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Direct analysis of anabolic steroids in urine using Leidenfrost phenomenon assisted thermal desorption-dielectric barrier discharge ionization mass spectrometry

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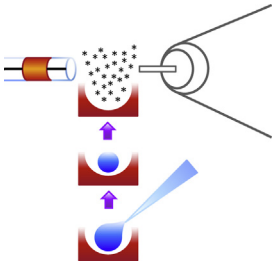
HIGHLIGHTS

- Urinary anabolic steroids were detected with high sensitivity using LPTD-DBDI-MS.
- Anabolic steroids were directly ionized using DBDI without any adduct formation.
- Analytical figures of merit are demonstrated using suitable internal standards.

GRAPHICAL ABSTRACT



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ABSTRACT

Rapid detection of trace level anabolic steroids in urine is highly desirable to monitor the consumption of performance enhancing anabolic steroids by athletes. The present article describes a novel strategy for identifying the trace anabolic steroids in urine using Leidenfrost phenomenon assisted thermal desorption (LPTD) coupled to dielectric barrier discharge (DBD) ionization mass spectrometry. Using this method the steroid molecules are enriched within a liquid droplet during the thermal desorption process and desorbed all-together at the last moment of droplet evaporation in a short time domain. The desorbed molecules were ionized using a dielectric barrier discharge ion-source in front of the mass spectrometer inlet at open atmosphere. This process facilitates the sensitivity enhancement with several orders of magnitude compared to the thermal desorption at a lower temperature. The limits of detection (LODs) of various steroid molecules were found to be in the range of 0.05–0.1 ng mL^{-1} for standard solutions and around two orders of magnitude higher for synthetic urine samples. The detection limits of urinary anabolic steroids could be lowered by using a simple and rapid dichloromethane extraction technique. The analytical figures of merit of this technique were evaluated at open atmosphere using suitable internal standards. The technique is simple and rapid for high sensitivity and high throughput screening of anabolic steroids in urine.

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1. Introduction

Anabolic steroids are considered as therapeutic agents used to promote the muscle growth and strength [1,2]. These compounds have wide applicability in the treatment of cancer and AIDS, where a rapid increase of muscle mass is highly desired [3]. However, administration of these compounds in a high dose is associated with adverse effects like permanent organ damage, reduced fertility, hypertension, psychiatric and behavioral disorders etc. [2,4–7]. Major concerns are the use of these compounds by athletes to increase their performance, which is popularly known as 'doping' [8]. These compounds stimulate the androgen receptors in brain and athletes enjoy a state of euphoria, increased aggressiveness and decreased fatigue [9,10]. Owing to the enhanced muscle power, doped athletes perform superiorly compared to their non-doped counterparts. In order to prohibit the illicit use of anabolic steroids and maintain a fair and healthy competition in sports ground, analytical strategies with high-throughput and high sensitivity spot detection of performance enhancing steroids in body fluids are highly desirable.

Analysis of urine is a convenient procedure to monitor the consumption of illicit compounds as urine can be obtained easily with large volumes [11,12]. However, challenges exist as the concentration levels of steroids in raw urine are very low [13]. Mass spectrometry is a dominant analytical tool for the analyses of doping compounds as it can accurately identify compounds based on their mass to charge ratios. Gas chromatography–mass spectrometry (GC–MS) is one of the most common and widely applied analytical techniques used for the detection of urinary steroids [13–16]. In order to enhance the volatility, ionization efficiency and detection limit, raw urinary samples need to undergo various sample pre-processing steps like hydrolyses, derivatization, pre-concentration etc. before injecting to the GC–MS system. These procedures are tedious, time consuming and need expert personnel to carry-on. Liquid chromatography–mass spectrometry (LC–MS) has also been used for the detection of steroids from urine [17–20]. But in this case also liquid–liquid extraction (LLE) or solid-phase extraction (SPE) is required for sample cleaning, pre-concentration and derivatization for increasing the ionization efficiency. On the other hand, ambient mass spectrometry (MS) is another choice where samples are being analyzed in high speed and specificity without or with minimal sample pre-treatment [21,22]. In 2007, reactive desorption electrospray ionization (DESI) was reported for the high throughput screening of anabolic steroids in urine [23]. Among various other mass spectrometric techniques, solvent assisted inlet ionization [24] and capillary photoionization [25] are significant for the high sensitivity screening of steroid molecules.

In this article, we report a new strategy for direct and high sensitivity analysis of anabolic steroids from urine with minimal pre-treatment using Leidenfrost phenomenon assisted thermal desorption (LPTD) coupled to dielectric barrier discharge ionization (DBDI) MS at open atmosphere. Earlier, we demonstrated that LPTD is a suitable desorption technique for high sensitivity detection of analytes based on their molecular ions and could be coupled with positive and negative mode MS operation [26,27]. In this technique a liquid droplet containing analyte is placed on a heated metallic sample holder situated in front of the inlet of mass spectrometer. The temperature of the sample holder must be higher than the Leidenfrost temperature of the liquid. Under this condition the liquid droplet levitates on the metallic surface for few seconds to minute based on the liquid's characteristic, metallic holder's temperature, and droplet volume and a slow solvent evaporation takes place. During the solvent evaporation process, the analyte molecules are enriched inside the liquid droplet and released at the final stage of droplet evaporation in a short time domain. Thus, the sensitivity of this technique becomes several times higher compared to the thermal desorption at a lower temperature. The desorbed neutral molecules are ionized in a post-ionization method at open atmosphere using DBD ion-source before entering the mass spectrometer inlet. The method was validated in terms of limit of detection (LOD), precision, linear range, correlation coefficient of linearity and recovery. Further, we coupled this whole process with a simple dichloromethane extraction technique for getting better sensitivity and reproducibility from urine sample. This is a rapid method and a single sample analysis time is around one minute.

2. Experimental

2.1. Reagents and solvents

Standard anabolic steroid samples (androstadienedione, androsterone hemisuccinate, 6-dehydrocholestenone, epitestoterone and stigmastadienone) were purchased from Steraloids Inc. (Newport, RI, USA). HPLC grade methanol and dichloromethane were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and Cica-Reagent (Tokyo, Japan). The water was purified with a Milli-Q purification system (Millipore, Bedford, MA, USA). All of the chemicals used for the preparation of artificial urine were obtained from Cica-Reagent (Tokyo, Japan), except bilirubin, D-glucuronic acid and human albumin, which were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Sample preparation

Stock solutions of all anabolic steroids (1 mg mL^{-1}) were separately prepared in methanol and stored in a refrigerator at −30 °C. Working standard solutions were prepared just before the experiments and dilutions were made using methanol. 'Artificial urine' was used to obtain the analysis data of anabolic steroids from urine. Artificial urine contains the major components present in human urine and prepared using the same procedure reported by Jacob et al. [28]. Water was used as a matrix for artificial urine and the concentrations of different components are as follows: ammonium sulfate (1.0 mg mL^{-1}), andosterone (0.02 mg mL^{-1}), ascorbic acid (0.02 mg mL^{-1}), bilirubin (0.005 mg mL^{-1}), citric acid (0.5 mg mL^{-1}), creatine (0.5 mg mL^{-1}), creatinine (1.5 mg mL^{-1}), cystine (0.1 mg mL^{-1}), glucuronic acid (0.5 mg mL^{-1}), hippuric acid (1.0 mg mL^{-1}), histidine (0.5 mg mL^{-1}), human albumin (0.1 mg mL^{-1}), magnesium sulfate (1.0 mg mL^{-1}), phenol (0.5 mg mL^{-1}), potassium phosphate monobasic (1.0 mg mL^{-1}), sodium chloride (10 mg mL^{-1}), urea (15 mg mL^{-1}), and uric acid (0.5 mg mL^{-1}). After preparation, the artificial urine was stored at −30 °C. The standard anabolic steroids were spiked in artificial urine just before the experiments. For the preparation of the working standard of urine, 10 μL of the diluted stock solutions of anabolic steroids were spiked in 1 mL of artificial urine. All of the experiments reported in this paper were performed using artificial urine.

2.3. Experimental setup

A home-made DBD ion-source was used for the ionization of the desorbed molecules. The ion source was placed in front of the mass spectrometer inlet in such a way that the desorbed molecules are ionized at open atmosphere before entering to the first vacuum chamber of the mass spectrometer. The detailed experimental set-up is illustrated in Fig. 1 and is similar to the reported one in our previous papers [26,27]. In short, the stainless steel sample holder was placed below the inlet of the mass spectrometer and the DBD ion-source made of glass tube with 3.0 mm o.d. and 1.5 mm i.d..

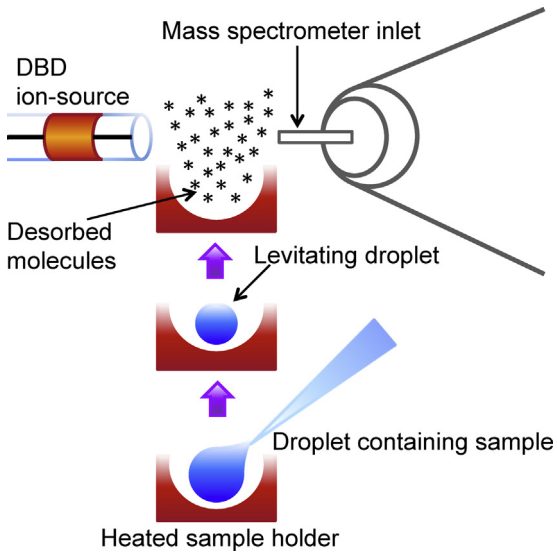

Fig. 1. Schematic diagram of the experimental procedure of LPTD-DBD-MS.

Copper tape was used as an outer electrode and a stainless steel wire as an inner electrode. Alternating high voltage of 15 kHz with 3.0 kV (V_{p-p}) was applied to the outer electrode with the inner electrode grounded and helium flow (450 mL min^{-1}) was passed through the glass tube of the DBD ion-source to generate helium plasma and initiate the ion-molecule reactions. The temperature of the sample holder was controlled by using a digital temperature controller (Arios Corporation, Tokyo, Japan) directly connected to the sample holder. Although a small variation of temperature was observed when the droplet was placed on the sample holder, the temperature reached to the set value within a few seconds and remained unchanged until the droplet evaporated completely.

All of the experimental results reported in this paper were collected using 'Exactive Orbitrap' (Thermo Fisher Scientific, Bremen, Germany) mass spectrometer and were processed using Xcalibur software (ver. 2.1) (also from Thermo Fisher Scientific). The Orbitrap mass spectrometer was used in high resolution mode (resolution: 50,000 at 2 Hz) and the capillary temperature and voltage were 250 °C and 30 V, respectively. Maximum injection time was kept at 100 ms and a tube lens voltage of 120 V and skimmer voltage of 20 V were maintained throughout the experiments. Other instrumental parameters are described elsewhere [26]. The droplet volume was maintained 50 μL for all of the experiments reported in this article. The droplet was placed manually using a calibrated pipette (Eppendorf, Germany) working in the range of 10–100 μL with replaceable tip. A fresh droplet was used for each of the experiment.

3. Results and discussion

3.1. Leidenfrost phenomenon on anabolic steroids

Leidenfrost phenomenon assisted thermal desorption (LPTD) is a high temperature thermal desorption technique for solutions and the temperature depends on the characteristics of the solvent. Despite being a high temperature thermal desorption technique, liquid droplets underwent LPTD experience much lower temperature due to the existence of heat insulating vapor layer between the liquid and hot solid surface. This unique property made this technique highly suitable for the high sensitivity detection of various small bio-molecules at open atmosphere using mass spectrometry. Fig. 2 shows the extracted ion chronogram (EIC) of epitestosterone (1 μg mL^{-1} in methanol) at Leidenfrost temperature (250 °C) and corresponding mass spectrum. The EIC clearly shows that the epitestosterone peak was not detected during the whole solvent evaporation time and at the last moment of solvent evaporation all of the epitestosterone was released in a short time span and a high intensity peak was observed. This technique could be regarded as a heat associated sample enrichment process under Leidenfrost condition. Thermal desorption of analytes does take place below the Leidenfrost temperature when the droplet is in contact with the heated substrate (no levitation). In such a case, less enrichment of nonvolatile analytes is anticipated and desorption of analytes will take place in a longer time domain during the liquid droplet vaporization compared to that at Leidenfrost temperature. As a proof of concept, we generated a normalized response vs temperature curve for epitestosterone (1 μg mL^{-1}) in methanol (Fig. 3). A gradual increase of response was observed with the increase of temperature up to 200 °C and a sudden increase of response occurred at 250 °C, which was the starting point of droplet levitation, i.e., Leidenfrost temperature. Although the levitation phenomenon exists at temperatures above 250 °C, a decrease of the target analyte ion intensity was observed due to the increase of background noise (Fig. S2, Supporting Information). Probably, the higher temperature of the metallic surface induces thermal desorption of adsorbed contaminants on the sample holder and increases the background ion-intensity. This curve clearly indicates that a high sensitivity ambient detection method for anabolic steroids is possible using LPTD combined with DBD ionization technique.

3.2. Analysis of standard solution of anabolic steroids

Anabolic steroids primarily contain carbonyl and hydroxyl groups which are not easily ionized due to their low proton affinities [3]. In order to increase the ionization efficiency, different types of derivatization techniques have been adopted so far prior to the mass spectrometric analysis. Most of these derivatization

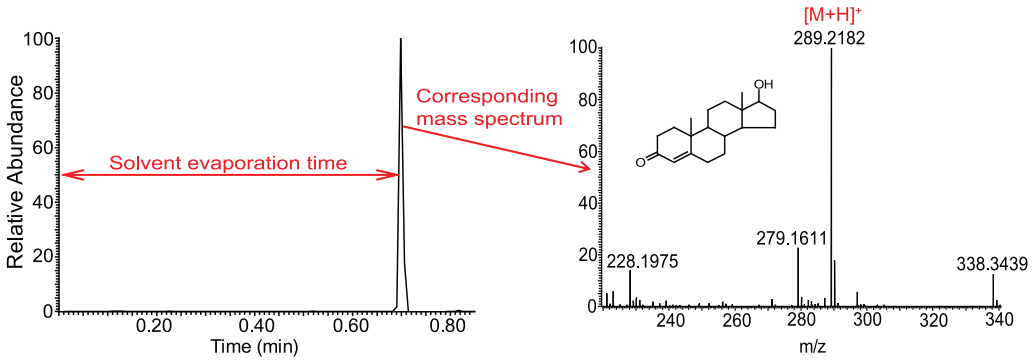

Fig. 2. Extracted ion chronogram (EIC) and corresponding mass spectrum of epitestosterone (1 μg mL^{-1}) using LPTD-DBD-MS.

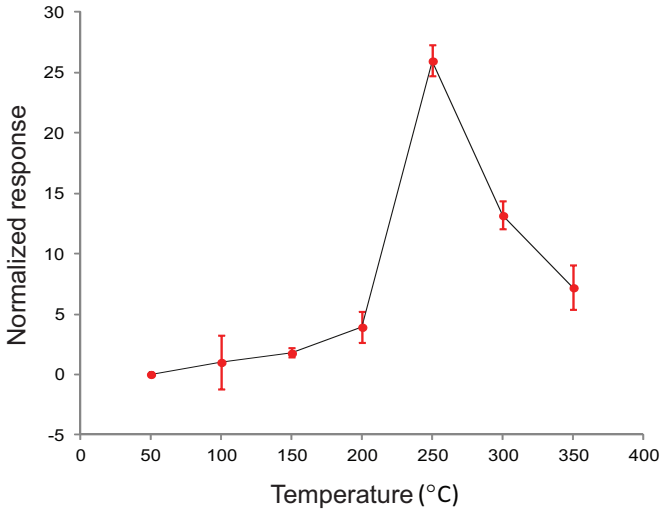

Fig. 3. Normalized response vs sample holder temperature plot of epitestosterone (1 μg mL^{-1} in methanol). Normalized response at each temperature was calculated by dividing the average epitestoterone peak intensity at that temperature by lowest average intensity of epitestosterone (i.e., 100 °C). The average was calculated from three independent experiments and error bars indicate the standard errors.

procedures or reactions deal with the binding of nitrogenous moiety to the anabolic steroid and increase the ionization efficiency as well as sensitivity of detection [3,23]. In the present study a DBD ion source was used for direct ionization of the desorbed steroid molecules without using any derivatization process. DBD was found to be a suitable ionization technique in this case as it is a soft atmospheric pressure ionization technique having a capability of ionizing wide range of compounds and helium DBD plasma is a field free source of reagents for atmospheric pressure ionization [26]. Using this technique, all of the targeted anabolic steroids show dominant $[M+H]^+$ peaks in their mass spectrum in positive ionization mode, except androsterone hemisuccinate (Fig. 4). Although a weak $[M+H]^+$ peak is present in the mass spectrum of androsterone hemisuccinate (Fig. 4d), the intensity of fragment ions $[M+H—C_4O_5H_8]^+$ and $[M+H—C_4O_4H_6]^+$ are much higher compared to the protonated molecule ion peak. The ion at *m/z* 273, $[M+H—C_4O_4H_6]^+$ was generated by the elimination of hemisuccinate group at the C-3 position of androsterone hemisuccinate. Further elimination of water molecule produced the ion at *m/z 255*, $[M+H—C_4O_5H_8]^+$. The protonated androsterone hemisuccinate must be relatively unstable and it is apt to produce fragment ions. As the intensity of $[M+H—C_4O_5H_8]^+$ is highest (Fig. 4d), it was used for the limit of detection (LOD) study for androsterone hemisuccinate. For other steroid molecules, $[M+H]^+$ peaks were used for the determination of LODs. Using this technique the LODs for various anabolic steroids were ranged in between 0.05 and 0.1 ng mL^{-1} (Table 1 and Fig. S3, Supporting Information). The LODs could be lowered using a higher droplet volume as the analyte enrichment takes place inside the droplet during the Leidenfrost condition, but 50 μL is the highest droplet volume that could be accompanied by the sample holder used for our experiments. The anabolic steroids and their fragment peaks were identified using their exact masses obtained by 'Exactive Orbitrap' mass spectrometer.

3.3. Analysis of anabolic steroids in urine

Urine is a common body-fluid used for the spot detection of doping. Direct analysis of urine by mass spectrometry is quite difficult as it contains high amounts of urea and creatinine, both of which have high proton affinities and show high intensity peaks in positive mode ionization. In the present experiment, urine showed Leidenfrost phenomenon at a temperature higher than 400 °C. But

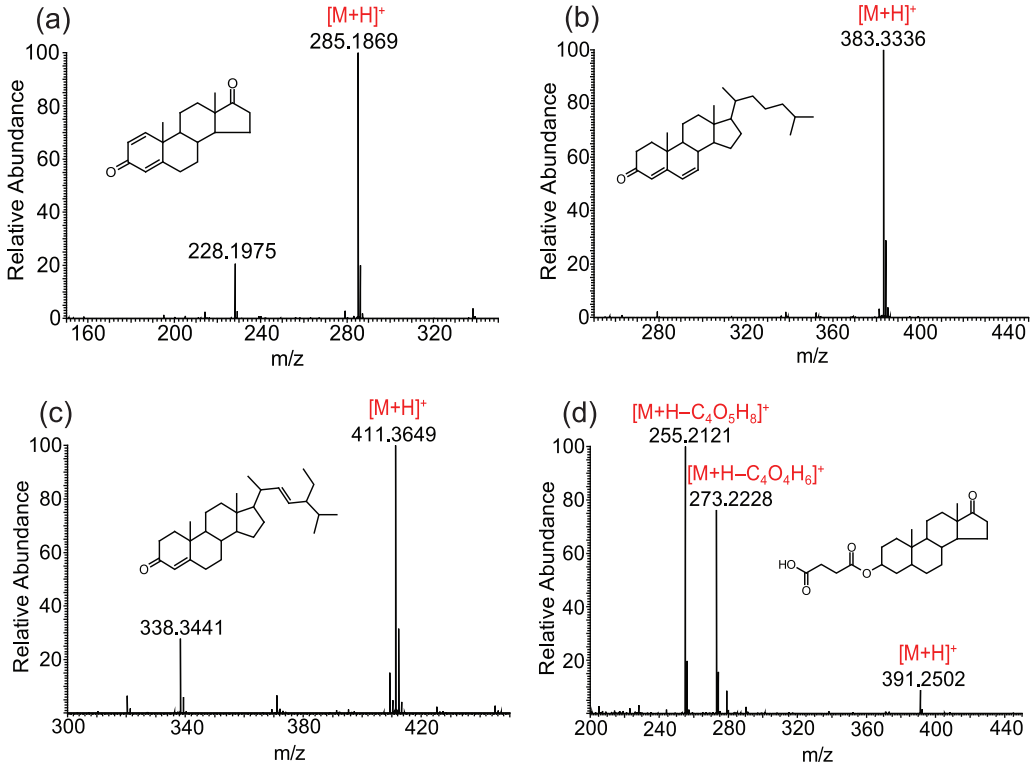

Fig. 4. Mass spectra obtained for standard solutions (1 μg mL^{-1} in methanol) of (a) androstadienedione, (b) 6-dehydrocholestenone, (c) stigmastadienone, (d) androsterone hemisuccinate using LPTD-DBDI-MS.

Table 1
Analytical figures of merit for the analysis of standard anabolic steroid samples by LPTD-DBDI-MS at open atmosphere.

Analyte	Linear range ($ng\,mL^{-1}$)	Correlation coefficient (R^2)	Limit of detection ($ng\,mL^{-1}$)	RSD[a] (%)
Androstadienedione	1–500	0.9964	0.1	8.27
Epitestosterone	1–500	0.9989	0.05	13.60
6-Dehydrocholestenone	1–500	0.9956	0.05	10.58
Stigmastadienone	1–500	0.9960	0.1	11.88
Androsterone hemisuccinate	1–500	0.9977	0.1	7.07

[a] RSD (%) was calculated at the LOQ level analyte concentration using $n = 3$ samples.

we observed that the addition of organic solvents like methanol or acetonitrile can reduce the Leidenfrost temperature of urine [26]. In this case artificial urine was first spiked with standard anabolic steroids, and then equal volume of methanol was added to the solution before conducting the experiments. Using this technique Leidenfrost phenomenon could be observed at 300 °C. Small and non-adherent particulate matter was observed on the sample holder after the evaporation of urine. The particulate matters were easily removed by blowing air and didn't give any effect on the instrument performance or successive analyses. Fig. 5a shows the mass spectrum of urine spiked with anabolic steroids mixture (concentration of each steroids: $1\,\mu g\,mL^{-1}$). In total mass spectrum (scan range, m/z: 50–500), urea, urea dimer and creatinine peaks were predominant and the intensity of these compounds were several orders of magnitude higher compared to the targeted anabolic steroids. The enlarged section of mass range, m/z: 250–420 (inset, Fig. 5a) shows that all of these targeted compounds were detected. Protonated molecule ion peaks were obtained for all of the steroids, except androsterone hemisuccinate. The $[M+H]^+$ peak was not detected for androsterone hemisuccinate, but it was identified by its characteristic peak at m/z: 255, which was predominant in the mass spectrum of standard solution. However, the intensity of androsterone hemisuccinate was much lower compared to the other steroids, likely to its relatively low proton affinity. This technique can detect as low as 2–50 $ng\,mL^{-1}$ anabolic steroids from urine (Table 2). The reported LODs for these steroid molecules in urine were around 300 $ng\,mL^{-1}$ using reactive DESI [23].

3.4. Method validation

In order to find out the quantitative capabilities of the present technique and its practical application to real world samples, various validation parameters like limit of detection (LOD), precision, linear range, correlation co-efficient of linearity (R^2), and analyte recovery

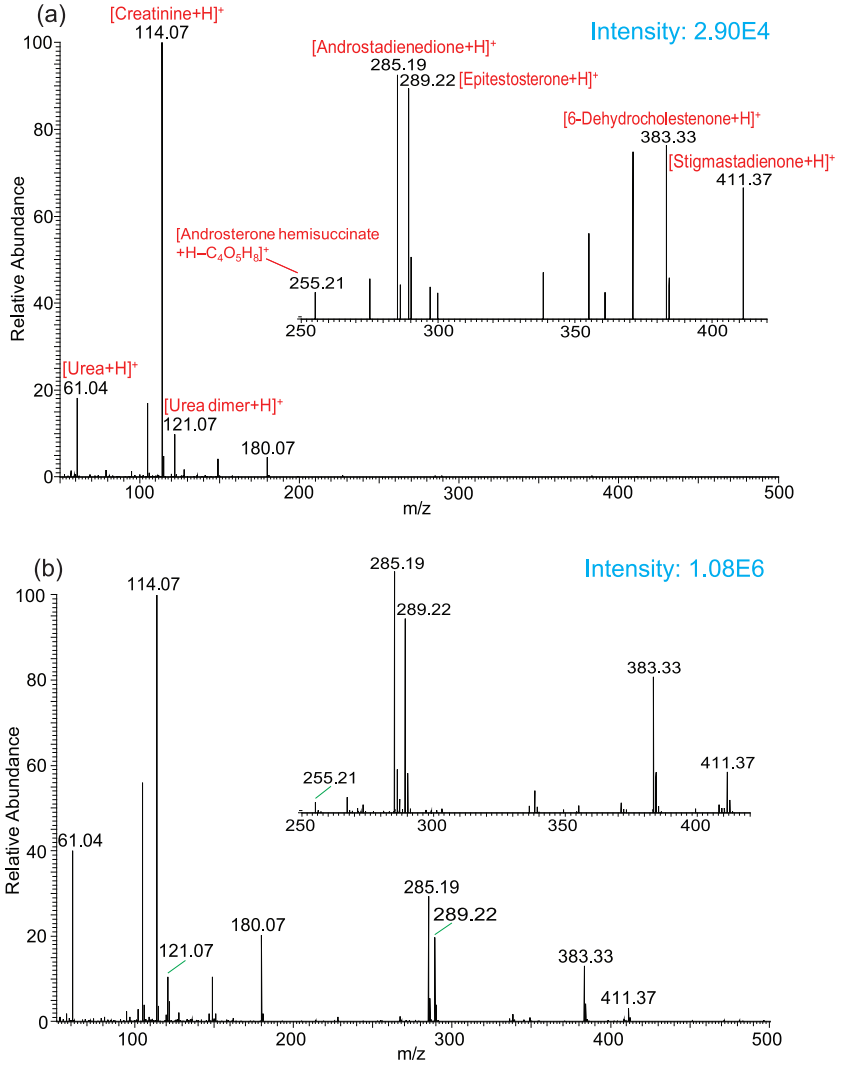

Fig. 5. Mass spectra of the mixture of anabolic steroids ($1\,\mu g\,mL^{-1}$ each) in artificial urine using LPTD-DBDI-MS (a) direct urine analysis, (b) urine analysis after dichloromethane extraction.

Table 2
Analysis of urine by LPTD-DBDI-MS at open atmosphere.

Analyte	Direct analysis of urine			Urine treated with dichloromethane		
	Limit of detection ($ng\,mL^{-1}$)	RSD[a] (%)	Recovery (%)	Limit of detection ($ng\,mL^{-1}$)	RSD[a] (%)	Recovery (%)
Androstadienedione	5.0	16.66	52.96	0.1	11.76	99.22
Epitestosterone	2.0	24.37	62.29	0.1	8.87	108.29
6-Dehydrocholestenone	10.0	16.30	64.88	0.1	9.71	112.31
Stigmastadienone	10.0	17.62	53.62	0.1	8.32	99.07
Androsterone hemisuccinate	50.0	24.83	47.41	1.0	13.43	94.47

[a] RSD (%) was calculated at LOQ level analyte concentration using $n = 3$ samples.

were investigated using suitable internal standards. Peak intensities were used for the evaluation of quantitative parameters. At LOD level each of the analyte showed a signal to noise ratio (S/N) of at least three. Around two orders of magnitude higher LODs were obtained for urine samples compared to the standard solutions of steroids (Tables 1 and 2). The probable reason could be the interference and ion-suppression effects generated by the high amount of urea and creatinine. The precision was expressed in terms of percentage of relative standard deviation (% RSD). The % RSD was calculated at the limit of quantitation (LOQ) level analyte concentration, where LOQ concentration was selected as three times of LOD level for each anabolic steroid. The three replicate analyses showed less than 14% RSD values for standard steroid solutions whereas for raw urine these were up to 25%. The complexity of urine is the main reason behind the higher % RSD values for urine samples compared to pure sample solutions. Linearity of the present technique was evaluated in a concentration range of 1–500 $ng\,mL^{-1}$ as the concentration level of these steroids in human urine is quite low [13]. For the preparation of calibration curve, a series of solutions were prepared for each anabolic steroid using an internal standard. Epitestosterone was used as an internal standard for other four steroids whereas androstadienedione was selected as internal standard for epitestosterone. A fix concentration (50 $ng\,mL^{-1}$) of internal standard was spiked in each solution and the target compound's concentration was varied. The linear calibration curves were generated by plotting the response factor against the concentration of target steroid where response factor was calculated as the ratio of intensities of target compound and internal standard at each concentration level. The 8-point calibration curves showed a good linearity for all steroids with correlation co-efficients (R^2) higher than 0.995 (Fig. S1, Supporting Information). Recovery of the present technique was determined by spiking a fix concentration of standard solutions (100 $ng\,mL^{-1}$) and internal standards (50 $ng\,mL^{-1}$) to the urine and calculated the amount from standard calibration curves. The percentage of recoveries are quite low as only 47–65% recoveries were obtained for various anabolic steroids from urine (Table 2). There could be several reasons for this low recovery. First of all, the calibration curves were made using pure methanolic solutions of steroids at 250 °C (Leidenfrost temperature of pure methanol) and for recovery study the urine samples mixed with methanol (1:1) were analyzed at the Leidenfrost temperature of the mixture, 300 °C. From Fig. 3, it is clear that the sample response is quite low at 300 °C compared to 250 °C. There is a probability that these anabolic steroids can interact with other components of urine and form different complexes, which could also decrease the steroids content in urine.

3.5. Coupling of dichloromethane extraction technique with LPTD-DBD-MS

According to the World Anti-Doping Agency (WADA) guideline, the detection limit of various anabolic steroids from urine should be 2–5 $ng\,mL^{-1}$ [29]. Although, using present technique epitestosterone and androstadienedione satisfy the criteria, but other three steroids showed the LOD of 10–50 $ng\,mL^{-1}$ from urine. At the same time using LPTD-DBDI-MS direct urine analysis showed the % RSD values up to 24.83% and the lowest recovery value was 47.41%. In order to overcome the disadvantages associated with the analysis of urine, we introduced a rapid dichloromethane extraction technique to suppress the matrix effect and increase the sensitivity and reproducibility of successive analyses. Dichloromethane is insoluble in water and can extract trace organic analytes from complex matrices [30,31]. As dichloromethane is a volatile solvent, it shows Leidenfrost phenomenon at a lower temperature (200 °C) compared to methanol (Fig. S4, Supporting Information). In this case, 500 μL steroids spiked urine was taken in a glass vial and 200 μL dichloromethane was added to that. The whole solution was vortexed for 5 s. A 50 μL droplet was pipette out from the lower layer (dichloromethane layer) and was placed on the sample holder (200 °C). The total procedure takes about 1 min to analyze a single sample. Fig. 5b shows the mass spectrum of dichloromethane extract for a mixture of five anabolic steroids (concentration of each steroids: 1 $\mu g\,mL^{-1}$) in urine. The peak intensities indicate that at least two orders of magnitude higher sensitivity were obtained compared to direct urine analysis. Using this technique the LODs for anabolic steroids from urine were ranged in between 0.1 and 1.0 $ng\,mL^{-1}$, which is sufficient to analyze these compounds by following WADA guideline [29]. Dichloromethane extraction technique also showed a better precision and recovery data compared to direct urine analysis. The % RSD values were less than 14% using this technique and the % of recoveries were 94–112% (Table 2). Basically, urine is a viscous liquid and contains a large amount of salt, which hinders the easy vapor formation. Here, using the dichloromethane extraction technique, not only the target compounds were extracted, but also were pre-concentrated using less volume dichloromethane. Steroid molecules are lipophilic in nature; therefore, these compounds are more soluble in dichloromethane compared to the urea and creatinine. The dichloromethane droplet showed good levitation property as the vapor formation is easy. Thus, the whole system showed better reproducibility compared to the direct urine analysis. The LODs obtained using dichloromethane extraction technique is comparable with the standard LC–MS or GC–MS methods for steroid analysis [13,15,17,18].

4. Conclusions

A new technique has been developed for the screening of anabolic steroids in urine by coupling LPTD and DBDI at open atmosphere and using MS as a detection system. Present technique is a high sensitivity detection technique which can detect anabolic steroids directly from urine without using any derivatization or adduct formation. In most of the cases steroids are unambiguously identified based on their $[M+H]^+$ peaks obtained by Orbitrap MS. It

was found that the coupling of LPTD-DBDI-MS with a dichloromethane extraction technique can increase the sensitivity of urinary detection of anabolic steroids by about two orders of magnitude and at the same time better reproducibility could also be achieved. The most fascinating aspect of this technique is that the sample enrichment takes place inside a droplet by using thermal desorption at a particular condition without using any external devices. The various validation parameters studied in this work showed quite promising results for ambient MS detection of anabolic steroids. The present technique is a viable alternative of conventional GC–MS or LC–MS for rapid and high-throughput spot detection of urinary anabolic steroids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2014.05.009.

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