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REVIEW

The abuse of diuretics as performance-enhancing drugs and masking agents in sport doping: pharmacology, toxicology and analysis

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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 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. This review provides an overview of the pharmacology and toxicology of diuretics and discusses their application in sports. The most common analytical strategies currently followed by the anti-doping laboratories accredited by the WADA are discussed along with the challenges laboratories face for the analysis of this diverse class of drugs.
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Keywords: diuretics; masking agents; doping analysis; GC/MS, LC/MS-MS

Abbreviations: AMS, high-altitude mountain sickness; CA, carbonic anhydrase; GC, gas chromatography; GFR, glomerular filtration rate; HPLC, high-performance liquid chromatography; IOC, International Olympic Committee; ISRP, internal surface reversed-phase; L/L, liquid/liquid; LC, liquid chromatography; MR, mineralocorticoid receptor; MRPL, minimum required performance levels; MS, mass spectrometry; PRA, plasma renin activity; SPE, solid phase extraction; UV-DAD, ultraviolet-diode array detection; VO_2, oxygen uptake; WADA, World Anti-Doping Agency

Introduction

For as long as sporting events have existed, the desire to gain a competitive edge has been present as well. With the huge financial incentives and the subsequent pressures to excel associated with the international sporting industry, attempts to achieve a competitive edge especially with the use of performance-enhancing drugs have only been increasing (Barroso *et al.*, 2008). Despite centuries of reports of using substances to enhance athletic performance, testing athletes for the use of performance-enhancing drugs began only in 1968 (Barroso *et al.*, 2008; Botrè, 2008). Since that time, a list of banned substances has been constantly updated by the International Olympic Committee (IOC) and the World Anti-doping Agency (WADA). Compounds and methods included on the list are those that can be used by an athlete to provide an unfair advantage (WADA, 2009b). Substances on the Prohibited List include anabolic androgenic steroids, glucocorticosteroids, peptide hormones and their modulators, hormone antagonists and their modulators, stimulants, β2-agonists, narcotics, alcohol, β-blockers, cannabinoids and diuretics and masking agents (WADA, 2009b). The objective of this paper is to review the pharmacology of diuretics and the applications of diuretics to sports doping, as well as detail the analytical methodologies currently described to detect and identify diuretics in urine. All the classes of diuretics (detailed later in this paper) are banned in sport.

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 (Jackson, 2006). They are used in clinical therapy for the treatment of various diseases and syndromes,

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Table 1 Statistics of positive findings of diuretics by all World Anti-Doping Agency (WADA) laboratories (WADA, 2004; 2005; 2006; 2007; 2008a; 2009a)

Year	*% of all positive samples*	*Occurrences*	*Most common drug detected (occurrences)*	*Second most common drug detected (occurrences)*
2003	5.2	142	Furosemide (48)	Hydrochlorothiazide (42)
2004	4.8	157	Furosemide (62)	Hydrochlorothiazide (44)
2005	5.7	246	Furosemide (91)	Hydrochlorothiazide (67)
2006	6.7	290	Furosemide (90)	Hydrochlorothiazide (88)
2007	7.4	359	Furosemide (111)	Hydrochlorothiazide (103)
2008	7.9	436	Hydrochlorothiazide (137)	Furosemide (104)

including hypertension, heart failure, liver cirrhosis, renal failure, kidney and lung diseases, as well as a more general reduction of the adverse effects of salts and/or water retention (Jackson, 2006). Diuretics were first banned in sport (both in competition 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 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 (Ventura and Segura, 1996; Goebel *et al*., 2004; Trout and Kazlauskas, 2004; Furlanello *et al*., 2007).

In 2008, diuretics represented 7.9% of all Adverse Analytical Findings reported by WADA laboratories, with a total number of 436 cases (WADA, 2009a). All classes of diuretics were represented in the positive cases; hydrochlorothiazide was the most common diuretic detected, with 137 cases. Table 1 summarizes the statistics of positive diuretic findings by all WADA laboratories from 2003 to the present. In all six of the past years, all classes of diuretics have been represented in the positive findings (WADA, 2004; 2005; 2006; 2007; 2008a; 2009a). Over the years, the total number of occurrences has been increasing; this trend of increasing positive findings may be due not only to an increase in abuse, but is likely due to improved methods of detection.

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 (Jackson, 2006). 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 (Jackson, 2006). In the following section, this review will briefly summarize the pharmacology and toxicology of diuretic classes based on mechanism of action. Figure 1 shows examples of diuretic structures grouped by mechanism of action: carbonic anhydrase (CA) inhibitors, inhibitors of the $Na^+/K^+/2Cl^-$ symporter (loop diuretics), inhibitors of the Na^+/Cl^- symporter (thiazide and thiazide-like diuretics), osmotic diuretics, inhibitors of renal epithelial Na^+ channels (some potassium-sparing diuretics) and mineralocorticoid receptor (MR) antagonists; note the variety of molecular structures. Figure 2 details the site and mechanism of the diuretic classes in the nephron (Figure 2A).

The identification and quantification of prohibited compounds and/or their metabolic products has been a major task in sports drug testing (Cowan and Kicman, 1997). Historically, the detection of diuretics in biological samples was achieved using high-performance liquid chromatography (HPLC) with ultraviolet-diode array detection (UV-DAD). However, the HPLC-DAD detection technique is not specific for the unequivocal identification of substances. Therefore, mass spectral methodology is required for confirmation according to international anti-doping regulations (Trout and Kazlauskas, 2004; Thevis and Schanzer, 2007; WADA, 2009c). Gas chromatography/mass spectrometry (GC/MS) after appropriate sample preparation and derivatization has been, since the last decade, the most widely used analytical technique for the detection of diuretics. Recently however, due to the heterogeneity of the chemical structures and physicochemical properties of diuretics and the advent of more economical instrumentation, the use of liquid chromatography/MS (LC/MS) has become popular (Thevis and Schanzer, 2007). The sample preparation before LC/MS analysis is simpler than with GC/MS and no derivatization is required. Ventura and Segura published a comprehensive review of diuretic analysis in 1996 (Ventura and Segura, 1996). This review will mainly focus on the developments and techniques that have been developed since that time. This article is an extension of the British Journal of Pharmacology special themed issue, Drugs in Sport (McGrath and Cowan, 2008).

Pharmacology and toxicology of diuretics

Carbonic anhydrase inhibitors

Carbonic anhydrase inhibitors (Figure 1A) by definition are a class of substances that act as inhibitors of CA (carbonate dehydratase, carbonate hydrolase, E.C.4.2.1.1) in proximal tubule cells of the nephron (Figure 2B). CA is a zinc metalloenzyme expressed in humans as a family of at least 15

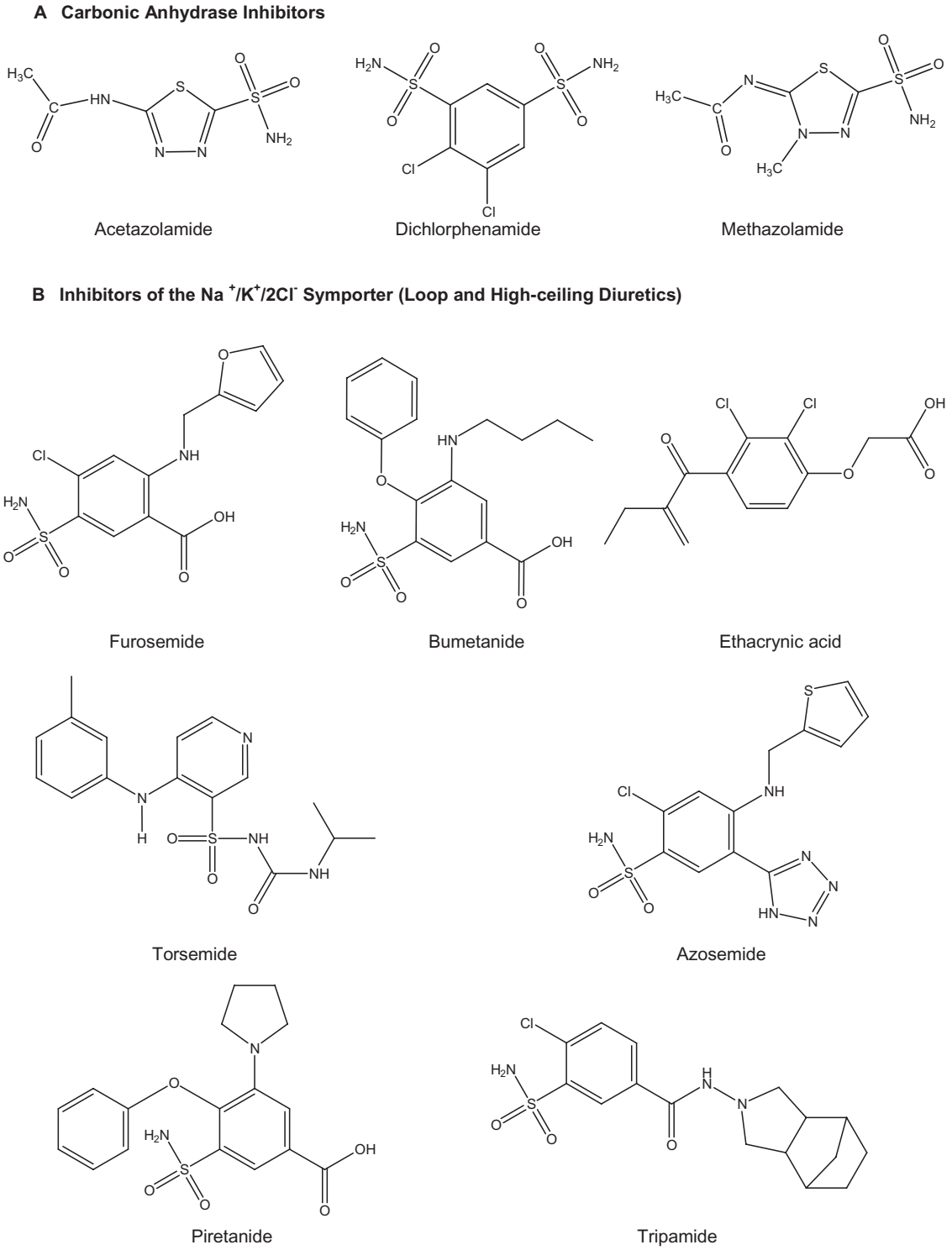
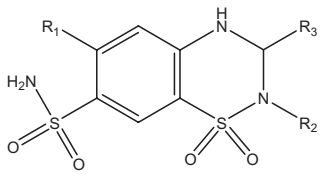
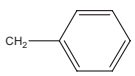


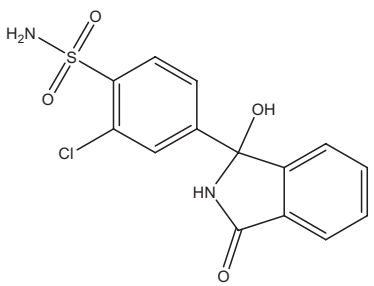
Figure 1 Examples of diuretic structures grouped by mechanism of action. (A) Carbonic anhydrase inhibitors; (B) inhibitors of the $Na^+/K^+/2Cl^-$ symporter (loop diuretics); (C) inhibitors of the Na^+/Cl^- symporter (thiazide and thiazide-like diuretics); (D) osmotic diuretics; (E) inhibitors of renal epithelial Na^+ channels (some potassium-sparing diuretics); (F) mineralocorticoid receptor (MR) antagonists (aldosterone antagonists and some potassium-sparing diuretics).

isoenzymes (Tashian, 2000), four of them (CA II, CA IV, CA XII and CA XIV) are present in the kidney (Schwartz, 2002). Type II CA, the most potent isoenzyme, represents 95% of total CA in the kidney and it is found as a soluble protein in the cytoplasm. Type IV CA, a membrane-bound isoenzyme, is found in the luminal and basolateral membranes. This enzyme plays a key role in bicarbonate reabsorption and acid secretion in the nephron by reversibly catalysing the hydration reaction of CO_2 with the production of H^+ and bicarbonate ions. Both CA II and CA IV are inhibited by

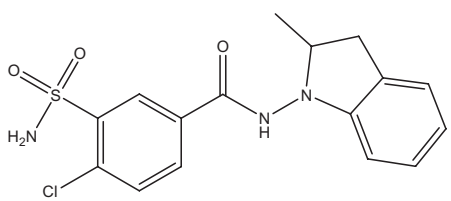
C Inhibitors of the Na^+/Cl^- Symporter (Thiazide and Thiazide-like Diuretics)



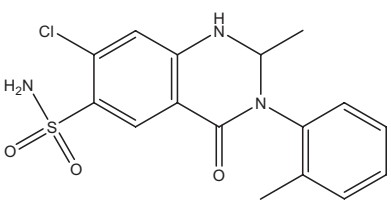
Compound	R_1	R_2	R_3
Bendroflumethiazide	—CF_3	—H	
Chlorothiazide	—Cl *unsaturated between C3 and C4	—H	—H
Hydrochlorothiazide	—Cl	—H	—H
Hydroflumethiazide	—CF_3	—H	—H
Methyclothiazide	—Cl	—CH_3	—CH_2Cl
Polythiazide	—Cl	—CH_3	—$C_2SCH_2CF_3$
Trichlormethiazide	—Cl	—H	—$CHCl_2$



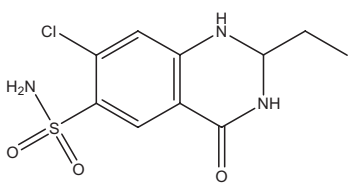
Chlorthalidone



Indapamide



Metolazone



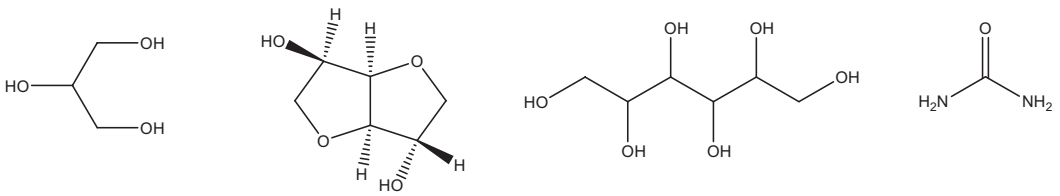
Quinethazone

Figure 1 *Continued.*

sulphonamides, and especially by aromatic sulphonamides with the unsubstituted $–SO_2NH_2$ functional group. The reduced capacity to exchange Na^+ with H^+ in the presence of these diuretics determines weak diuretic action. In addition, bicarbonate is kept in the lumen with the consequent increase of urinary pH to approximately 8 and subsequential development of a metabolic acidosis. Phosphate excretion is also increased by a mechanism not fully clarified. Ca^{2+} and Mg^{2+} excretion is not affected.

Currently, there are three main CA inhibitors available as diuretics (see Figure 1A for structures), acetazolamide (the prototype of the class, a sulphonamide without antibacterial activity), dichlorphenamide and methazolamide. They all show an oral bioavailability of 100% with a half-life of 6–14 h. Acetazolamide and dichlorphenamide are excreted by the kidneys as intact drugs while methazolamide is extensively metabolized. The major therapeutic indication of CA inhibitors is open-angle glaucoma. Acetazolamide is often used for the prevention of high-altitude mountain sickness (AMS), a pathological effect of high altitude on the body caused by acute exposure to low partial pressure of oxygen at high altitude that can progress to high-altitude oedema

D Osmotic diuretics

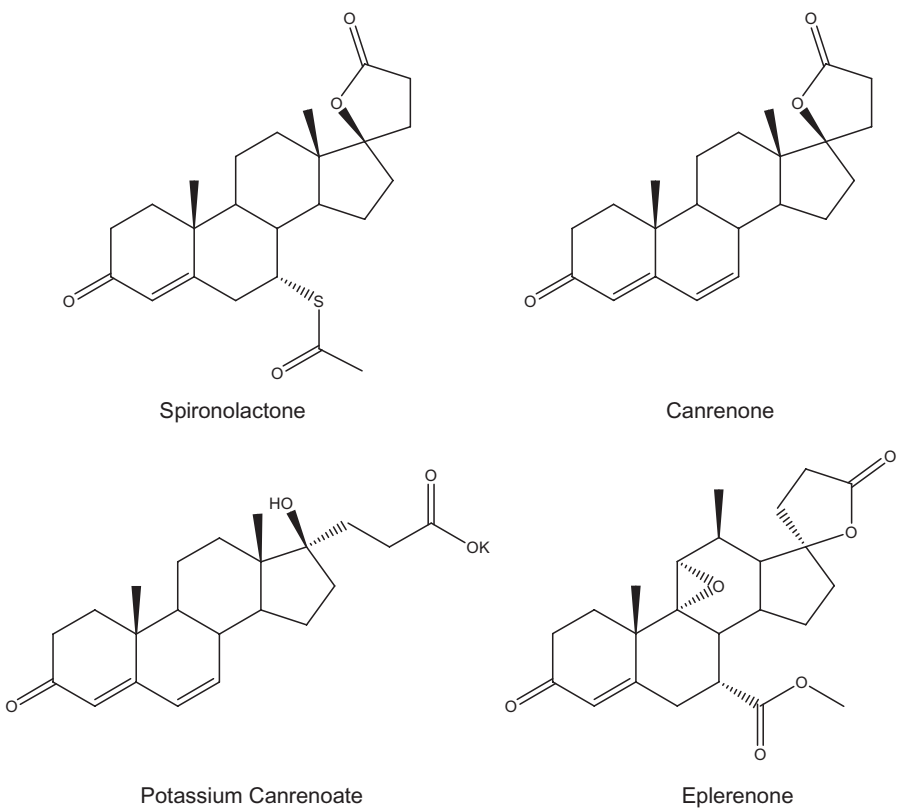


Glycerin Isosorbide Mannitol Urea

E Inhibitors of Renal Epithelial Na^+ Channels (Some Potassium-sparing Diuretics)

Amiloride Triamterene

F Mineralocorticoid Receptor (MR) Antagonists (Aldosterone Antagonists and Some Potassium-sparing Diuretics)



Spironolactone Canrenone

Potassium Canrenoate Eplerenone

Figure 1 *Continued.*

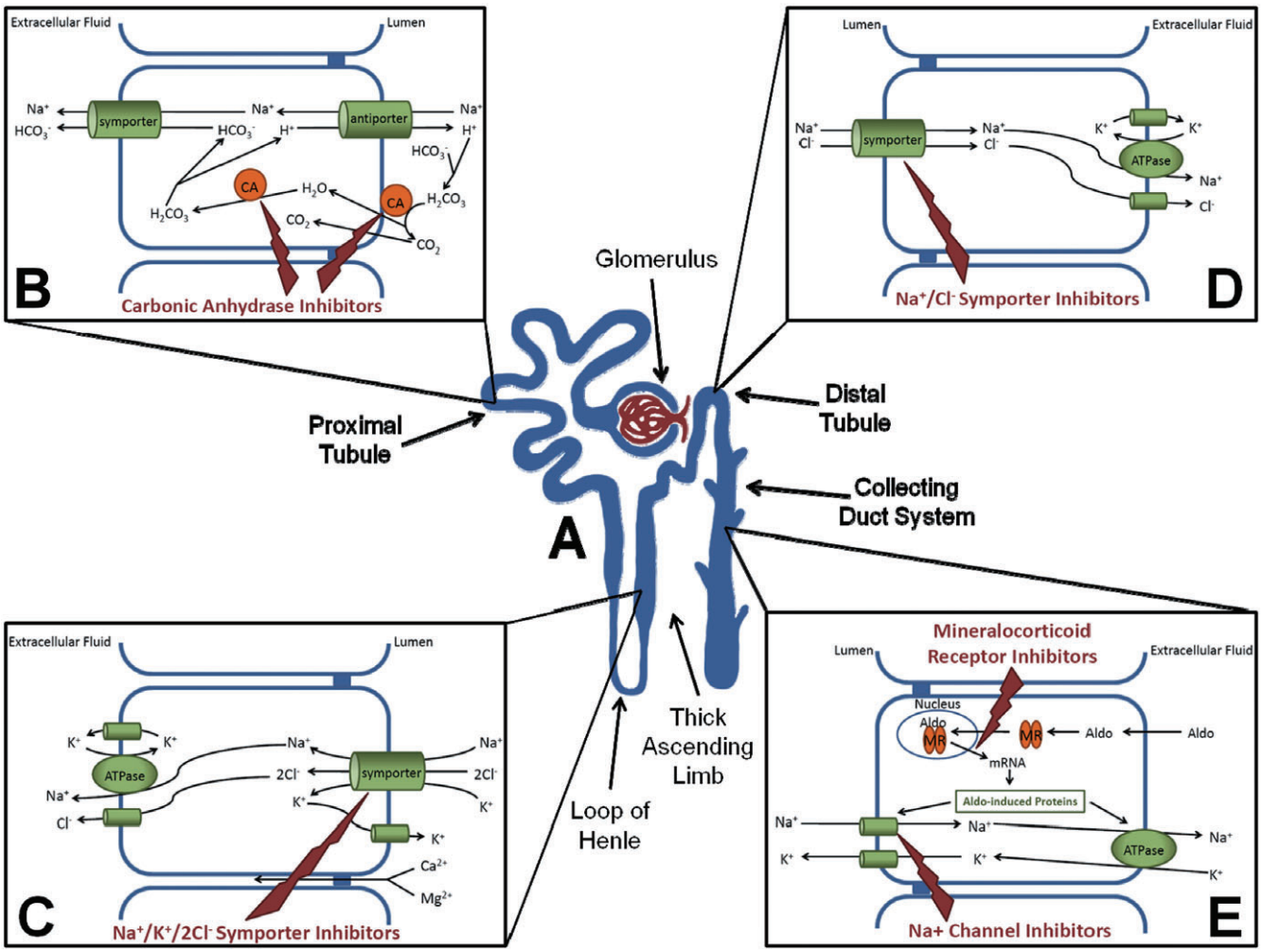


Figure 2 Site and mechanism of action of diuretics. (A) The nephron with major divisions labelled. (B) Mechanism of carbonic anhydrase inhibitors in the proximal tubule. (C) Mechanism of the $Na^+/K^+/2Cl^-$ symporter inhibitors in the thick ascending limb of the loop of Henle. (D) Mechanism of the Na^+/Cl^- 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).

(pulmonary and cerebral). (Coote, 1991; Botrè and Botrè, 1993). Acetazolamide increases bicarbonate excretion in urine, making the blood more acidic and increasing ventilation, thus aiding in acclimatization to high altitude. Acetazolamide is also used for the treatment of oedema. CA inhibitors may also be used therapeutically for the treatment of pre-menstrual fluid retention.

Carbonic anhydrase is present in a number of extrarenal tissues, including the eye, gastric mucosa, pancreas, central nervous system and erythrocytes. Because of the varied location around the body, CA inhibitors are typically used for non-diuretic indications, such as glaucoma, to decrease the rate of formation of aqueous humour and consequently reduce intraocular pressure. It has been demonstrated that topical administration of dorzolamide, a CA inhibitor abolishing the enzymatic activity in the ciliary body, does not produce any diuretic effect (Mazzarino *et al*., 2001). CA inhibitors are also used as antiepileptic drugs due in part to the production of metabolic acidosis.

Most adverse effects, contraindications and drug interactions are a consequence of urinary alkalinization or metabolic acidosis. Infrequent adverse effects are similar to those of sulphonamides. The diversion of ammonia of renal origin from urine into the systemic circulation, calculus formation and ureteral colic causing precipitation of calcium phosphate salts in alkaline urine, worsening of metabolic or respiratory acidosis and reduction of the urinary excretion rate of weak organic bases are also adverse effects of CA inhibitors.

The efficacy of CA inhibitors as single agents is low and the long-term usefulness of CA inhibitors is often compromised by the development of compensatory processes such as metabolic acidosis. Additionally, the continuous use of CA inhibitors may result in the diminution of the desired therapeutic effect. Acetazolamide accounted for 1.4% of the positive diuretic findings in 2008 (WADA, 2009a).

Inhibitors of the $Na^+/K^+/2Cl^-$ symporter (loop diuretics)

Inhibitors of the $Na^+/K^+/2Cl^-$ symporter (Figure 1B) are a highly potent short-acting diuretic class that bind to the Cl^- binding site located in the transmembrane domain of

$Na^+/K^+/2Cl^-$ symporter, which is found in the thick ascending limb of the loop of Henle (Figure 2C). Blocking the function of this symporter results in a significant reduction in the ability of the kidney to concentrate urine and a consequent significant increase in the urinary excretion of Na^+ and Cl^-. There is also a marked increase in the excretion of Ca^{2+}, Mg^{2+} and K^+. Uric acid excretion is also increased with acute administration while chronic administration has the converse effect.

Inhibitors of the $Na^+/K^+/2Cl^-$ symporter are furosemide, bumetanide, ethacrynic acid, torsemide, axosemide, piretanide and tripamide (structures pictured in Figure 1B). Greater than 90% of drug binds to plasma proteins. They are quickly and widely absorbed from the gastrointestinal tract (65–90%) but have a very short half-life (less than 1 h for bumetanide and piretanide and a maximum of 3.5 h for torsemide). These symport inhibitors undergo partial metabolism (hepatic for bumetanide and torsemide, renal glucuronation for the others) with renal excretion as intact drugs (Shankar and Brater, 2003).

Because of their sulphonamide-based structure, some loop diuretics have weak CA-inhibiting activity that further increases the diuretic effect of these drugs. Moreover, they have direct vascular effects (Dormans *et al*., 1996) that acutely increase systemic venous capacitance and decreases left ventricular filling pressure. This effect, particularly evident for furosemide, benefits patients with pulmonary oedema even before diuresis ensues.

A major indication of loop diuretics is in the treatment of acute pulmonary oedema. They are also used for the treatment of chronic congestive heart failure. This leads to a significant reduction in mortality, a decrease in the risk of worsening heart failure and an improvement in exercise capacity (Faris *et al*., 2002). Loop diuretics are also widely used for the treatment of hypertension (van der Heijden *et al*., 1998). Inhibitors of the $Na^+/K^+/2Cl^-$ symporter are also indicated in the treatment of oedema and ascites of liver cirrhosis, in the treatment of the oedema of nephrotic syndrome and for life-threatening hyponatremia.

Adverse effects are all correlated to fluid and electrolyte imbalance. They include hyponatremia and/or extracellular fluid volume depletion (associated with hypotension, circulatory collapse and thromboembolic episodes), hypochloremic alkalosis, hypokalemia (which induces cardiac arrhythmias), hypomagnesemia, hyperuricemia (occasionally leading to gout) and hyperglycaemia. Furthermore, they increase plasma levels of low-density lipoprotein cholesterol and triglycerides while decreasing plasma levels of high-density lipoprotein cholesterol. Loop diuretics can cause ototoxicity, especially ethacrynic acid. This class of diuretics has drug–drug interactions with several substances including aminoglycosides, anticoagulants, digitalis glycosides, lithium, propranolol, sulphonylureas, cisplatin, probenecid and amphotericin B. The synergism of diuretic activity of associated loop and thiazide diuretics leads to profound diuresis.

In 2008, inhibitors of the $Na^+/K^+/2Cl^-$ symporter accounted for 24.6% of positive diuretic doping samples. Furosemide was the second most frequently detected diuretic with 104 samples testing positive (23.9%) (WADA, 2009a).

Inhibitors of the Na^+/Cl^- symporter (thiazide and thiazide-like dugs)

Inhibitors of the Na^+/Cl^- symporter (Figure 1C) have optimal diuretic action in the early distal convoluted tubule and a lesser diuretic effect in the proximal tubule. In addition, some thiazide diuretics also are weak inhibitors of CA. They reduce the Na^+ reabsorbtion by inhibition of Na^+/Cl^- co-transportion (Figure 2D). Na^+ or Cl^- binding to the Na^+/Cl^- symporter modifies thiazide-induced inhibition of the symporter, suggesting that the thiazide-binding site is shared or altered by both Na^+ and Cl^- (Monroy *et al*., 2000).

Some examples of the drugs included in this class are the following (see the structure in Figure 1C): bendroflumetazide, chlorothiazide, hydrochlorothiazide, hydroflumethiazide, methyclothiazide, polythiazide, trichlormethiazide, chlortalidone, indapamide, metolazone and quinethazone. In general, all of them show good bioavailability after oral administration (100% for bendroflumetazide and polythiazide, at least 50% for hydroflumethiazide and the others). They are partially metabolized by unknown pathways and are partially excreted as intact drugs by the kidney. Plasma protein binding varies considerably among the class. The wide ranges of half-lives vary from 1.5 h for chlorothiazide to almost 50 h for chlortalidone.

Although this class of diuretic is expected to greatly enhance Na^+ and Cl^- excretion, this effect is moderate as approximately 90% of the filtered Na^+ is reabsorbed before reaching the distal convoluted tubule. As with loop diuretics, inhibitors of the Na^+/Cl^- symporter affect K^+ and uric acid excretion by the same mechanisms; K^+ excretion is markedly increased after administration and uric acid excretion is increased after acute administration and decreases after chronic administration. They, however, decrease Ca^{2+} excretion (Friedman and Bushinsky, 1999).

Thiazide diuretics are the most widely used diuretics. They are employed as a first-line therapy for hypertension either alone or in combination with other antihypertensive medications (Chobanian *et al*., 2003). They are also used for the treatment of oedema associated with heart, liver and renal diseases. Thiazide diuretics are frequently used because of their low cost, high tolerance, good compliance (once daily administration), few contraindications, efficacy comparable to other classes of antihypertensive agents and proven benefits in reducing cardiovascular morbidity and mortality.

Again, similar to loop diuretics, most adverse effects of Na^+/Cl^- symport inhibitors are due to abnormalities of fluid and electrolyte balance and include: extracellular volume depletion, hypotension, hypokalemia (which compromises the antihypertensive effect), hyponatremia, hypochloremia, metabolic alkalosis, hypomagnesemia, hypercalcemia, hyperuricemia and hyperglycaemia (latent diabetes mellitus may be unmasked during therapy) (Wilcox *et al*., 1999). However, unlike loop diuretics, Na^+/Cl^- symport inhibitors increase plasma levels of low-density lipoprotein cholesterol, total cholesterol and total triglycerides and the incidence of erectile dysfunction is greater.

Thiazide and thiazide-like diuretic drug–drug interactions cause a diminished effect of anticoagulants, uricosuric agents, sulphonylureas and insulin and increase the effects due to

synergism of action between anaesthetics, diazoxide, digitalis glycosides, lithium, vitamin D and loop diuretics.

Inhibitors of the Na^+/Cl^- symporter were the most abused class of diuretics in 2008 according to WADA statistics, accounting for 38.7% of positive samples. Hydrochlorothiazide was the most detected diuretic, found in 31.4% (137) of positive samples (WADA, 2009a).

Osmotic diuretics

Osmotic diuretics are a class of non-metabolizable low-molecular-weight compounds. Only four compounds are included in this diuretic class, glycerin, isosorbide, mannitol and urea. The molecular structures are shown in Figure 1D. These compounds are relatively pharmacologically inert, freely filterable by the glomerulus and non-diffusible across the nephron. They are administered in large doses, not only orally (glycerine, isosorbide) but also intravenously (mannitol, urea). Such administration significantly enhances the osmolality of plasma and tubular fluid and, in turn, causes an increase of urine osmolality with consequent reduction of water reabsorption in the distal nephron/collecting ducts. Osmotic diuretics act both in the proximal tubule and the loop of Henle, with the latter being the primary site of action. These diuretics also work via an osmotic effect in the tubules and by reducing medullary tonicity. The half-lives range from less than 1 h in the case of glycerin and mannitol to almost 10 h for isosorbide.

By extracting water from intracellular compartments, osmotic diuretics expand the extracellular fluid volume, decrease blood viscosity and inhibit renin release. This results in an increase of the urinary excretion of all electrolytes, Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, HCO_3^- and PO_4^{3-}. Their use is limited to well-defined clinical situations, for instance, mannitol is used to reduce cerebral oedema and brain mass before and after neurosurgery, in acute tubular necrosis as a renal protector (Levinsky and Bernard, 1988), and for the treatment of dialysis disequilibrium syndrome. Because osmotic diuretics extract water from the eye and brain, they are all used to control intraocular pressure during acute attacks of glaucoma and in ocular surgery.

Osmotic diuretic therapy can cause hypernatremia and dehydration due to the loss of water in excess of electrolyte loss. Conversely, their use can lead to hyponatremia, which is responsible for the common adverse effects (headache, nausea and vomiting). Hyperglycaemia can occur as a result of glycerin metabolism.

Inhibitors of renal epithelial Na^+ channels

Inhibitors of renal epithelial Na^+ channels (Figure 1E) act in the late distal tubule and collecting duct cells of the nephron by inhibiting the Na^+ reabsorbtion and K^+ and H^+ secretion (Figure 2E). The molecular mechanism is the blockage of epithelial Na^+ channels in the luminal membrane by competition with Na^+ for negatively charged areas within the pore of the Na^+ channel.

The only two drugs of this class in clinical use are triamterene and amiloride (structures pictured in Figure 1E). Both drugs show a modest diuretic effect on their own and a small increase in Na^+ and Cl^- excretion. Typically, they are used in combination with other diuretics to offset their severe kaliuretic effects and preserve potassium levels in patients at risk of hypokalaemia. In the treatment of oedema or hypertension, the combination of a Na^+ channel inhibitor with a thiazide or loop diuretic enhances the diuretic and antihypertensive effect.

Na^+ channel inhibitors show low oral bioavailability and great differences in half-life (more than 20 h for amiloride, less than 5 h for triamterene). The route of elimination is predominantly renal for intact amiloride while triamterene is extensively metabolized into the active 4-hydroxytriamterene sulphate and excreted in urine. The most common adverse effects of Na^+ channel inhibitors are nausea, vomiting, diarrhoea, headache, leg cramps and dizziness. The most dangerous adverse effect of Na^+ channel inhibitors is hyperkalemia. Triamterene can also reduce glucose tolerance and induce photosensitization.

Amiloride and triamterene were detected in 3% of positive diuretic doping samples in 2008 (WADA, 2009a).

Mineralocorticoid receptor antagonists

Mineralocorticoid receptor antagonists (Figure 1F) are competitive inhibitors of aldosterone that bind to and inhibit cytosolic MRs found in the epithelial cells of the late distal tubule and collecting duct of the nephron (Figure 2E).

The MR is a member of the steroid superfamily of nuclear receptors. Normally, aldosterone enters the epithelial cell and binds to MRs. The MR–aldosterone complex then translocates to the nucleus where it binds to specific sequences of DNA (hormone responsive elements) and thereby regulates the expression of multiple gene products called aldosterone-induced proteins. Unlike the MR–aldosterone complex, the MR–antagonist complex is not able to induce the synthesis of aldosterone-induced proteins.

The compounds belonging to this class (see Figure 1F for molecular structures) are spironolactone, canrenone, potassium canrenoate and eplerenone. The oral availability of spironolactone, the prototype molecule of the class, is about 65%; it is metabolized extensively, undergoes enterohepatic recirculation, highly binds to plasmatic proteins and has a short half-life (approximately 1.6 h) (Beermann, 1984). Canrenone is an active metabolite of spironolactone with a 10-fold greater half-life (16.5 h) that prolongs the effect of the parent compound. Canrenoate is not active, but is converted to canrenone in the body. Eplerenone has good oral availability and is extensively metabolized.

Mineralocorticoid receptor antagonists have effects on urinary excretion similar to renal epithelial Na^+ channel inhibitors. The clinical efficacy of MR antagonists strictly depends on endogenous levels of aldosterone; higher levels cause greater effects.

This group of diuretics is very useful as an alternative to potassium replacement therapy. Usually, they are employed in cases of high-aldosterone concentrations. In the treatment of oedema and hypertension, these drugs are often co-administered with thiazide or loop diuretics, as well as the other K^+-sparing diuretics. Spironolactone is useful in the treatment of primary hyperaldosteronism and refractory

oedema associated with secondary aldosteronism (Ouzan *et al*., 2002). Similar to Na^+ channel inhibitors, the most common adverse effect of MR antagonists is hyperkalemia.

Because of its molecular structure (Figure 1F), spironolactone has some affinity for progesterone and androgen receptors that causes some side effects such as gynecomastia, impotence and menstrual irregularities. Conversely, owing to the 9,11-epoxide group, eplerenone has very low affinity for progesterone and androgen receptors (<1% and <0.1%, respectively) compared with spironolactone. Chronic administration of spironolactone can induce malignant tumours; in particular, breast cancer has been observed. With regard to drug–drug interactions, salicylates reduce the tubular secretion of canrenone and decrease the diuretic efficacy of spironolactone, while spironolactone alters the clearance of digitalis glycosides.

Canrenone and spironolactone together accounted for 4.3% of positive diuretic doping samples in 2008 (WADA, 2009a).

Diuretics and sports

General remarks

As previously mentioned, diuretics are commonly prescribed in clinical medicine to treat hypertension and other cardiovascular disorders. These compounds are also frequently encountered illicitly in sports. 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 (WADA, 2009f) permits the therapeutic use of diuretics when athletes and their physicians apply for therapeutic use exemptions (TUE) according to the International Standard for TUE (WADA, 2009d). TUE is defined as 'the permission to use, for therapeutic purposes, substances or methods contained in the List of Prohibited Substances or Methods, whenever approved by a Therapeutic Use Exemption Committee based on a documented medical file before the use of the substance in sports'. For diuretics, the primary permitted therapeutic use is for hypertension (WADA, 2008b). It should be noted that a TUE is not valid if an athlete's urine contains a diuretic in association with a threshold or subthreshold level of another exogenous substance included on the Prohibited List. Because of the TUE, some athletes do use diuretics for legitimate medicinal purposes; in many cases, however, diuretic use is illicit (Clarkson and Thompson, 1997).

Diuretics and doping

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') (WADA, 2009b).

Although there is little evidence of athletic performance enhancement following diuretic administration, their abuse is widespread among athletes who want to lose weight quickly. For example, diuretic(s) use can allow an athlete to transiently reduce body weight, which is a clear advantage in wrestling, boxing, judo and weight-lifting as well as in general sports where weight categories are involved and among athletes who want to maintain a low body weight, such as female gymnasts and ballet dancers. Skiers and mountain climbers, however, make legitimate use of acetazolamide (a CA inhibitor that also acts on sites different than the kidney) in preventing AMS.

As already stated, diuretics are banned in sport because they can be used: (i) directly, to produce a rapid weight loss that can be critical to meet a weight category in sporting events; and/or (ii) indirectly, to alter the normal metabolism/excretion profile of other doping drugs. In both cases and discussed in more detail below, the diuretic administration can be acute or chronic with administered doses that can markedly exceed therapeutic levels. In general, athletes can use diuretics in a single dose some hours before a competition (i.e. wrestlers or sportsmen for masking purposes) or chronically abuse them for months (i.e. female gymnasts). It is important to note that the diuretics most abused by athletes (furosemide, hydrochlorothiazide and triamterene) have a short half-life and are therefore undetectable in urine if samples are not collected within 24–48 h after the last administration.

Diuretics, exercise and weight loss

In an attempt to assess the significance of diuretic use in weight loss, Caldwell *et al*. (1984) compared the different effect of exercise-, sauna- and diuretic-induced acute dehydration on weight change. The results showed a decrease of 2.3 ± 0.8 kg after exercise, 3.5 ± 0.8 kg after sauna and 3.1 ± 0.8 kg after furosemide administration respectively. Additionally, diuretics are abused simultaneously with androgenic-anabolic steroids by bodybuilders to accentuate muscle definition and body tone. In the same study reported by Caldwell *et al*. it was demonstrated that the plasma volume change in athletes is –0.9% after exercise, –10.3% after sauna and –14.1% after furosemide administration (total amount of 1.7 $mg \cdot kg^{-1}$ in two divided doses, 16 h prior to testing) (Caldwell *et al*., 1984).

Diuretics can have variety of physiological effects on exercise physiology, including effects on metabolism (thermoregulation, potassium homeostasis), the cardiovascular system and the respiratory system [pulmonary actions, oxygen uptake (VO_2)]. Most of the effects are related to the consequences of volume depletion and electrolyte imbalance and depletion. Exercise can affect the action of diuretics as well, with consequences on both pharmacology and pharmacokinetics. At the level of the nephron, exercise can both complement and antagonize the effects of diuretics. Exercise acutely induces a negative water balance and long-term regular exercise lowers blood pressure, augmenting pharmacological properties of diuretics (Zappe *et al*., 1996). Exercise also influences specific actions of diuretics; it can cause an acute shift of intracellular potassium into the intravascular

Table 2 Effects of exercise and diuretics on renal physiology [adapted from Caldwell (1987) and Reents (2000)]

	GFR	*Urine Output*	*PRA*	*Aldosterone*
Exercise @ 25% VO_2 max	↑	↔	↑	↑
Exercise @ % VO_2 max	↓	↓	↑↑	↑↑
Thiazide diuretics	↓	↓	↑↑	↑↑
Loop diuretics	↔	↑↑	↑↑	↑↑
Spironolactone	↔	↑	↑↑	↑↑
Other K^+-sparing agents	↔	↑	↑↑	↑↑

One arrow indicates a moderate effect; two arrows indicate a profound effect.
GFR, glomerular filtration rate; PRA, plasma renin activity; VO_2 max, maximum oxygen uptake.

space (Young *et al*., 1992) and potentiate the kaliuretic effect of diuretics. While thiazide diuretics are associated with insulin resistance (Moser, 1998), exercise potentiates the opposite effect (Plasqui and Westerterp, 2007). In most cases, physical exercise is used as a therapy for insulin resistance because it activates the pancreatic β-cells via the neuroadrenergic system (Bordenave *et al*., 2008). This reduces blood insulin levels and consequently increases hepatic glucose release and decreases muscle utilization of insulin (Bonen *et al*., 2006). Although there is little information on how exercise affects diuretic pharmacokinetics, chlorothiazide, hydrochlorothiazide and triamterene have an elimination half-life short enough (1.5–4 h) to be affected by 1 h or more of sustained exercise (Somani, 1996), which decreases renal and hepatic blood flow. Therefore, these substances are not always detected in urine samples collected post-competition or at the end of an intense training session. It is notable that both exercise and diuretics can independently cause fluid and electrolyte loss. Table 2, adapted from Caldwell *et al*. (1984) and Reents (2000), summarizes the effects of both exercise and diuretics on renal physiology.

It is known that during exercise skeletal muscle temperature exceeds core temperature within several minutes, and alteration of the body's thermoregulatory systems is a major risk of diuretic abuse. The marked dehydration following diuretic intake exerts a detrimental effect on the cardiovascular and thermoregulatory systems of the body during exercise and can lead to exhaustion, irregular heartbeat, heart attack and death. Both acetazolamide (Brechue and Stager, 1990), a mild diuretic, and furosemide (Claremont *et al*., 1976), a potent diuretic, have been shown to impair adaptive increases in skin blood flow during exercise.

Diuretics affect potassium homeostasis in exercising muscle; intracellular potassium and the resting membrane potential of the cell both decrease. All diuretics except the potassium-sparing agents increase kaliuresis, accelerating the depletion of intracellular potassium. The resultant hypokaliemia can lead to muscle cramps and to cardiac arrhythmias secondary to electrolyte shifts/losses. On the other hand, overuse of potassium-sparing diuretics such as spironolactone, triamterene and amiloride can lead to hyperkalaemia and consequently may expose athletes to malignant arrhythmias (Appleby *et al*., 1994). Moreover, the interference of most diuretics with uric acid metabolism can cause a gout attack, which can be very painful (Koutlianos and Kouidi, 2006).

Diuretic-induced dehydration influences exercise heart rate. In particular, at lower exercise intensity a higher heart rate results, while during maximal exercise exertion, the effect is lower or almost absent (Stager *et al*., 1990). This is especially true for acetazolamide (Brechue and Stager, 1990) and to a lesser extent, furosemide abuse (Claremont *et al*., 1976). Studies performed on CA inhibitors and thiazide diuretics demonstrated that after administration of acetazolamide (Brechue and Stager, 1990) or a hydrochlorothiazide–triamterene combination (Nadel *et al*., 1980) plasma volume and stroke volume are significantly decreased. Loss of plasma volume and stroke volume disrupts thermoregulation via peripheral vasodilation (radiation cooling) and perspiration (evaporative cooling), impairing both the acute and the long-term physiological vasodilatory response to aerobic exercise. Furthermore, aldosterone antagonists, in particular spironolactone, interfere with the increase in aldosterone receptor sensitivity due to exercise-induced hypervolemia (a consequence of normal adaptation to regular exercise).

Additional effects of specific classes of diuretics

Because CA plays a key role in the acid-base regulation mechanisms, CA inhibitors are the only class of diuretics that can affect pulmonary function. Acetazolamide has been shown to impair CO_2 elimination during exercise (Scheuermann *et al*., 1999), but also impairs CO_2 efflux from inactive muscle (Kowalchuk *et al*., 1992). In AMS, acetazolamide enhances alveolar oxygenation by increasing arterial oxygen pressures and lowering arterial carbon dioxide pressures (Bradwell *et al*., 1986). The cellular metabolic effects of acetazolamide may override its pulmonary effects and cause an inhibition of VO_2 during maximal exercise (Stager *et al*., 1990; Kowalchuk *et al*., 1992). Furosemide decreases tidal volume, minute ventilation and the respiratory exchange ratio at aerobic threshold (Caldwell *et al*., 1984). Conversely, clinical data indicate that inhaled furosemide reduces the exercise-induced bronchoconstriction in asthmatic children (Munyard *et al*., 1995). The effects of diuretics on VO_2 are variable. Furosemide causes a dose-dependent effect; furosemide has no influence on VO_2 at low doses (Armstrong *et al*., 1985; Baum *et al*., 1986), but VO_2 significantly decreases at higher doses (Caldwell *et al*., 1984). Acetazolamide affects VO_2 only during maximal exercise (Stager *et al*., 1990; Kowalchuk *et al*., 1992) as VO_2 is not affected under normoxic conditions (Brechue and Stager, 1990), but it is greatly improved under hypoxic conditions (Schoene *et al*., 1983). Acetazolamide effects on performance depends on altitude; at sea level (Heigenhauser *et al*., 1980) and under normoxic conditions (Schoene *et al*., 1983; Stager

et al., 1990) it may impair aerobic performance, but under hypoxic conditions it decreases the time to exhaustion during submaximal exercise (Stager *et al*., 1990).

Finally, thiazide diuretics are derivatives of sulphonamides and can cause photosensitivity if exercising outdoors during midday hours.

Caldwell *et al*. performed a diuretic-induced cycling workload reduction study to assess the effects of hypohydration on cycle ergometer performance. In this study, VO_2 max (maximum oxygen uptake) and workload while cycling decrease in athletes after furosemide intake. Even after rehydration, muscular endurance and performance are greatly compromised by diuretic use (Caldwell *et al*., 1984). Additional studies performed on middle-distance runners (Armstrong *et al*., 1985) and wrestlers (Caldwell, 1987) confirmed that diuretics decrease the effects on overall athletic performance. Although insufficient data are available to establish the effect of long-term diuretic treatment on exercise capacity, it has been clearly shown that both single dose and short-term diuretic treatment adversely affect maximal exercise capacity and the duration of prolonged submaximal exercise (Fagard *et al*., 1993). For the multitude of reasons mentioned above, the drawbacks related to diuretic administration outweigh the potential advantages of lowering of weight and urine dilution; dehydration drastically impairs aerobic capacity and muscular strength and decreases metabolic efficiency. This results in a detrimental effect on overall sport and exercise ability and especially on athletic performance (Caldwell *et al*., 1984; Armstrong *et al*., 1985). In addition, a potential effect of diuretics abuse is the possible alteration of the glomerular filtration size, which depends on a series of parameters (Edwards *et al*., 1999), most of which can markedly be affected by the mechanism of action of the different classes of diuretics. Finally, it should be noted that disqualification from competition as well as the other, previously mentioned detrimental effects of diuretic abuse offset any perceived benefits.

Although many of the studies highlighted above were published in the 1980s and 1990s, diuretics are still widely abused in sport (and among the most prescribed therapeutic agents). Few studies of the effects of diuretics on athletes have been published recently because in recent times, most studies assessing doping agents and exercise and sport have focused on newer drugs and methods of performance enhancement. Diuretic use for the masking of other prohibited substances remains a serious problem, however.

Analysis of diuretics

General remarks

For the detection of diuretics in urine in sports doping, a single minimum required performance level (MRPL) of 250 $ng \cdot mL^{-1}$ is fixed by WADA for accredited laboratories (WADA, 2009e). 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 250 $ng \cdot mL^{-1}$ 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 of diuretics, primarily among them HPLC-UV-DAD, GC/MS, LC/MS and LC/MS-MS, micellar electrokinetic 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 hydrochlorthiazide and althiazide. This phenomenon is more relevant when there is a delay between collection of the sample and the laboratory analysis (Thieme *et al*., 2001; Goebel *et al*., 2004; Deventer *et al*., 2009).

In the 1980s and 1990s, GC/MS was the most common analytical technique used by the anti-doping laboratories for the analysis of xenobiotics in urine (Maurer, 1992; Hemmersbach and de la Torre, 1996). Historically, diuretics were also screened for by GC/MS [extensively reviewed by Ventura and Segura, 1996 (Ventura & Segura, 1996)]. The recent evolution towards LC/MS (see below) has been driven by a series of concomitant causes that make the GC/MS-based approach less preferred than it was in the past two decades: (i) the number of target substances, and especially of low-molecular-weight xenobiotics, to be screened for in anti-doping analysis increased dramatically in the period 2002–2007 promoting the development of more 'universal' analytical techniques aimed to reduce the ratio of resources/assay; (ii) the need to simplify sample pretreatment due to the increased number of analytical procedures running simultaneously in anti-doping laboratories; and (iii) the technological advances in the field of analytical instrumentation, and, more specifically, the availability of benchtop LC/MS and LC/MS-MS systems at an affordable price. All of the above occurrences promoted a progressive shift from GC/MS to LC/MS.

Gas chromatography/mass spectrometry

Gas chromatography/mass spectrometry is still used by many anti-doping laboratories and can still represent a valid alternative for the anti-doping analysis of diuretics. A general analytical procedure based on GC/MS is structured as a series of pretreatment steps (at the minimum: extraction of the diuretics from the biological matrix and chemical derivatization) to be carried out before the chromatographic run.

Sample pretreatment. As it is known, the GC/MS analysis of biological samples to screen for diuretics requires a series of pre-instrumental procedures aimed to make the sample suitable for the analysis. Basically, the critical steps are represented by the extraction of the diuretics from the biological matrix and the chemical derivatization performed to increase volatility and thermal stability of the target compounds.

Different methods have been published for the detection of diuretics in urine using liquid/liquid (L/L) and solid phase

Table 3 Properties of diuretics important for analytical method development [adapted from Ventura and Segura (1996) and Jackson (2006)]

	pKa	*logP*	*Relative potency**	*Elimination route*	*Metabolism %*
Carbonic anhydrase inhibitors					
Acetazolamide	7.4, 9.1 7.2, 9.0	–0.3	1	Renal	0
Dichlorphenamide	7.4, 8.6	1.0	30	NA	NA
Methazolamide	7.3	–0.2	>1, <10	Renal	75, Hepatic
$Na^+/K^+/2Cl^-$ symporter inhibitors (loop diuretics)					
Furosemide	3.8, 7.5	2.0	1	Renal	35, Renal
Bumetanide	3.6, 7.7	2.6	40	Renal	38, Hepatic
Ethacrynic acid	3.5	3.7	0.7	Renal	33, Hepatic
Torsemide	7.1	2.5	3	Renal	80, Hepatic
Azosemide	6.8	3.3	1	Renal	63, Hepatic
Piretanide	4.1	1.9	3	Renal	50, Hepatic
Tripamide	NA	NA	NA	NA	NA
Na^+/Cl^- symporter inhibitors (thiazide and thiazide-like diuretics)					
Bendroflumethiazide	9.0	1.9	10	Renal	70, Hepatic
Chlorothiazide	6.85	–1.9	0.1	Renal	0
Hydrochlorothiazide	9.5, 11.3	–0.1	1	Renal	0
Hydroflumethiazide	8.9	0.4	1	Renal	20–60, Hepatic
Methyclothiazide	9.4	1.4	10	Renal	100, Hepatic
Polythiazide	NA	1.9	25	Renal	25–75, Unknown
Trichlormethiazide	8.6	0.6	25	Renal	0
Chlorthalidone	9.4	0.8	1	Renal, bile	25. Unknown
Indapamide	8.8	2.7	20	Renal	100, Hepatic
Metolazone	NA	1.8	10	Renal, bile	10, Hepatic
Quinethazone	NA	0.2	1	NA	NA
Osmotic diuretics					
Glycerin	NA	–2.3	NA	Renal	80, Hepatic
Isosorbide	NA	1.3(di) –0.2 (mono)	NA	Renal	0
Mannitol	NA	–3.1	NA	Renal, bile	20, Renal
Urea	NA	–2.1	NA	Renal	0
Renal epithelial Na^+ channel inhibitors (some potassium-sparing diuretics)					
Amiloride	8.7	0.3	1	Renal	0
Triamterene	6.2	1.0	0.1	Renal	100, Hepatic
Mineralocorticoid receptor antagonists (aldosterone antagonists and some potassium-sparing diuretics)					
Spironolactone	NA	2.8	NA	Renal	100, Hepatic
Canrenone	NA	3.5	NA	Renal	100, Hepatic
Potassium canrenoate	NA	NA	NA	Renal	100, Hepatic
Eplerenone	NA	2.3	NA	Renal	100, Hepatic

*Potency is relative to diuretics within the same class.
NA, data not available.

(SPE) extraction procedures. SPE can allow the recovery of diuretics with higher yields, but at the same time the use of disposable cartridges increases the overall cost of the pretreatment procedure, especially in the case of more complex supports, like internal surface reversed-phase supports (ISRP-size exclusion). Commercially available pre-activated columns have been tested for their efficacy and the best choice should depend on the characteristics of the matrix and on the expected composition of the sample [reviewed by Ventura and Segura in 1996 (Ventura and Segura, 1996)].

On the other hand, L/L extraction generally requires multiple extraction procedures. When the detection of all diuretics (basic, acidic and neutral) is desired, the optimal solution is a process based on two separate L/L extraction procedures (one in neutral or basic medium, and another in acidic medium) using ethyl acetate or a mixture of organic solvents. Anhydrous sodium sulphate can be added to promote a salting-out effect. Particular care has to be dedicated to the study of potential degradation processes that could involve target compounds. Oxidation of thiazides (althiazide, benzthiazide and polythiazide) has been demonstrated in the presence of ethyl acetate, so the efficacy and the non-reactivity of different extraction solvents has to be preliminarily assessed.

In some instances, two or more pretreatment steps can be combined, as in the case of extractive methylation in which both the extraction and the derivatization steps are combined in a single procedure.

Derivatization procedures. As mentioned above, derivatization is necessary prior to GC/MS analysis as most diuretics are not sufficiently volatile, lipophilic or thermally stable to be directly assayed with this analytical technique. The most common derivatization procedures are silylation and methylation, but the latter is usually preferred as it allows sufficient yields of more stable derivatives for most diuretics to be obtained [reviewed by Carreras *et al*. in 1994 (Carreras *et al*., 1994)]. Methylation can be performed 'statically' (by a mixture of methyl iodide and acetone under thermal

heating) or 'dynamically' by either extractive methylation (Lisi *et al*., 1991; Lisi *et al*., 1992) or 'on column' methylation (flash methylation) (Beyer *et al*., 2005). When methylation is performed by a stand-alone process, the time can be drastically reduced by microwave irradiation, either in combination or as an alternative to thermal incubation (Amendola *et al*., 2003).

Chromatographic and spectrometric conditions. The best stationary phase for the analysis of diuretic compounds is phenylmethylsilicone, allowing for the effective separation of all diuretics within a reasonable time (<15 min). Drastically shorter times can be obtained by fast-GC systems, in which last generation columns and mass spectrometric detection relying on fast electronics are successfully coupled. Fast-GC systems provide a 10-fold reduction of the overall chromatographic run (Morra *et al*., 2006). Electron impact ionization and MS detection are the most described methods used [reviewed in Ventura and Segura, 1996 and by Müller *et al*. in 1999 (Ventura and Segura, 1996; Müller *et al*., 1999)]. The mass spectra of the methyl derivatives of diuretics have been described by different authors, and the fragmentation profiles have been also interpreted by comparison with the deuterated methyl derivatives (Yoon *et al*., 1990).

Liquid-chromatography/mass spectrometry

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 (Ventura and Segura, 1996). 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 (Ventura *et al*., 1991) in confirmation analyses. The lack of robustness of the equipment did not permit a daily running screening method based on these instruments.

Thieme *et al*. (Thieme *et al*., 2001) described a method for the analyses of 32 diuretics in human urine by LC/MS/MS using an electrospray ionization technique. This technique has the advantage that the traditional LC flow rates and reverse-phased LC columns (octadecylsilane-ODS columns with 5 or 3 μm particle size) usually used in LC-UV methods can be used. Additionally, both positive and negative ionization modes can by used simultaneously permitting the detection of both acidic and basic compounds included among diuretics. The analysis by tandem MS with triple stage quadrupoles were selective and sensitive enough compared with previous methods and made simplification of sample preparation possible as the cleanness of the urinary extracts was less critical compared with previously designed LC-UV methods.

The development of new analysers (ion traps) coupled to LC created additional alternatives for the analysis of diuretics by LC/MS (Deventer *et al*., 2002). Even more recently, the need for more universal strategies for doping agents analysis introduced the use of time-of-flight analysers (Georgakopoulos *et al*., 2007) that can be coupled to LC. For some compounds and for identifications purposes, ionization by atmospheric pressure chemical ionization (another possible ionization technique of LC/MS interfaces) is of interest as it produces additional fragmentation (Qin *et al*., 2003).

The selectivity and sensitivity of these techniques allowed the inclusion of additional non-diuretic drugs, also forbidden in sports, in the same screening procedures (Deventer *et al*., 2005; Mazzarino *et al*., 2008). Additionally, different approaches for sample preparation have been explored. In the past, classical double extractions with organic solvents at acidic and basic pH were used to permit the recovery of diuretics presenting different physicochemical properties.

The new features of the instruments and the extension of the screening methods to other compounds expand the possibilities of sample preparation. Specific SPE procedures can be performed in robotic systems (Goebel *et al*., 2004) and some analytical procedures require no sample preparation, just a dilution the urine sample and subsequent direct injection into the LC/MS system (Politi *et al*., 2007; Thorngren *et al*., 2008). The improvements in the scanning speed of the mass spectrometers as well as better performing LC columns and LC pumps allow an increase in the speed of analysis (UPLC or fast LC) and of more heterogeneous screening procedures by LC/MS/MS. Currently, there are analyses that includes diuretics among other doping substances where more than 100 different compounds can be analysed in less than 10 min (Thorngren *et al*., 2008; Ventura *et al*., 2008).

Summary and conclusion

The members of the diuretic class of drugs vary greatly in structure, physicochemical properties and site and mechanism of action. In the 1990s the analysis of diuretics in doping (by LC-UV or GC/MS) was a challenge for anti-doping laboratories due to the heterogeneity of the substances included. Since the advent of robust and reliable LC/MS instruments their detection in urine samples is no longer a problem. Future goals of diuretic analysis include developing more efficient and more economical detection methods. Increasing the sensitivity of the methods and the number of compounds in the screen while decreasing the analysis time and cost to laboratories would be welcome improvements. Additionally, the development of methods that combine the detection of diuretics with other prohibited substances will enhance the ability of laboratories to monitor abuse and doping in sports.

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Statement of conflict of interest

None.

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