

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.

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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 pro-

cesses 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).

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

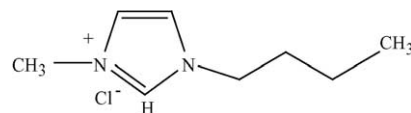


Fig. 2. Chemical structure of [C₄mim]Cl.

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 10¹⁸ 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 ([C₄mim]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 ([C₄mim]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 C₁₈ 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-

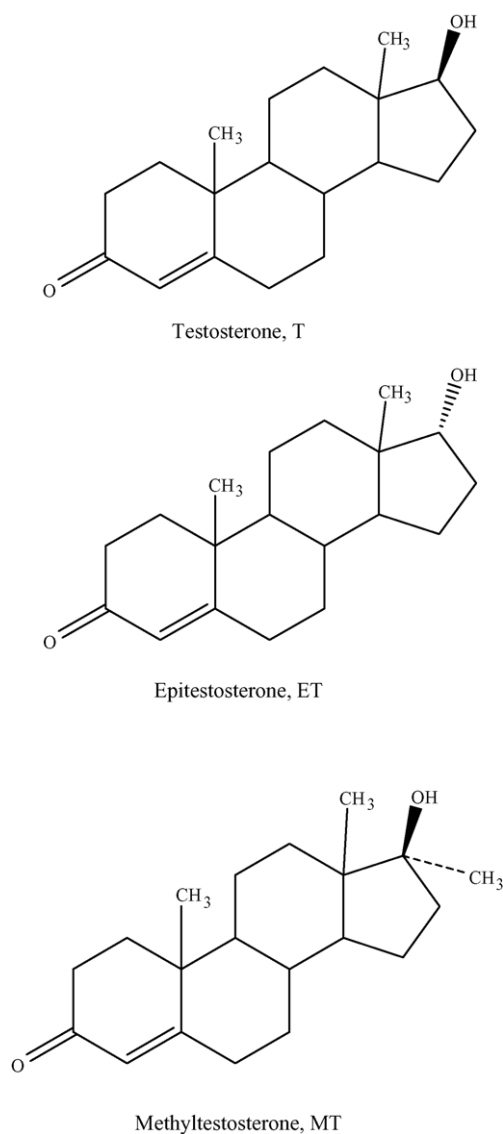


Fig. 1. Chemical structures of steroids.

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