ISSN : 0974-7419 Volume 13 Issue 7

Analytical CHEMISTRY
An Indian Journal

Full Paper

ACAIJ, 13(7) 2013 [270-283]

# Comprehensive screening of diuretics in human urine using liquid chromatography tandem mass spectrometry

Shobha Ahi[1], Alka Beotra[1]*, G.B.K.S.Prasad[2]
[1]National Dope Testing Laboratory, Ministry of Youth Affairs and Sports, CGO Complex, Lodhi Road, New Delhi,-110003, (INDIA)
[2]SOS in Biochemistry, Jiwaji University, Gwalior, (INDIA)
E-mail : drabeotra@gmail.com

## ABSTRACT

Diuretics are drugs that increase the rate of urine flow and sodium excretion to adjust the volume and composition of body fluids. There are several major categories of this drug class and the compounds vary greatly in structure, physicochemical properties, effects on urinary composition and renal haemodynamics, and site mechanism of action. Diuretics are often abused by athletes to excrete water for rapid weight loss and to mask the presence of other banned substances. Because of their abuse by athletes, diuretics have been included in the World Anti-Doping Agency's (WADA) list of prohibited substances. The diuretics are routinely screened by anti-doping laboratories as the use of diuretics is banned both in-competition and out-of-competition. This work provides an improved, fast and selective liquid chromatography–tandem mass spectrometric (LC/MS/MS) method for the screening of 22 diuretics and probenecid in human urine. The samples preparation was performed by liquid-liquid extraction. The limit of detection (LOD) for all substances was between 10-20 ng/ml or better. The method was successfully applied to 21,916 routine doping control samples.
© 2013 Trade Science Inc. - INDIA

## KEYWORDS

Doping, diuretics;
LC-MS/MS; WADA;
Drugs of abuse.

## INTRODUCTION

Diuretics are therapeutic agents that are used to increase the rate of urine flow and sodium excretion in order to adjust the volume and composition of body fluids or to eliminate excess of fluids from tissues. They are used in clinical therapy for the treatment of various diseases and syndromes, including hypertension, heart failure, liver cirrhosis, renal failure, kidney and lung diseases[1]. Diuretics were first banned in sport (both incompetition and out of competition) in 1988 because they can be used by athletes for two primary reasons. First, their potent ability to remove water from the body which can cause a rapid weight loss that can be required to meet a weight category in sporting events. Second, they can be used to mask the administration of other doping agents by reducing their concentration in urine primarily because of an increase in urine volume. The urine dilution effect of diuretics also allows them to be classified as masking agents and precludes their use both in and out of competition. Some diuretics also cause a masking effect by altering the urinary pH and inhibiting the passive excretion of acidic and basic drugs in urine[2-4].

Although the main application of diuretics is to enhance renal excretion of salt and water, their effects are not limited to sodium and chloride; they may also influence the renal absorption and excretion of other cations ($K^+$, $H^+$, $Ca_2^+$ and$Mg_2^+$), anions ($Cl^-$, $HCO_3^-$ and $H_2PO_4^-$) and uric acid. This pharmacological class of drugs includes compounds with a variety of pharmacological and physicochemical properties. Because of the variety of diuretic compounds, classification of these drugs can be based on different criteria. The most common classification categories are by site of action in the nephron, relative efficacy, chemical structure, effects on potassium excretion, similarity to other diuretics and mechanism of action[1]

There are several classes of diuretic drugs based on their mechanism of action—Thiazides (e.g., benzthiazide), loop diuretics (e.g., bumetanide), potassium sparing diuretics (e.g., amiloride), carbonic anhydrase inhibitors (e.g., acetazolamide), osmotic diuretics (e.g., mannitol)and mercurial diuretics (e.g., mersalyl) (Figure 1).

Diuretics are relatively polar, hence are amenable to analysis by high performance liquid chromatography (HPLC) using C18 type phases with diode array (DAD) or fluorescence detection[5,6]. However, whilst this method may be suitable for screening purposes it suffers from significant interferences due to the background occurring in urine samples. The information provided by HPLC is insufficient for confirmation as alternative mass spectral confirmation of identity is needed for doping control. To overcome this problem, detection of diuretics by gas chromatography mass spectrometry (GC-MS) was introduced. In order to improve the volatility of the diuretics either methylation or silylation was performed prior to GC–MS. The methylation of these polar drugs has been the most suitable proce-

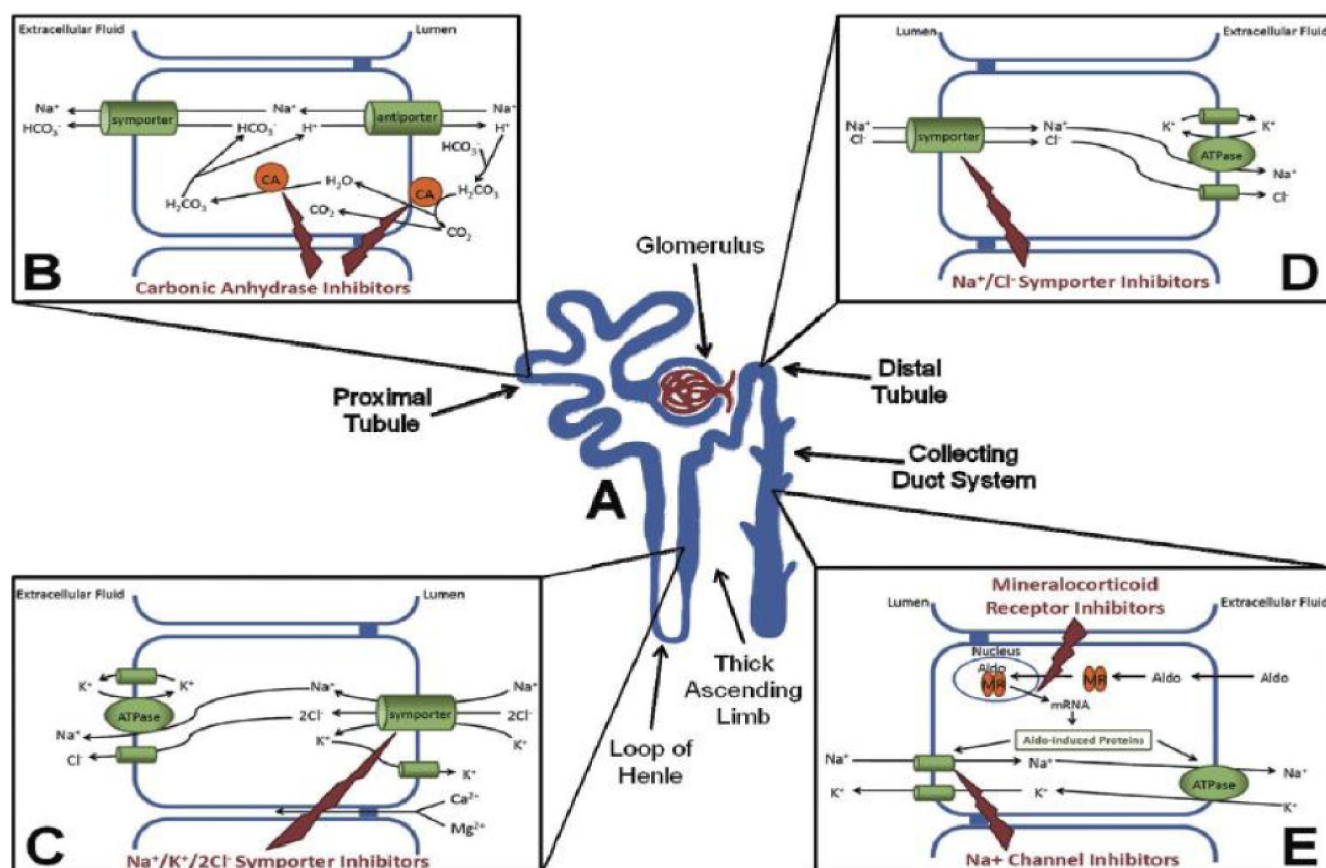


**Figure 1 : Site and mechanism of action of diuretics: (A) The nephron with major divisions labeled. (B) Mechanism of carbonic anhydrase inhibitors in the proximal tubule. (C) Mechanism of the Na+/K+/2Cl- symporter inhibitors in the thick ascending limb of the loop of Henle. (D) Mechanism of the Na+/Cl- symporter inhibitors in the distal tubule. (E) Mechanism of renal epithelial Na+ channel inhibitors and mineralocorticoid receptor antagonists in the collecting duct. Aldo, aldosterone; CA, carbonic anhydrase; MR, mineralocorticoid receptor. Figure modified from Jackson (2006).**

dure[7-9] and has allowed GC–MS to be used as a screening and confirmatory method by replacing the less selective HPLC procedures.

Later, a rapid method was published using microwaves to assist the methylation after extraction[10]. Few problems encountered with the methylation procedure prior to GC–MS approach were the difficulty in methylating some diuretics, and the toxicity of the methyl iodide used in the derivatization process. With the advent of liquid chromatography tandem mass spectrometry (LC–MS/MS) instrumentation, a technique particularly suited to the detection of polar substances, the opportunity has arisen to develop methods for the detection and confirmation of diuretics without the need for derivatization[11-13].

The aim of this work was to develop a fast and simple LC–MS/MS method for the detection of diuretics and probenecid that could replace the existing GC–MS method based on derivatisation following methylation. The method was also required to reliably detect additional diuretics which could not be detected by existing method.

## EXPERIMENTAL

### Chemicals and reagents

All reagents were analytical grade or HPLC grade: Acetonitrile, and ethyl acetate were purchased from Qualigens Mumbai, India, tertiary butyl methyl ether, and formic acid 98% were purchased from Merck, Mumbai, India. HPLC mobile phases were filtered through a 0.2 µm PTFE filter. Ultra high purity nitrogen was obtained from nitrogen generator plant installed at the laboratory. Standards of the following diuretics were obtained from reliable sources: acetazolamide, hydrochlorthiazide, chlorthiazide, chlorthalidone, indapamide, furosemide, bumetanide, bendroflumethiazide, ethacrynic acid, probenecid, mefruside, canrenone, spironolactone, triametrene and amiloride (Sigma–Aldrich, USA), and benzthiazide, cyclopenthiazide, cyclothiazide, epithiazide, hydroflumethiazide, polythiazide, eplerenone, 2-amino-4-chloro-1,3-benzenedisulphonamide (ACB), 4 amino-6-trifluoromethyl-benzene-1,3-disulphonamide (ATFB) (courtesy of the World Association of Anti-Doping Scientists-WAADS) and metolazone (courtesy of the Institute of Biochemistry, Cologne, Germany). Water was purified using a Milli-Q water purification system installed in the laboratory (Millipore, Bedford, USA).

### Sample preparation

The sample preparation was performed using the pre-set method for extraction of various categories of drugs in the laboratory involving enzymatic hydrolysis followed by liquid-liquid extraction[14]. To two or four ml of urine sample aliquots (based on specific gravity), 250 ng/ml of internal standard (mefruside) was added. The urine samples were hydrolysed by β–glucuronidase *(E.coli)* enzyme at 60°C for an hour at pH 7.0 using 0.2 M phosphate buffer. The pH was adjusted to 9-10 with 7% $K_2CO_3$ and liquid-liquid extraction was performed using 5 ml TBME. After mixing for 15 minutes and centrifugation for 10 minutes at 3000 rpm, the organic layer was separated. The pH of the aqueous layer was adjusted to 2-3 by 6 N HCL and second extraction was done using 4 ml ethyl acetate. After mixing for 15 minutes and centrifugation for 10 minutes at 3000 rpm, the organic layer was mixed with the first one and evaporated under nitrogen gas at 60°C. Finally, the residue was reconstituted in 100 µl of mobile phase (1% Formic acid and Acetonitrile) (50:50) *(v/v)* and transferred into conical autosampler vials for analysis.

### Instrumentation

### Negative polarity

A Waters Aquity ultra performance liquid chromatography (UPLC) separation module equipped with a binary pump and C18 column (Acquity BEH, 1.7µX2.1X100mm) was used for the LC separation. The mass spectrometer used was API 4000 QTrap triple stage quadrupole platform. The following binary mobile phase gradient was formed by solvent A (1% aqueous formic acid) and solvent B (acetonitrile) at a flow rate of 0.3ml/min; 40% B to 80% B in 4.00 min and then 40% B in 5.00 min. The injection volume was 5 µl. The spray conditions of the API interface were performed under Electro Spray Ionization (ESI) where the interface temperature was 450°C and IS voltage was 4500 Volts.

### Positive polarity

An Agilent 1100 series, high-pressure gradient pumping system and autosampler (Agilent Technolo-

gies, Waldbronn, Germany) and an API 3200 ™ tandem mass spectrometer (AB Sciex, Canada), operating with an electrospray ionization source. A C18 column (Intersil- C-18 ODS-3 (3.0 mm, 50mm x 4.6mm) was used. The mobile phase, delivered at a flow rate of 0.7 ml/min, consisted of Solvent A (1% formic acid in water) and Solvent B (acetonitrile). The gradient program was: 0min – 15% B; 4.00 min – 60% B; 7.00 min – 100% B; 11 min – 15% B. The injection volume was 10 µl. The mass spectrometer operating conditions consisted of a source heater probe of 550 °C, with a TurboIonspray voltage of 5500 V, entrance potential of 10, curtain gas setting of 10 and CAD setting of 4. The compound dependent parameters were optimized for each compound and nitrogen was used as the collision gas. Collision energies were different for different analytes and are listed in TABLE 1.

## Preparation of reference solutions and quality control samples

A stock standard solution was prepared of each individual compound at a concentration of 1 mg/ml in ethanol and stored at -20 ºC. The reference working solution mix of the compounds was prepared at the concentration level of 10 µg/ml in ethanol. Urinary quality control samples were prepared with every batch at a concentration level of 200 ng/ml.

## Method development and validation

The analytical method was developed and validated as per the WADA guidelines for the anti-doping laboratories[14]. For validation the parameters specificity, ion suppression, intra and inter-day precision, limit of detection (LOD) and robustness were determined.

## Recovery

The recoveries of the diuretics excreted as parent compounds were determined by spiking five replicates of blank urine with each analyte at a concentration of 200 ng/ml and comparing these results with an unextracted standard.

## Specificity

Evaluation of specificity was carried out by analyzing six different spiked and six different blank urine samples collected from healthy volunteers to test for interfering signals in the selected MRM chromatograms at expected retention times of the analytes.

## Ion suppression/ion enhancement

The extent of ion suppression or enhancement was investigated by analysing six different blank urine samples via post-column continuous infusion of a mixture of the reference compounds (1 mg/ml, 20 ml/min)[15].

## Precision

Intra-day precision was determined at 200 ng/ml for each compound using five replicates of spiked urine samples. The corresponding inter-assay precision was calculated from samples prepared and analyzed at three different days (n=5/day). The precision of the method was determined by calculation of the coefficient of variation (CV) of the area ratio of the ion transition of the analytes and the internal standard.

## Limit of detection (LOD)

The LOD was defined as the lowest concentration of analyte that can be identified, measured and reported. It was calculated using two diagnostic ions with a signal-to-noise ratio greater than 3. The LOD was estimated via signal to noise ratio (S/N) of the respective ion traces using ten blank samples and ten fortified samples at concentration levels from 10 to 50 ng/ml.

## Applicability to excretion study samples/routine doping control samples

A total of 21,916 doping control samples received in National Dope Testing Laboratory (NDTL), India from 2008 to 2012 were analyzed by the developed method for diuretics and probenecid. The samples of major events viz. III Commonwealth Youth Games, I Singapore Youth Olympic Games and XIX Commonwealth Games were also included in the study. The method was also applied to excretion study samples after oral administration of spironolactone and eplerenone to human volunteers. The study was duly approved by the ethics committee of NDTL, India.

# RESULTS

A total of 22 diuretics and the masking agent probenecid were detected by the method (Figure 2). As the certified reference compounds for all the diuret-

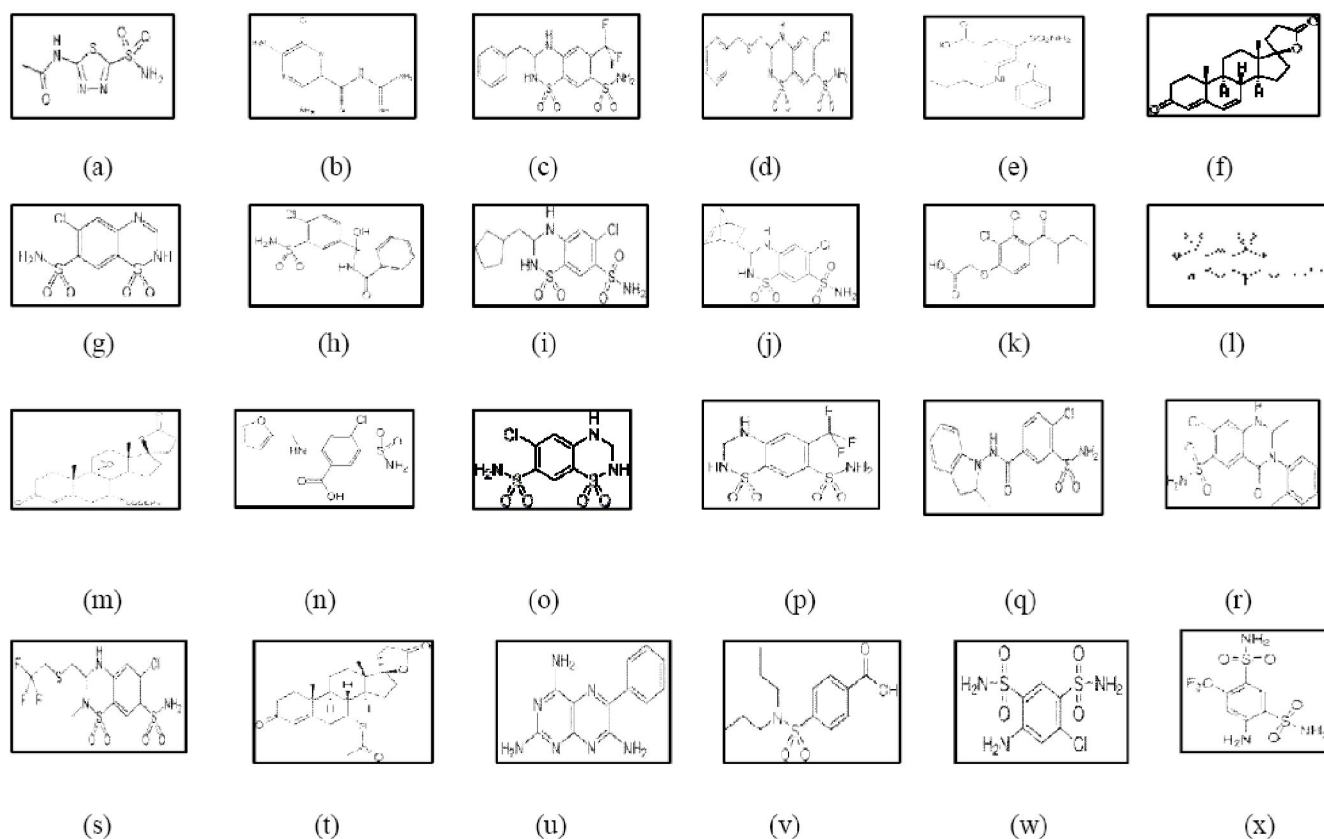


**Figure 2 : Chemical structure of following diuretics: (a) Acetazolamide, (b) Amiloride, (c) Bendroflumethiazide, (d) Benzthiazide, (e) Bumetanide, (f) Canrenone, (g) Chlorthiazide, (h) Chlorthalidone, (i) Cyclopenthiazide, (j) cyclothiazide, (k) Etacrynic acid, (l) Epithiazide, (m) Eplerenone, (n) Furosemide, (o) Hydrochlorthiazide, (p) Hydroflumethiazide, (q) Indapamide, (r) Metolazone, (s) Polythiazide, (t) Spironolactone, u) Triametrene, (v) Probenecid. (w), ACB, (x) ATFB**

ics and probenecid were available, direct infusion analysis was opted to obtain instrumental conditions, and reference product ion spectra.

Both positive and negative modes of ionization were used for each analyte to obtain mass spectra (TABLE 1). The MS–MS data were obtained by choosing a precursor ion and measuring the product ion transitions for each compound for both modes of ionization. The most appropriate precursor to product ion transition in each ionization mode was selected for each compound and this was optimized to obtain maximum sensitivity and specificity. The chromatographic run was optimized taking into account the chemical versatility of the analytes, resulting in a wide range of polarities. The detection of 22 diuretics and probenecid was accomplished. While 4 analytes were detected in positive ion mode as protonated quasi-molecular ions $[M+H]^+$, 19 analytes were detected in negative ionmode as deprotonated quasi-molecular ions $[M-H]^-$. All analytes were clearly detectable at required concentration levels (Figure 3). No interfering signals of the matrix were detected at the expected retention times of the analytes proving the specificity of the method.

Stable retention times are of utmost importance for reliable evaluation. Analysis of QC samples over four weeks yielded stable retention times (CV < 2%) for all of the compounds. No significant decrease or increase of the electrospray response at the expected retention times of the analytes was observed when the urinary matrix was injected disproving the phenomenon of ion suppression or enhancement in the developed method.

The LOD of different compounds in the developed method is listed in TABLE 2. The linearity was evaluated from 10 to 200 ng/ml in urine for all parent drug analytes. All gave a linear response with correlation coefficients ($R^2$) ranging from 0.975 to 0.994. The recovery percentage for all the analytes was found to be between 88.7-110%, intra-and inter-day precisions showed coefficients of variation less than 15% for all analytes (TABLE 2).

TABLE 1 : Mass spectrometric parameters

| S. No. | Compound | Ionization mode | Molecular weight | Precursor Ion (m/z) | Product Ion (m/z) | Collision Energy (eV) | Declustering potential (V) |
|---|---|---|---|---|---|---|---|
| 1. | Acetazolamide | - | 222.25 | 221 | 83; 142 | -40; -35 | -40; -40 |
| 2. | Amiloride | + | 229.6 | 230 | 171; 116 | 36; 43 | 39; 39 |
| 3. | Bendroflumethiazide | - | 421.4 | 420 | 289; 328 | -41; -36 | -40; -40 |
| 4. | Benzthiazide | - | 431.94 | 430 | 228; 308 | -38; -35 | -48; -48 |
| 5. | Bumetanide | - | 364.42 | 363 | 319; 80 | -20; -40 | -40; -40 |
| 6. | Canrenone | - | 340.46 | 341 | 187; 107 | 47; 51 | 48; 48 |
| 7. | Chlorthiazide | - | 295.7 | 294 | 214; 179 | -30; -42 | -40; -40 |
| 8. | Chlorthalidone | - | 338.7 | 337 | 190; 146 | -41; -40 | -35; -35 |
| 9. | Cyclopenthiazide | - | 379.91 | 378 | 205; 269 | -39; -36 | -44; -44 |
| 10. | Etacrynic acid | - | 303.13 | 301 | 243; 207 | -38; -43 | -40; -40 |
| 11. | Epithiazide | - | 425 | 424 | 268; 404 | -39; -18 | -41; -41 |
| 12. | Eplerenone | - | 414.50 | 415 | 163; 337 | 48; 39 | 51; 51 |
| 13. | Furosemide | - | 330.7 | 329 | 285; 205 | -30; -35 | -40; -40 |
| 14. | Hydrochlorthiazide | - | 297.7 | 296 | 269 | -25 | -40 |
| 15. | Hydroflumethiazide | - | 331.2 | 330 | 239; 302 | -33; -21 | -39; -39 |
| 16. | Indapamide | - | 365 | 364 | 189; 132 | -40; -45 | -40; -40 |
| 17. | Metolazone | - | 365.8 | 366 | 259; 277 | 34; 48 | 47; 47 |
| 18. | Polythiazide | - | 439 | 438 | 324; 418 | -38; -32 | -43; -43 |
| 19. | Spironolactone | + | 416.5 | 314 | 187; 107 | 47; 51 | 48; 48 |
| 20. | Probenecid | + | 285.3 | 286 | 244; 185 | 25; 38 | 33; 33 |
| 21. | Triamterene | + | 253.2 | 254 | 237; 195 | 25; 38 | 36; 36 |
| 22. | ACB | - | 285.73 | 284 | 207; 169 | -25; -38 | -30; -30 |
| 23. | ATFB | - | 319.28 | 318 | 214; 239 | -38; -32 | -34; -34 |

**- negative; + positive**

The method was successfully applied to the analysis of 21,916 routine samples received in NDTL from 2008 to 2012. A total of 5.08 % adverse analytical findings (AAFs) for various drugs of abuse were reported during the period (Figure 4). Out of the total adverse analytical findings, 6.47 % of AAFs were constituted for diuretics (Figure 5). In 2009, a steep rise in AAFs of diuretics was observed after the inclusion of method in the routine screening procedure which may be due to the improved method. The drug-wise breakup shows that most abused diuretic was furosemide (Figure 6).

Eplerenone is methyl hydrogen 9,11 α-epoxy-17 α -hydroxy-3-oxopregn-4-ene-7 α,21-dicarboxylate,γ-lactone and ahighly selective aldosterone blockerand potassium-sparing diuretic used in the therapy of hypertension. The excretion study samples of eplerenone were collected for 100 hours after oral administration of single dose of Eptus, 25 mg (Glenmark, India) to 3 healthy male volunteers (age-25±2 years). The excretion study rate of the parent compound was quantitated in urine using a five level calibration curve with r= 0.9987. The highest concentration of the drug was found at 6 hours (Figure 7). Additionally, the metabolite 6 β-OH eplerenone was also monitored and was seen till 75 hours post administration (Figure 8). The precursor to product ion transitions of m/z 415-163 and m/z 431-337 were used to monitor eplerenone and 6 β-OH eplerenone, respectively. Acceptable precision and accuracy were obtained for concentrations over the standard curve range.

Spironolactone is a mineralocorticoid receptor antagonist (aldosterone antagonist) and a potassium-sparing diuretic. The excretion study samples of spironolactone were collected for 100 hours after oral administration of single dose of Lasilactone, 20 mg (Aventis

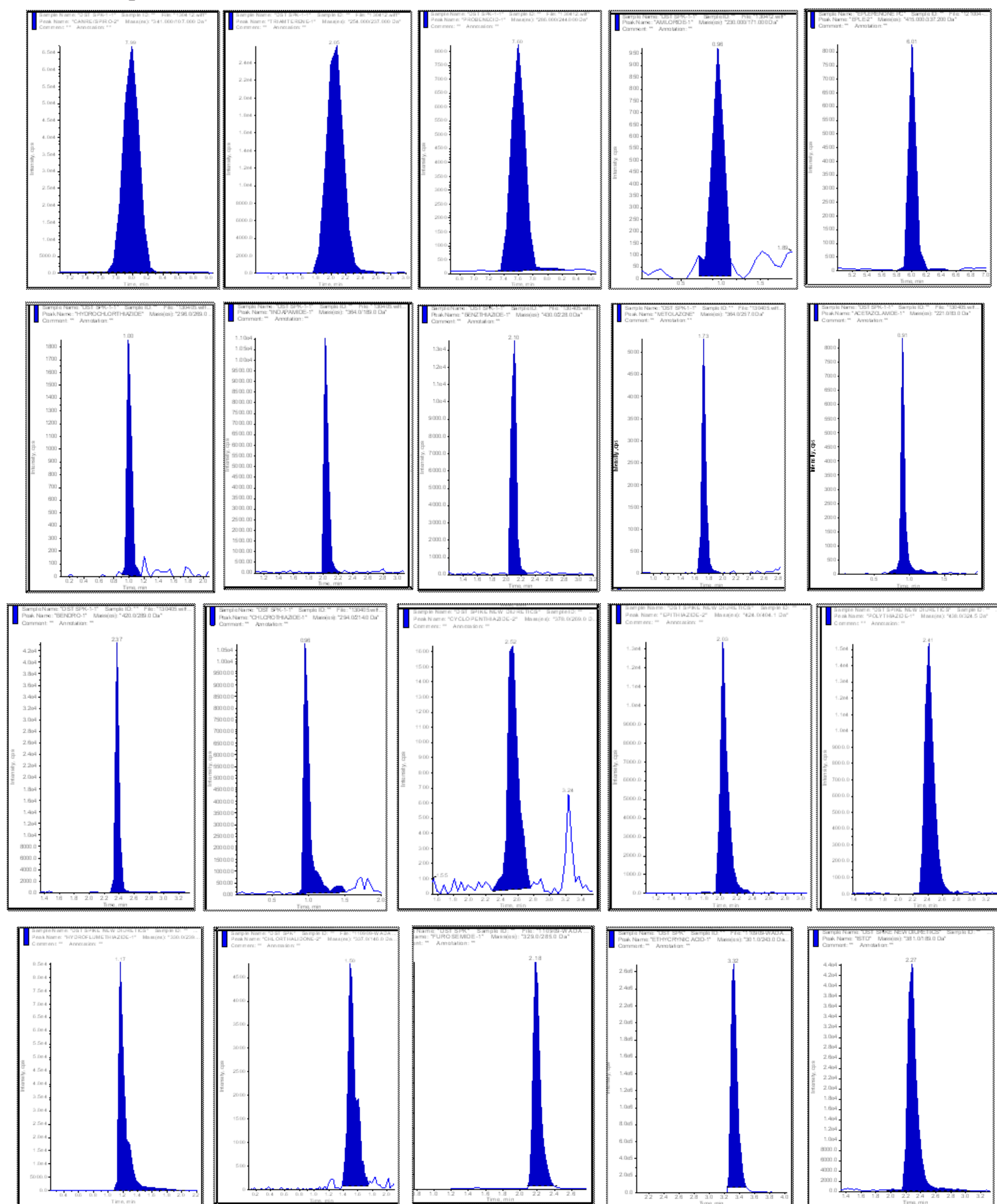

**Figure 3 : Detectability of the method showing ion chromatogram of all analytes**

Pharma Ltd, India) to 3 healthy male volunteers (age-25±2 years). During electrospray evaporation and ionization process, spironolactone readily loses the 7á-acetylthiogroup being transformed to canrenone, additionally it is readily metabolised in humans to canrenone, so it was quantified in that form. The excretion study rate was quantitated in urine using a five level calibration curve with r = 0.9971. The highest concentration

of canrenone was found at 12 hours (Figure 9). The precursor to product ion transitions of m/z 341-187 was used to monitor canrenone in urine samples. Acceptable precision and accuracy were obtained for concentrations over the standard curve range.

## DISCUSSION

As previously mentioned, diuretics are commonly prescribed in clinical medicine to treat hypertension and other cardiovascular disorders. Diuretics are banned in all sports because they can cause rapid weight loss and can act as masking agents (to hide the effects of other prohibited substances) both in and out of competition. However, the World Anti-Doping Code permits the therapeutic use of diuretics when athletes and their physicians apply for therapeutic use exemptions (TUE) according to the International Standard for TUE[28]. For diuretics, the primary permitted therapeutic use is for hypertension. It should be noted that a TUE is not valid if an athlete's urine contains a diuretic in association with a threshold or sub-threshold level of another exogenous substance included on the Prohibited List.

Reasonably, the most effective use of diuretics in sport doping would be before an anti-doping test. Diuretics increase urine volume and dilute any doping agents as well as their metabolites present in the urine and make their detection more problematic by conventional anti-doping analysis. For this reason, diuretics are classified as masking agents on the WADA Prohibited List (class S5: 'Diuretics and other masking agents')[4].

For the detection of diuretics in urine in sports doping, a single minimum required performance level (MRPL) of 200 ng/ml is fixed by WADA for accredited laboratories[17]. Even though the relative potencies, metabolism and elimination properties vary dramatically and result in different urinary levels between the classes of diuretics (TABLE 3), the MRPL at 200 ng/ml is sufficient to detect acute diuretic abuse by athletes. Lower dosages of diuretics are likely to be insufficient at causing the masking effect or dramatic and acute weight loss abusers seek.

Several analytical techniques have been proposed for the analysis in doping control, primarily among them are HPLC-UV-DAD, GC-MS, LC-MS and LC-MS.MS[17-26]. However, the best solution for a comprehensive screening method capable of detecting the presence in a biological sample, at the same time satisfying the WADA's MRPL is represented by the methods based on LC-MS or LC-MS/MS[1-13,18,19].

When diuretics were introduced on the list of forbidden substances, by the International Sports Authorities, the first attempts, to create a screening method for their detection were based on HPLC with UV diode array as detector as it facilitated peak identification[5,6]. According to WADA the confirmation procedure data to support a positive case should be based on mass spectrometry. This made the use of GC-MS a method of choice[15]. As a matter of fact, GC-MS is still used by various anti-doping laboratories for the detection of diuretics. The analytical procedure based on GC-MS is structured into the series of following events, sample pre-treatment, chemical derivatization and injection on GC-MS. Derivatization is necessary prior to GC-MS analysis as most of the diuretics are not sufficiently volatile, lipophillic or thermally stable to be directly assayed with the analytical technique. The most common derivatization procedures are silylation and methylation but the latter is preferred as it allows sufficient yields of more stable derivatives[30]. However, the method faces some pitfalls, because of the tedious and hazardous methylation step and not-so-sensitive GC-MS analysis to give lower limit of detection (LOD).

The attempts to use LC-MS (MS) for the detection of diuretics started in early 1990's. In the past decade, LC-MS/MS has proven to be the best method of choice for the analysis of diuretics for the following reasons; first, it does not require the chemical derivatization of the samples prior to the analysis and second, it does not require the sample to be converted into gaseous phase before thereby improving sensitivity and lowering the LOD. The use of simultaneous positive and negative ionization method allowed the detection of acidic as well as basic diuretics. The analysis with tandem MS with triple stage quadruples proved to be highly sensitive and specific. The improvements in the scanning speed of the mass spectrometers as well as better performing LC columns and LC pumps (UPLC) allowed an increase in speed of analysis[11-13,18,19].

Due to the compatibility of the LC-MS/MS system with the aqueous matrix of urine and high sensitivity the

Full Paper

**TABLE 2 : Method validation results showing recovery and precision**

| S. No. | Compound | LOD (ng/ml) | Recovery (%) | RSD (%) | RRT-precision (n=5) | Inter-day Precision (CV%) (n=5X3) | Intra-day precision (CV%) (n=5) |
|---|---|---|---|---|---|---|---|
| 1. | Acetazolamide | 20 | 92.4 | 10.9 | 0.12 | 2.3 | 3.2 |
| 2. | Amiloride | 20 | 88.7 | 7.1 | 0.21 | 4.5 | 4.1 |
| 3. | Bendroflumethiazide | 10 | 98.5 | 14.2 | 0.12 | 5.6 | 4.8 |
| 4. | Benzthiazide | 10 | 101.8 | 12.4 | 0.8 | 1.2 | 2 |
| 5. | Bumetanide | 10 | 108.4 | 13.2 | 0.23 | 2.2 | 2.1 |
| 6. | Canrenone | 10 | 94.5 | 7.8 | 0.24 | 4.8 | 4.1 |
| 7. | Chlorthiazide | 20 | 92.5 | 8.9 | 0.15 | 5.9 | 4.9 |
| 8. | Chlorthalidone | 20 | 87.4 | 9.8 | 0.22 | 5.5 | 4.4 |
| 9. | Cyclopenthiazide | 20 | 98.1 | 7.6 | 0.9 | 3.9 | 3.2 |
| 10. | Etacrynic acid | 20 | 87.4 | 7.4 | 0.15 | 2.8 | 2.5 |
| 11. | Epithiazide | 20 | 95.6 | 9.4 | 0.13 | 3.3 | 3.1 |
| 12. | Eplerenone | 10 | 99.3 | 10.1 | 0.23 | 3.4 | 2.8 |
| 13. | Furosemide | 10 | 105.2 | 12 | 0.12 | 4.8 | 4 |
| 14. | Hydrochlorthiazide | 10 | 94.2 | 11.8 | 0.12 | 5.8 | 5.2 |
| 15. | Hydroflumethiazide | 20 | 96 | 11.2 | 0.13 | 6.8 | 6.5 |
| 16. | Indapamide | 10 | 104.5 | 8.5 | 0.09 | 5.2 | 5 |
| 17. | Metolazone | 10 | 110 | 9.4 | 0.13 | 4.4 | 5 |
| 18. | Polythiazide | 20 | 94.7 | 12.5 | 0.14 | 2.5 | 3.8 |
| 19. | Spironolactone | 10 | 98.7 | 13 | 0.17 | 3.5 | 3.2 |
| 20. | Probenecid | 10 | 94.7 | 9.5 | 0.26 | 4.4 | 4.3 |
| 21. | Triametrene | 10 | 89.5 | 8.9 | 0.17 | 5.5 | 5.2 |
| 22. | ACB | 15 | 78.1 | 7.7 | 0.1 | 4.8 | 4.4 |
| 23. | ATFB | 15 | 81.2 | 4.2 | 0.12 | 3.9 | 4.1 |

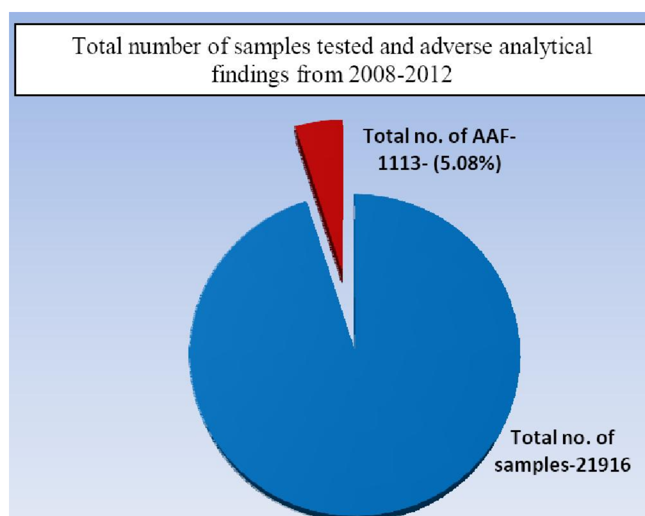


**Figure 4 : Total number of samples and AAFs during 2008-2012**

identification of prohibited substances without pre-concentration steps is possible, resulting in very simple and fast 'dilute-and-shoot' methods. Additionally, omission of sample pre-treatment steps leads to savings in terms of labour and reagent costs, error-prone and time. Diuretics are excreted as parent compound in humans hence, are suitable candidates for direct "dilute and shoot" urine analysis. Various direct urine analysis methods for diuretics were developed in the past[28,29]. However, the dilute and shoot approach possess few pitfalls like shifted retention times due to unwanted matrix effects, frequent degradation of LC column, contamination of source and analysers of the mass spectrometer etc. Hence, the dilute and shoot methods may be a good choice for confirmatory procedures but they could not be adapted for routine screening purposes.

The members of diuretic class vary greatly in terms of structure, physicochemical properties and site of mechanism of action. In the 1990s the analysis of diuretics in doping by LC-UV and GC-MS methods was a challenge for the anti-doping laboratories due the heterogeneity of the substances included. Since, the ad-

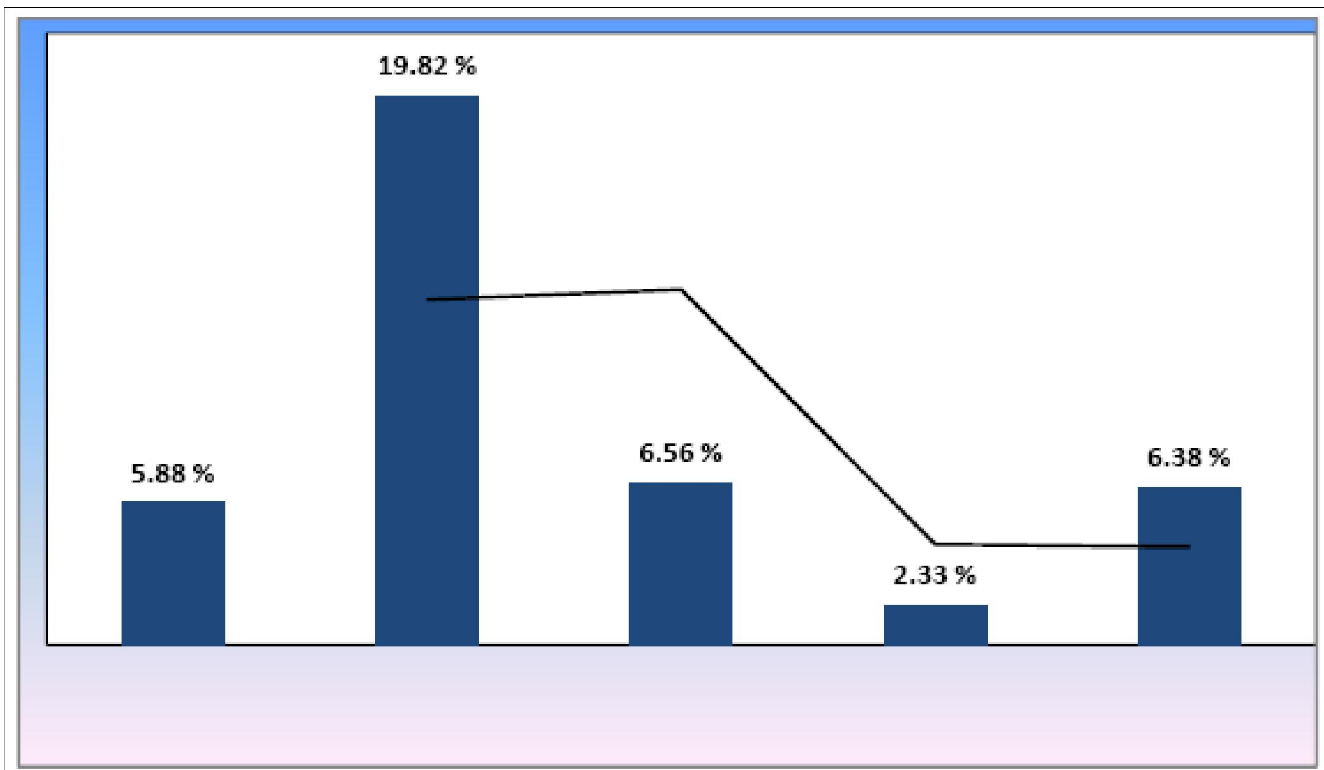


**Figure 5: Year wise distribution of AAFs for diuretics in NDTL (2008-2012)**

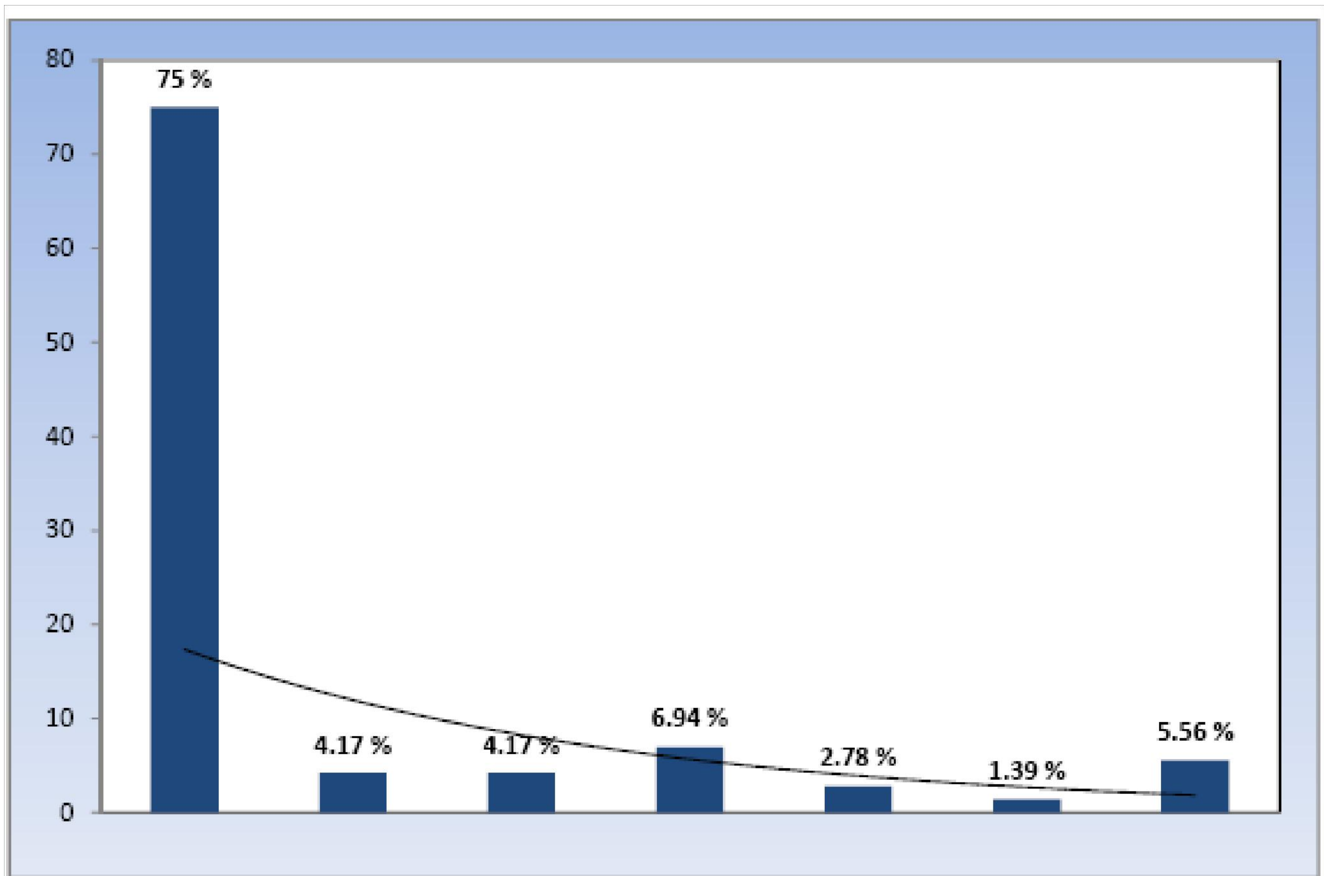


**Figure 6 : Drug wise distribution of AAFs for diuretics in NDTL (2008-2012)**

Full Paper

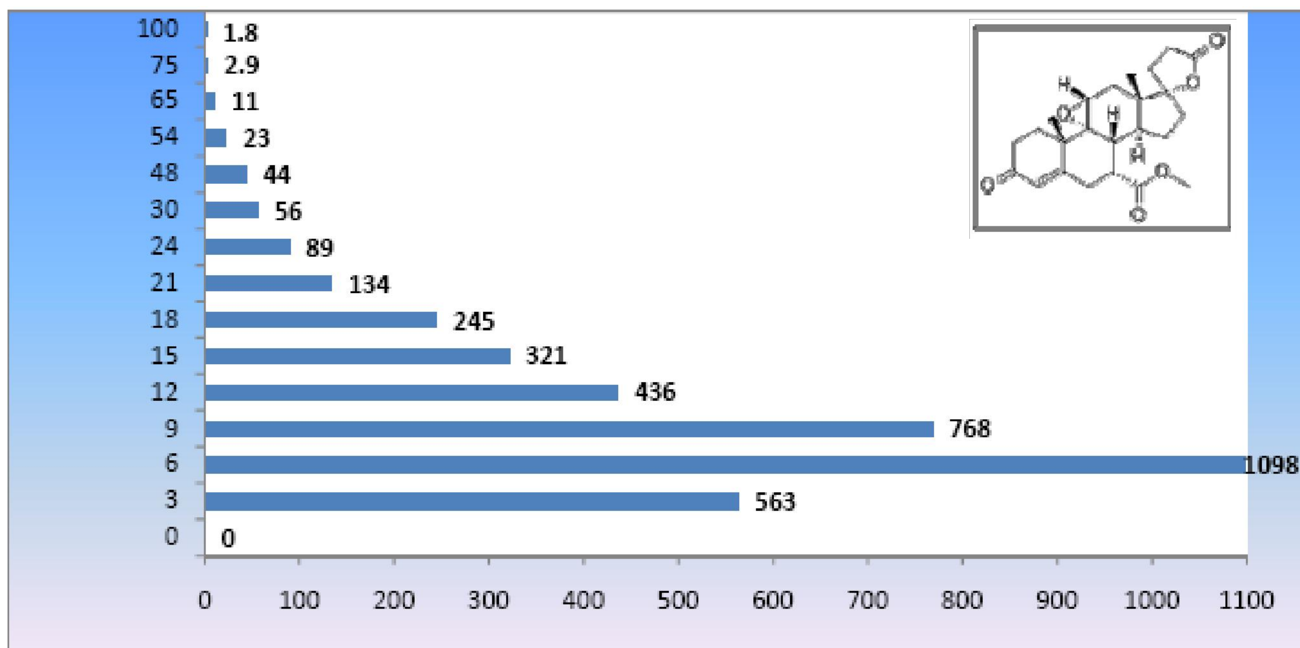


**Figure: 7: Urinary excretion profile of eplerenone after oral administration**

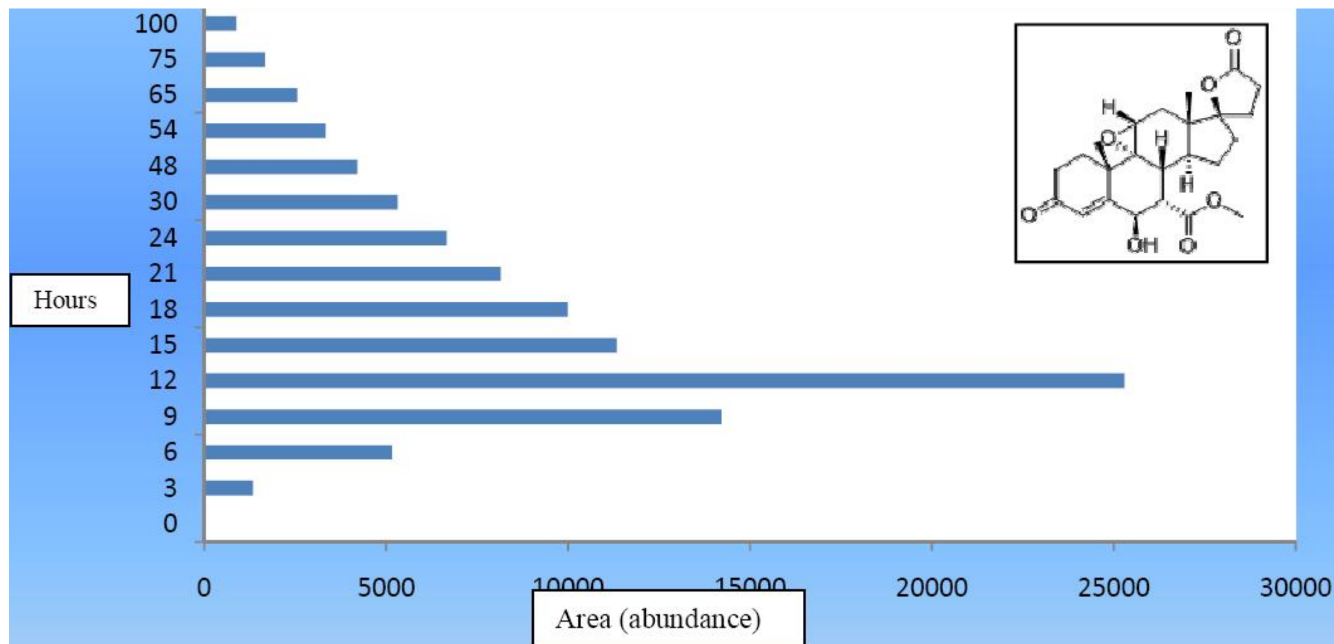


**Figure 8 : Urinary excretion profile of 6 β-OH eplerenone after oral administration**

vent of robust and reliable LC-MS/MS instrumentation, their detection in human urine is no longer a problem. This has increased the sensitivity of the method and the number of compounds in the screening procedure whereas, decreased the analysis time and cost to the laboratories.

The method described in this study has confirmed that both positive and negative ionization are required for detection of diuretics on LC-MS/MS. For the compounds included in the study, 04 were detected in positive ionization mode while 19 were included in the negative ionization mode. The use of solvent systems using water with added ammonium acetate, formic acid, and acetic acid combined with methanol or acetonitrile was evaluated. The best combination of ionisation efficiency and chromatographic peak shape was found with the 1

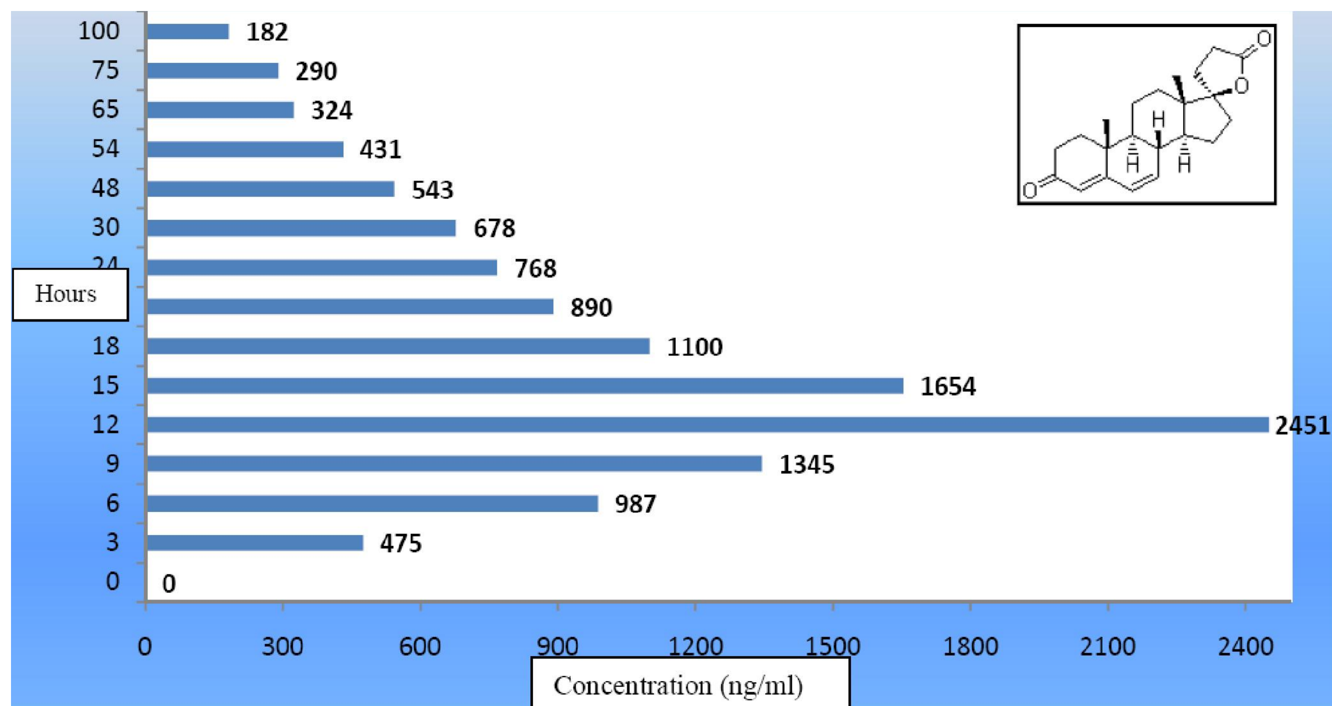


**Figure 9 : Urinary excretion profile of spironolactone/canrenone after oral administration**

% formic acid acetonitrile combination. Until, the development of this method, diuretics were screened and confirmed in NDTL, India using GC–MS methylation method. However, screening of diuretics was shifted to LC-MS/MS after full and successful method development and validation. All the compounds were found to have minimum three product ions of sufficient intensity to enable effective confirmation by comparing their ion ratios.

The current screening method on LC-MS/MS takes only 5 min. of runtime to analyze 1 sample against the 15 min runtime of the traditional GC-MS method. This has significantly improved the throughput where 12 samples could be detected in an hour against 4 samples per hour using the GC-MS method. The high-throughput of the method proved to be beneficial during the testing of major events viz. I Singapore Youth Olympic Games and XIX Commonwealth games.

The method developed has shown itself to be simple, robust and reliable in the detection of diuretics for sports drug testing. It has been in routine use for more than 5 years involving the analysis of over 20,000 urine samples. Only four UPLC columns were needed for this period. Use of the GC–MS diuretics screening proceeding had been discontinued after running both methods in parallel for 1 month. A rise in AAFs for diuretics was observed in 2009, after employing the improved method in routine testing procedure. Maintenance of the API 4000 Qtrap has been minimal merely involving occasional washing of the curtain plate to restore sensitivity. Apart from the intended advantages of screening diuretics with faster, simpler and safer sample preparation it has also been found that the interpretation of the data is much easier as interfering peaks are very rarely found in samples.

## CONCLUSION

A fast, generic and sensitive method was developed for the analysis of 22 diuretics and probenecid. The experiments presented in this work were based on ultra-high-pressure liquid chromatography coupled to hybrid quadruple tandem mass spectrometry. The method was validated according to the International Standard for Laboratories described in the World Anti-Doping Code and was selective enough to comply with the World Anti-Doping Agency recommendations.

## ACKNOWLEDGMENT

The support of Ministry of Youth Affairs and Sports is duly acknowledged for financial grant.

**TABLE 3 : Properties of diuretics important for analytical method development**

| S. No. | Compound | pKa | logP | Relative potency* | Elimination route | Metabolism % | Category |
|---|---|---|---|---|---|---|---|
| 1. | Acetazolamide | 7.4, 9.1 | 0.3 | 1 | Renal | 0 | Carbonic anhydrous inhibitor |
| 2. | Bumetanide | 3.6, 7.7 | 2.6 | 40 | Renal | 38, Hepatic | |
| 3. | Furosemide | 3.8, 7.5 | 2.0 | 1 1 | Renal | 35, Renal | $Na^+/K^+/2Cl^-$ symporter inhibitors (loop diuretics) |
| 4. | Etacrynic acid | 3.5 | 3.7 | 0.7 | Renal | 33, Hepatic | |
| 5. | Bendroflumethiazide | 9.0 | 1.9 | 10 | Renal | 70, Hepatic | |
| 6. | Chlorthiazide | 6.85 | -1.9 | 0.1 | Renal | 0 | |
| 7. | Chlorthalidone | 9.4 | 0.8 | 1 | Renal, bile | 25. Unknown | |
| 8. | Cyclopenthiazide | 9.13 | 2.07 | 9 | Renal | 0 | |
| 9. | Epithiazide | 8.8 | NA | 1 | Renal | 15, Renal | |
| 10. | Benzthiazide | 4.60 | NA | 1 | Renal | 0 | $Na^+/Cl^-$ symporter inhibitors (thiazide and thiazide-like diuretics) |
| 11. | Hydrochlorthiazide | 9.5, 11.3 | -0.1 | 1 | Renal | 0 | |
| 12. | Hydroflumethiazide | 8.9 | 0.4 | 1 | Renal | 20–60, Hepatic | |
| 13. | Indapamide | 8.8 | 2.7 | 20 | Renal | 100, Hepatic | |
| 14. | Metolazone | -1.6, 9.54 | 1.8 | 10 | Renal, bile | 10, Hepatic | |
| 15. | Polythiazide | -3.1, 9.31 | 1.9 | 25 | Renal | 25–75, Unknown | |
| 16. | Amiloride | 8.7 | 0.3 | 1 | Renal | 0 | Renal epithelial Na+ channel inhibitors (potassium-sparing diuretics) |
| 17. | Triametrene | 6.2 | 1.0 | 0.1 | Renal | 100, Hepatic | |
| 18. | Spironolactone | -4.9, 18.01 | 2.8 | NA | Renal | 100, Hepatic | |
| 19. | Canrenone | NA | 3.5 | NA | Renal | 100, Hepatic | Mineralocorticoid receptor antagonists |
| 20. | Eplerenone | 15.11 | 2.3 | NA | Renal | 100, Hepatic | |
| 21. | Probenecid | 3.53 | 3.21 | NA | Renal | 90, Hepatic | Sulfonamide-derived uricosuric |

***Potency is relative to diuretics within the same class. ;**

## REFERENCES


[1] E.K.Jackson; Diuretics In: L.Brunton, J.Lazo, K Parker, (Eds);. 11th Edition. Goodman and Gilman's The Pharmacological Basis of Therapeutics, McGraw-Hill: New York, **(2006)**.

[2] G.J.Trout, R.Kazlauskas; Chem.Soc.Rev., **33**,1 **(2004)**.

[3] R.Ventura, J.Segura; J.Chromatogr.B Biomed.Appl., **687**, 127 **(1996)**.

[4] The List, World Anti-Doping Agency (WADA). The 2012 Prohibited List, International Standard. URL: http://www.wada-ama.org/Documents/World_Anti- Doping_Program/WADP-Prohibited-list/To_be_effective/WADA_Prohibited_List_2013_EN.pdf(Accessed on January 1, 2013), **(2013)**

[5] S.F.Cooper, R.Masse, R Dugal; J.Chromatogr., **489**, 65 **(1989)**.

[6] H.J.Guchelaar, L.Chandi, O.Schouten, W.A.Van Den Brand, Fresenius; J.Anal.Chem., **363**, 700 **(1999)**.

[7] S.J.Park, H.S.Pyo, Y.J.Kim, M.S.Kim, J Park; J.Anal.Toxicol., **14**, 84 **(1990)**.

[8] A.M.Lisi., R.Kazlauskas, G.J.Trout; J.Chromatogr., **581**, 57, **(1992)**.

[9] A.Beotra, S.Jain, T.Kaur; Diuretics testing in sports

by GC-MSD: Excretion studies of bendroflumethiazide, diclofenamide and etacrynic acid" Paper presented at the International Congress on frontiers in pharmacology and therapeutics in 21st century, New Delhi, 3-5 Dec **(1999)**.

**[10]** L.Amendola, C.Colamonici, M.Mazzarino, F.Botre; Anal.Chim.Acta., **475**, 125 **(2003)**.

**[11]** V.Sanz-Nebot, I.Toro, R.Berges, R.Ventura, J.Segura, J.Barbosa; J.Mass Spectrom., **36**, 652 **(2001)**.

**[12]** K.Deventer, F.T.Delbeke, K.Roels, P.Van Eenoo; Biomed.Chromatogr., **16**, 529 **(2002)**.

**[13]** D.Thieme, J.Grosse, R.Lang., R.K.Mueller, A.Wahl; J.Chromatogr.B, **757**, 49 **(2001)**.

**[14]** M.I.Reddy, A.Beotra, S.Jain, S.Ahi; **41(2)**, 80 **(2009)**; WADA International Standards for Laboratories (ISL), Version 7, available online at http://www.wada-ama.org/documents/world_anti-doping_program/wadp-is aboratories/isl/wada_int_standard_laboratories_2012_en.pdf. (Accessed on January 1, 2013)

**[15]** T.M.Annesley; Clin.Chem., **49**, 1041 **(2003)**.

**[16]** World Anti Doping Agency, Technical document on Minimum required performance limits.http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/Technical_Documents/WADA-TD2013MRPL-Minimum-Required-Performance-Levels-v1-2012-EN.pdf(Accessed on January 1, 2013)

**[17]** G.J.Murray, J.P.Danaceau; J.Chromatogr.B, **877**, 3857 **(2009)**.

**[18]** M.Thevis, W.Schanzer, H.Schmickler; J.Am.Soc.Mass.Spectrom., **14**, 658 **(2003)**.

**[19]** M.B.Barroso, H.D.Meiring, A.De Jong, R.M.Alonso, R.M.Jime´nez; J.Chromatogr.B, **690**, 105 **(1997)**.

**[20]** S.J.Park, H.S.Pyo, Y.J.Kim, M.S.Kim, J.Park; J.Anal.Toxico., **14**, 84 **(1990)**.

**[21]** F.Y.Tsai, L.F.Lui, B.Chang; J.Pharma.Bio.l Anal., **9**, 1069 **(1991)**.

**[22]** L.Amendola, C.Colamonici, M.Mazzarino, F.Botrè; Anal.Chim.Acta., **475**, 125 **(2003)**.

**[23]** J.Beyer, A.Bierl, F.Peters, T Maurer; Ther.Drug.Monit., **27**, 509 **(2005)**.

**[24]** T.Goto, E.Mikami, T.Ohno, H.J.Matsumoto; Food Hygie.Soci.Japan, **43**(2), 95 **(2002)**.

**[25]** M.L.Riekkola, J.H.Jumppanen; J Chromatogr A., **31**(735), 151 **(1996)**.

**[26]** M.Lu, P.Tong, H.Xiao, S.Xia, X.Zheng, W.Liu, Zhang L, G.Chen; Electrophoresis, **28(9)**, 1461 **(2007)**.

**[27]** World Anti Doping Agency, International Standard for therapeutic use exemption, Available online at. Version 5, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS

**[28]** TUE/2011/WADA_ISTUE_2011_revJanury-2012_EN.(Accessed on January 1, 2013)

**[29]** S.Guddat, E.Solymos., A.Orlovius, A.Thomas, G.Sigmund, H.Geyer,M.Thevis, W.Schänzer; Drug Test.Anal., **3**, 836 **(2011)**.

**[30]** K.Deventer, O.J.Pozo, P.V.Eenoo, F.T.Delbeke; J.Chromatogr.A, **1216(31)**, 5819 **(2009)**.