. Chromatogr. A. 1066 (2005) 27.
[28] M. Mank, B. Stahl, G. Boehm, Anal. Chem. 76 (2004) 2938.
[29] K.E. Gutowski, G.A. Broker, H.D. Willauer, J.G. Huddleston, R.P. Swatloski, J.D. Holbrey, R.D. Rogers, J. Am. Chem. Soc. 125 (2003) 6632.
[30] H. Weingärtner, W. Schröer, J. Mol. Liq. 65–66 (1995) 107.
[31] K.D. Collins, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 5553.
[32] R. Itri, W. Caetano, L.R.S. Barbosa, M.S. Baptista, Braz. J. Phys. 34 (2004) 58.
[33] K.D. Collins, Biophys. J. 72 (1997) 65.
[34] K.D. Collins, M.W. Washabaugh, Quart. Rev. Biophys. 18 (1985) 323.
[35] J.A. Dean, Lange's Handbook of Chemistry, 15th ed., McGraw-Hill Co., New York, 1999.