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Liquid chromatography tandem mass spectrometry determination of prohibited diuretics and other acidic drugs in human urine: A Review

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Abstract
This paper reviews liquid chromatographic-mass spectrometric (LC-MS) procedures for the screening, identification and quantification of doping agents in urine and other biological samples and devoted to drug testing in sports. Reviewed methods published approximately within the last five years and cited in the PubMed database have been divided into groups using the same classification of the 2004 World Anti-Doping Agency (WADA) Prohibited List. Together with procedures specifically developed for anti-doping analysis, I.C-MS applications used in other fields (e.g., therapeutic drug monitoring, clinical and forensic toxicology, and detection of drugs illicitly used in livestock production) have been included when considered as potentially extensible to doping control. Information on the reasons or potential abuse by athletes, on the requirements established by WADA for analysis, and on the WADA rules for the interpretation of analytical finding. The basis of human sports doping control is set by the World Anti-Doping Agency (WADA) within the World Anti-Doping Code. It defines doping as mainly the presence of prohibited substances in a specimen; and the use or attempted use of a prohibited substance/method. The list of Prohibited substances covers all classes that are used in doping.

Keywords: diuretics, liquid chromatography tandem mass spectrometry, world anti-doping agency (WADA), doping

Introduction
Doping control is a particularly demanding task for both analytical and interpretive reasons: Diuretics are wide spectrum substances in doping control, in terms of molecular weight, polarity, PKa, and chemical/thermal stability. High sensitivity of detection in urine samples is necessary for many substances that are, being administered before competition, during the competition and out of competition and are expected to be present at the low micrograms-per-litre level. Discrimination is possible in doping of doping from other possible reasons; drugs are used for the masking purposes or physiological/pathological changes in the diuretics and other acidic drugs of different classes. Because of its Parameter like robustness, precision, accuracy and high level of standardization, liquid chromatography-mass spectrometry (LC-MS) has a lot of features that can be effectively exploited in sports drug testing or that offer good perspectives of application in the future [1, 2]. First, LC-MS allows minimal sample preparation, thus increasing the sample throughput by means of:

1. Direct analysis of conjugated metabolites.
2. Chromatographic separation of polar compounds
3. Online sample preparation, because of the compatibility between aqueous sample and analytical system, at least in the reversed-phase (RP) mode. Furthermore, LC-MS makes it possible to detect the whole metabolic profile of drugs, from the parent compound to very polar conjugated metabolite or in the discrimination between doping and recreational use of drugs such as stimulants or narcotics. LC-MS can be helpful also in the analysis of chemically unstable substances [3]

List of prohibited substances by wada
The WADA prohibits any substances for use in sports on the basis of following three criteria:

- The potential to enhance performance,
- An actual or potential health risk, and,
- Whether it violates the spirit of sport.

If a substance is prohibited, the entire class of the substance is usually banned, regardless of

whether the specific substance is named with a few exceptions. It is the athlete's personal responsibility to avoid consuming a banned substance. The List is updated annually based on several factors like pattern of use of a substance in preceding year, potential of abuse/enhancement effects based of pharmacological studies, ill health effects, etc and finally following an extensive consultation process facilitated by WADA.

The currently effective list of prohibited substance and methods in human sports was issued by WADA on first January, 2017.

The list is broadly categorized in fifteen classes based on either their pharmacological properties or effects. Each category contains more several representative examples of individual drugs. There are more than 350 drug and/or metabolites enlisted in the list.

Substances prohibited at all times-

- S0. Non-approved Substances
- S1. Anabolic Agents
- S2. Peptide Hormones, Growth Factors and Related Substances
- S3. Beta-2 Agonists
- S4. Hormone and Metabolic Modulators
- S5. Diuretics and Other Masking Agents

Substances prohibited in-competition-

- S6. Stimulants
- S7. Narcotics
- S8. Cannabinoids
- S9. Glucocorticosteroids

Substances prohibited in particular sports-

- P1. Alcohol
- P2. Beta-Blockers

Prohibited Methods

- M1. Manipulation of blood and blood components
- M2. Physical and chemical manipulation
- M3. Gene Doping [4, 5].

Diuretics metabolism in body

Diuretics increases 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 haemodynamic, and site and 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 on The World Anti-Doping Agency's (WADA) list of prohibited substances; the use of diuretics is banned both in competition and out of competition and diuretics are routinely screened for by anti-doping laboratories [6, 7].

Site and mechanism of action of diuretics (8).

Diuretics	Site of Action	Mechanism
Osmotic diuretics	1. Proximal tubules 2.Loop of Henle 3.Collecting duct	Inhibition of water and sodium reabsorption
Carbonic anhydrase inhibitor(CA-I)	Proximal tubules	Inhibition of bicarbonate reabsorption
Loop Diuretics	Loop of Henle (thick ascending limb)	Inhibition of sodium, potassium, chloride cotransport
Thiazide	Early distal tubule	Inhibition of sodium, chloride co-transport
Potassium sparing diuretics	Late distal tubule collecting duct	Inhibition of sodium reabsorption and potassium secretion

Diuretics profiling in doping control analysis

Diuretics are prohibited/banned in every sport discipline in, as well as out, of competition and during the competition Thus, all urine samples of athletes taken for doping are screened for many agents e.g. : diuretic agents. As we know diuretics are drugs that help in weight reduction as it causes increased renal elimination of water, thus giving it a great importance in sports. On the other hand, it also helps in an increased urine flow and can decrease the concentration of renal excreted compounds that are prohibited in WADA list. It is also marked in mind that the drugs being administered from the body should not be off an increased cut off limit if so, is considered banned. Therefore, the possibility of manipulation of doping control samples by administration of diuretic agents exists with all specimens requiring a comprehensive doping analysis. The wide variety of diuretics and their heterogeneous chemical structures and physico- chemical properties have proven to complicate the development of broad and capacious screening procedures for doping control purposes. Various substances/ drugs of this category bear acidic functions (e.g., bumetanide, piretanide, furosemide, and ethacrynic acid), but others are basic compounds such as amiloride and triamterene. In addition, numerous benzothiadiazines (e.g., ethiazide, epithiazide, and althiazide) demonstrate poor GC behavior even after derivatization, and osmotic diuretics such as mannitol are hardly compatible with most of the sample preparation and extraction procedures. Several approaches based on GC–MS, HPLC–UV, and HPLC–plasma spray were developed in the past [9,10-12] to cope with the complex class of diuretic agents by means of procedures including extractive alkylation, solid-phase extraction (SPE), multiple liquid–liquid extractions under different pH conditions, and derivatization. Employing LC–ESI–MS–MS or LC–APCI–MS–MS, most of the analyses belonging to the class of diuretics are identified in human urine at reasonable detection limits with high specificity as demonstrated in several studies [13-15]. Exceptions are generally the osmotic diuretics (such as mannitol), which are neither isolated from urine by commonly accepted sample preparation procedures, nor are these compounds efficiently ionized by ESI or APCI under the given conditions. Different strategies here have been evaluated in order to identify mannitol and its stereoisomers in human urine [16]. Because of the heterogeneity of diuretics regarding acidity and basicity, the ionization mode (positive or and (B) acetonitrile at. a flow rate of 800 µL/min. A single run is completed within 8 min.

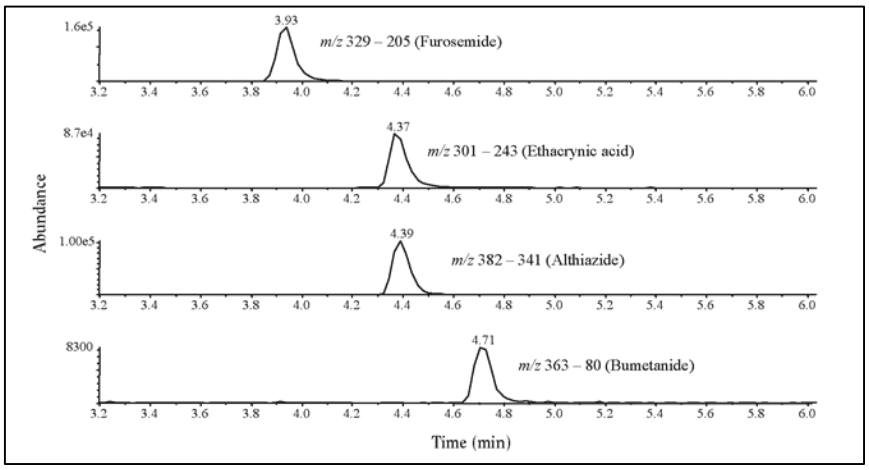

Fig 1: extracted ion chromatogram of urine sample fortified with 100ng/ml of furosemide, ethacrynic acid, althiazide, and bumetanide, analysed on agilent 1100 series LC interfaced by APCI to an Applied biosystems API2000 triple quadrupole MS. Ionization was performed by deprotonation negative mode [16]

Why diuretics are banned in sports

Athletes use diuretics for three reasons. And that's why WADA prohibited the use of diuretics

1. For acute reduction of weight which may offer a potential advantage in sports such as boxing, judo or weightlifting where competition is in weight categories.
2. To overcome fluid retention induced by anabolic androgenic steroids (AAS). This could be useful to body – builders trying to obtain a cut look.
3. To speed up the elimination of drugs from the system [17].

Diuretics profiling for clinical diagnosis

It can sound anomalous that, whereas diuretics abuse in sport is widely treated, their clinical usefulness for patients with severe hypertension, edema, chronic renal failure. For example, furosemide has maximal natriuretic effect is much greater than that of other classes, it is active even in patients with relatively several renal failure. Bumetanide is 40 times for more potent than furosemide used in renal haemodynamic changes and may be tolerated by patient allergic to furosemide [18, 19]. The analysis of diuretics plays an important role in clinical laboratories for the investigation of renal and hypertension disorders [20, 21]. Diagnostics also plays an important role in disorders of edema- cardiac, hepaticor renal. They also play an important role in acute pulmonary edema, cerebral edema, hypertension, hypercalcaemia of malignancy. Diuretics profiling is a very effective method for distinguishing almost all diuretics related disorders [22]. It allows accurate diagnosis and is very useful in many clinical situations [23, 24]. LC/MS technique is highly advantageous as only small amounts of urine enable determination of a whole profile of diagnostically important diuretics [25-31]. For example, the presence of diagnostically important metabolites in plasma steroid profile is particularly helpful in the diagnosis of ambiguous genitalia in new-borns and of disorders associated with virilisation. To reduce the number of unnecessary tests several groups implemented a second tier test for diuretics profiling by LC–MS/MS [31-34].

Carbonic anhydrase is an enzyme which catalyses the reversible reaction. Carbonic acid spontaneously ionizes in to bicarbonate and hydrogen ion. Carbonic anhydrase thus function in co_2 and HCO_3^- transport and in H^+ ion secretion. The enzyme is present in renal tubular cells (especially PT) gastric mucosa, exocrine pancreas, ciliary body of eye, brain and RBC. In these tissues a gross excess of Case is present.

Human matrices analyzed

URINE: As mentioned, the biological fluid most used in the determination of diuretics is urine, preferred to blood because not only the sampling is less invasive but mainly diuretics and metabolites are more concentrated here, due to the slower diuretic metabolism rate in urine. But when athletes take diuretics over a long period during the training months, and then they stop the consume prior to competition. The behaviour may prevent diuretics detection in urine. Urine sampling procedure for doping control is described in detail in the Olympic Movement Anti-Doping Code. The urine sample is divided between two bottles, ideally containing about 70 mL for screening purpose and 30 mL for confirmatory analysis. The samples are usually maintained at ambient temperature without addition of preservative. Sample degradation can therefore occur: markers of degradation are for example an increase of pH (due to ammonia formation) that reaches values above that physiologically possible (8.3). However, the absence of signs of general degradation is not a conclusive proof of the integrity.

Methods and progresses in the determination of diuretics

Initially, this double extraction methodology was kept after the introduction of LC–MS in our laboratory. Currently, a single step LLE at pH 7 using ethyl acetate enables detection of all diuretics at or below the minimum required reporting level (MRPL) of 250ng/mL imposed by WADA [35].

Several papers describe the extraction of diuretics at pH 9.5 prior to LC–MS or LC–MS/MS analysis. Although this pH is not optimal for the extraction of (weak) acidic diuretics, the salting out effect, the polar solvent ethyl acetate, the pre - concentration step and in particular the high sensitivity of tandem mass spectrometry allow for the detection of all these compounds at the required MRPL. High sensitivity of tandem mass spectrometry for the detection of diuretics has also been observed in an approach using a non-cationic/anionic polystyrene-divinyl benzene solid-phase extraction method [36-38]. The excellent sensitivity of LC–MS for diuretics and their relatively high MRPL make these compounds considerable for direct urinalysis. As a result, the detection of diuretics by direct injection (DI) of the urine has been presented recently. Several analytical techniques have been proposed for the analysis of diuretics, primarily among them HPLC-UV-DAD, GC/MS, LC/MS and LC/MS-MS, micellar electro kinetic chromatography and capillary electrophoresis. However, the

best solution for a comprehensive screening method capable of detecting the presence in a biological sample of any diuretic, at the same time satisfying the WADA fixed MRPL is represented by methods based on GC/MS, LC/MS and LC/MS-MS. Typically, the use of GC/MS, LC/MS and LC/MS-MS instrumentation detects diuretic parent compounds and/or the most diagnostic and abundant metabolites. However, in some instances, the target analyte may not be the parent compound or its metabolites, but one or more degradation products formed after the hydrolysis of the diuretics in aqueous media. This is the case of thiazide diuretics, and primarily among them hydrochlorothiazide and althiazide. This phenomenon is more relevant when there is a delay between collection of the sample and the laboratory analysis. 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. At that time, UV diode array was used as detector as it facilitated peak identification. According to IOC/WADA requirements, the confirmation procedures needed to support a positive case should be based on MS. Because of this, a GC/MS method after methylation of the compounds was, in most of the cases, the technique of choice. For the reasons explained in previous sections, at the end of the 1990s, when more robust, reliable and affordable LC/MS instruments became available, major changes in the strategies for the detection of diuretics in the doping field were introduced. First attempts to use LC/MS for the detection of diuretics started in the beginning of 1990s using thermospray or particle beam interfaces in confirmation analysis. Advancements in the field of liquid chromatography and tandem mass spectrometry has provided effective means for detection of drugs of various chemistries in a single comprehensive analytical method. Fast chromatography, effective ionization and polarity switching have been key factors for high throughput screening method for acidic, basic and neutral substances.

However, the time and cost involved in sample preparation has been an area of concern in the few years in analytical chemistry in the field of bio analysis. Direct urine injection on LC-MS/MS without a clean-up has been widely used in drugs of abuse analysis. Direct injection of the urine sample after appropriate dilution in water or LC mobile phase can provide optimum sensitivity without any significant problems associated with matrix. Several works have been carried on methods involving dilute and shoot direct urine analysis of LC-MS/MS.

Table 1: Characteristics fragment ions of selected diuretics using electron spray ionization and CID

Compounds	Mol. Wt	Chemical class	$(M-H)^-$	$(M-H)^-SO_2$-HCN	$(M-H)^-CO_2$					
Buthiazide	363	Thiazide	352	261	-	269	205	190	126	78
Cyclopenthiazid-e	379	Thiazide	378	287	-	269	205	190	126	78
Ethiazide	325	Thiazide	324	283	-	269	205	190	126	78
Hydrochlorothi-azide	297	Thiazide	296	-	-	269	205	190	126	78
Hydroflumethiazide	331	Thiazide	330	-	-	269	205	190	126	78
Bumetanide	364	Sulfonyl benzoic acid	363	-	319	306	271	238	207	80
Furosemide	330	Sulfonyl benzoic acid	329	-	285	249	205	-	126	78
Piretanide	362	Sulfonyl benzoic acid	361	-	317	-	269	225	205	80
Clopamide	345	Sulfonyl benzamide	344	-	-	308	280	189	80	78
Indapamide	365	Sulfonyl benzamide	364	-	-	233	216	189	80	78
Xipamide	354	Sulfonyl aniline	353	-	-	274	273	206	127	78

Conclusion

The detection of the misuse of diuretics in sports is routinely achieved by mass spectrometric techniques. Depending on the class of diuretics, which influences the molecular structure and thereby also to some extent the analytical properties, preceding separation by liquid chromatography is favoured. As lots of diuretics are extensively metabolised, doping control analysis predominantly concentrates on the detection of specific metabolites tracing the prohibited administration. The administration of diuretics and other acidic drugs like(stimulants and anabolic androgenic steroids) can be determine by the various traditional methods for the detection are recently complemented by supplemental assays to detect the misuse of unknown substances. The use of GC/MS, LC/MS and LC/MS-MS instrumentation detects diuretic parent compounds and/or the most diagnostic and abundant metabolites.

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