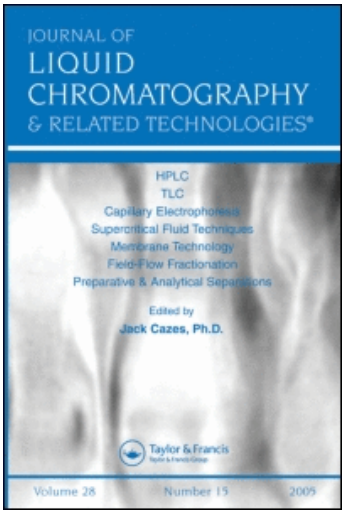

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Liquid Chromatographic Screening Test for Some Diuretics of Doping Interest in Human Urine

Vanessa Moreira[a]; Regina Lúcia Moraes Moreau[a]
[a] College of Pharmaceutical Sciences, Laboratory of Analytical Toxicology, University of São Paulo, São Paulo, Brazil

To cite this Article Moreira, Vanessa and Moreau, Regina Lúcia Moraes(2005) 'Liquid Chromatographic Screening Test for Some Diuretics of Doping Interest in Human Urine', Journal of Liquid Chromatography & Related Technologies, 28: 17, 2753 — 2768
To link to this Article: DOI: 10.1080/10826070500225036
URL: http://dx.doi.org/10.1080/10826070500225036

Journal of Liquid Chromatography & Related Technologies®, 28: 2753–2768, 2005

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070500225036

Liquid Chromatographic Screening Test for Some Diuretics of Doping Interest in Human Urine

Vanessa Moreira and Regina Lúcia Moraes Moreau
College of Pharmaceutical Sciences, Laboratory of Analytical Toxicology, University of São Paulo, São Paulo, Brazil

Abstract: Despite being considered ergolytic drugs, diuretics have been included on the lists of prohibited substances of the International Olympic Committee since 1986 due to their capacity of masking doping agents in urine. As diuretics are also prone to be misused regarding weight categories, screening procedures to check their presence in urine, whenever doping control is involved, are mandatory. A simple screening method using liquid-liquid extraction was validated for the compounds: acetazolamide, amiloride, bumetanide, chlorthalidone, clopamide, furosemide, hydrochlorothiazide, piretamide, spironolactone, and triamterene. Urine samples were extracted in both acidic and basic media. HPLC analyses were performed with a Spherisorb ODS column and diode array UV detector, set at 260, 270, and 360 nm wavelengths. A gradient mobile phase was used. Limits of detection varied from 0.09 to 0.75 μg/mL. Recoveries ranged from 55.26 to 94.82%. Inter and intra-assay precision tests showed good values.

Keywords: Screening test, Diuretics, Doping, Human urine

INTRODUCTION

The non-medical use of therapeutic drugs to artificially alter the performance of athletes in certain sports activities has been of great concern for more than thirty years. Besides being an ethically condemned practice, the risk to

Address correspondence to Regina Lúcia Moraes Moreau, College of Pharmaceutical Sciences, Laboratory of Analytical Toxicology, University of São Paulo, USP, São Paulo, Av. Prof. Lineu Prestes, 580, CEP 05508-900, Brazil. E-mail: rlmoreau@usp.br

the athletes' health has to be considered, since they are generally self-administered in a wrong manner: overdoses, interactions with other drugs, or even the use of drugs of illicit origin.[1,2]

Diuretic drugs are largely used in therapeutics, especially for the treatment of hypertension and oedemas. Whenever used prior to sports activities, they are considered ergolitic agents decreasing the athletes' performance due to a rapid reduction of plasmatic volume.[3] Even so, these drugs were included in the list of prohibited substances by the International Olympic Committee in 1986. Since then, diuretics have been subject to doping control in sports activities. Nowadays, The World Anti-doping Agency (WADA), in its 2005 version, includes diuretics in the substances and methods prohibited at all times (in-and out-of-competition) (class S5).[4]

The prohibition is due to the different mechanisms of action involved in diuretics: they increase urinary flux and volume. As a consequence, whenever other drugs used as doping are present, their concentrations in urine can be so drastically reduced under the limit of detection of the method used leading the way to false negative results in urinalyses. Diuretics that inhibit carbonic anhydrase act as urinary alkalinasing agents, reducing the excretion of weak basic compounds (mostly stimulants) of prohibited use. However, there are some sports activities in which diuretics are prone to be misused and abused where weight categories are involved with positive effects. Weightlifting, wrestling, judo, karate, and boxing are some of the sports categories in which a rapid reduction of bodyweight is mandatory. Nevertheless, the control of water retention caused by the use of anabolic steroids is another frequent use of diuretics.[3,5,6]

Gas chromatography coupled with mass spectrometric detection (GC/MS), as well as high performance liquid chromatography (HPLC), are the techniques used for screening diuretics in doping control urinalyses.

HPLC is the method of choice for the analysis of diuretics due to the time consumed, cost of the analysis, and some limitation involving the GC/MS technique to detect diuretics in urine (low volatility of the compounds and the necessity of the additional step of derivatization).[7] In this work a simple screening HPLC method, coupled a with diode array detector, was chosen to validate detection of ten of the most important diuretics of doping interest in human urine.

EXPERIMENTAL

Reagents and Chemicals

Diuretic drugs used as standards of reference were kindly provided by the Brazilian branches of the following international pharmaceutical industries: acetazolamide (Wyeth-Whitehall); amiloride (Prodome); bumetanide (Sintofarma); clopamide and chlorthalidone (Novartis); spironolactone

(Searle); furosemide and hydrochlorthiazide (Prodotti); piretanide (Hoescht-Marion-Roussel), and triamterene (Sanofi-Wintrop). The internal standard caffeine was supplied by (Novartis). Methanol, acetonitrile (Merck), and ethylacetate (EM Science) were HPLC grade. All other reagents used were of analytical grade.

Apparatus

HPLC analyses were performed on a Hewlett-Packard 1100 series chromatograph coupled with a diode array UV detector and equipped with a G1322A series vacuum degasser (G1322A), binary pump (G1312A), autosampler (G1313A), and thermostatized column compartment (G1316A). The column was a Spherisorb ODS (125 × 4 mm I.D.) 5 μm particle size (Hewlett-Packard).

The mobile phase consisted of a 0.05 M buffer solution of ammonium acetate adjusted to pH 3 with concentrated orthofosforic acid and acetonitrile.[8] The solution was filtered over a 0.22 μm/47 mm cellulose ester GS membrane and degassed in a system equipped with a ultrasonic bath and vacuum pump. A gradient was used to increase acetonitrile from 10% to 15% in 2 min, to 30% in 4 min, and to 60% in 8 min, and to decrease to the initial condition. The base line was stabilized for 2 min before the next injection. Column temperature was set at 30°C flow-rate = 1.5 mL/min; volume injected = 20 μL; duration of each analysis was 10 min. The detector was set to monitor signals at 260, 270, 360 nm, once these wavelengths have been found to be the optimum for the screening for the diuretic studied. The column was maintained at 35°C.

Preparation of Stock and Working Solutions

Methanolic stock solutions of the diuretics and internal standard were prepared at concentrations of 2 mg/mL (exception to the solution of 0.4 mg/mL of triamterene) and stored at −20°C. Working solutions of each diuretic and internal standard were prepared at a concentration of 10 μg/mL and stored at −4°C when not in use.

Sample Extraction

Diuretic drugs are either weak organic acids or bases. Consequently, urine samples containing diuretic compounds need to be extracted under both acidic and basic conditions. For our studies the most convenient technique of extraction was that proposed by Cooper et al.,[5] in which aliquots of the same sample is extracted both in acidic and basic pH values. In short, for the acidic extraction, an aliquot of 2 mL of urine is treated with 0.5 g of

solid buffer (monopotassium phosphate 99: disodium phosphate 1 w/w) to reach pH values between 5.0 to 5.5 and extracted with ethyl acetate. In order to remove interfering substances (urinary pigments) which may be extracted in these conditions of pH, a 5% lead acetate solution is added. The organic layer is separated and preserved for further elaboration. For the basic extraction a 2 mL aliquot of the urine is treated with sodium bicarbonate 3 : potassium carbonate 2 w/w to reach final pH values from 9.0 to 9.5. The solution is extracted with 4 mL of ethyl acetate and the organic phase is separated. Both organic phases are evaporated to dryness separately under nitrogen flow. The residues are reconstituted with 200 μL of water: acetonitrile (85 : 15 v/v), and 20 μL of each is injected in the chromatograph.

Validation of the Method

A pool of blank urine was prepared with five samples from normal healthy volunteers who declared not to have used diuretics for at least five days before the urine collecting. Half of the volume obtained was spiked with the methanolic solutions of diuretics as described below to be used in the validation of the method. Aliquots of the other half volume were analysed both in acidic and basic medium to check the presence of interfering compounds.

Concentration Range of Diuretics Spiked in Urine Samples Used to Validate the Method

In recovery and inter and intra precision studies the following range of concentrations were employed: a low concentration corresponding to two times the LOD and a high concentration corresponding to two times the urinary concentration found 24 hours after the therapeutic administration of a single dose of the diuretic. These values are described in Table 1.

Limit of Detection

The limit of detection (LOD) for each diuretic was determined by an empirical method, which consists of analysing a series of urine samples containing decreasing amounts of the drug. The lowest concentration values were determined through the smallest peak are, which presented a coefficient of variation lower than 20% in ten replicates.[9]

Recovery and Inter- and Intra-Assay Precision

Urine samples spiked with the diuretics in concentrations according to Table 1, underwent the complete extraction procedure as described above. Recovery

Table 1. Urinary concentrations of diuretics (μg/mL) detected 24 hours after one single dose[5,6]

Diuretics	Dosage (mg)	Urinary concentration (μg/mL)
Acetazolamide	250	20
Amiloride	5	1.25
Bumetanide	5	0.5
Clopamide	5	0.75
Chlorthalidone	5	1.0
Spironolactone	25	0.3
Furosemide	25	3.0
Hydrochlorthiazida	25	2.0
Piretanide	6	1.44
Triamterene	50	1.25

was estimated by comparing the peak areas of each compound extracted with that obtained from the methanolic standard solution at a concentration equivalent to 100% recovery.

Precision defined as the relative standard deviation was determined by inter- and intra-assay.[9] Intra-assay precision was performed by analysing samples of urine spiked with the diuretics in six replicates in the same day. Inter-assay precision was determined by analysing spiked urine samples in six replicates during six days.

Reproducibility of the Validated Method

Application of the method was performed by analysing four urine samples of healthy male volunteers (athletes) who declared to have used diuretics some days or hours prior to the sample collection. The samples were positive for hydrochlorothiazide and furosemide. The samples were also analysed under the authorization of the College of Pharmaceutical Ethical Committee.

RESULTS

The chromatograms illustrated in Figure 1 were obtained from the injection of the methanolic solution containing a mixture of the ten diuretics (100 μg/mL) and the internal standard. The detector was set in the three different wavelengths: 260, 270, and 360 nm. The compounds could be resolved distinctly in 10 minutes.

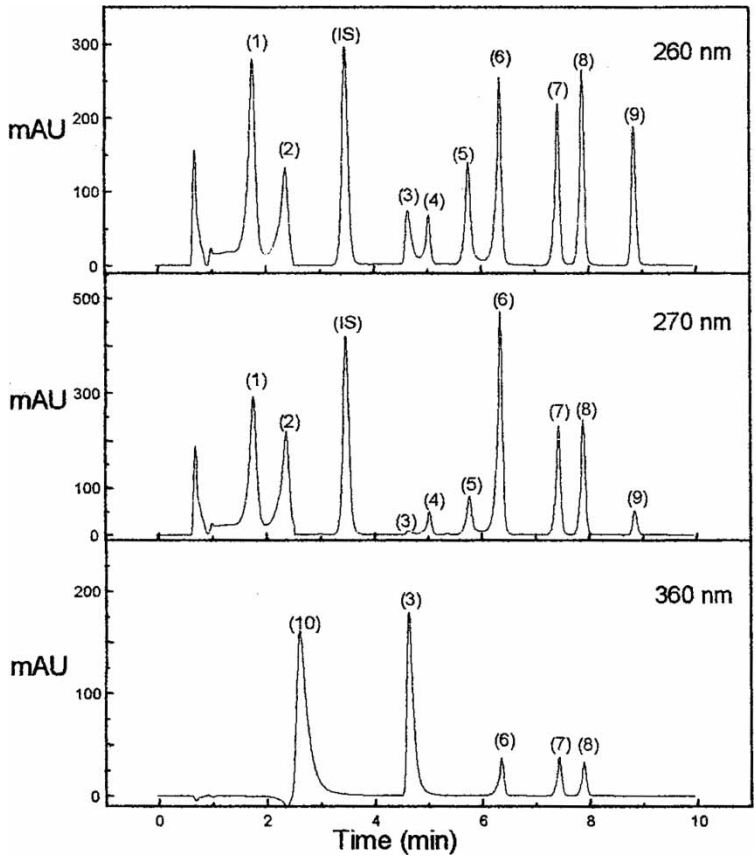

Figure 1. Chromatographic profile obtained by the analyses of a pool of ten diuretics in methanolic solution (100 μg/mL) at 260, 270 and 370 nm. Gradients of 10% acetonitrile at the beginning, 15% in 2 min, 30% in 4 min, and 60% in 8 min were applied. (1) acetazolamide; (2) hydrochlorthiazide; (3) triamterene; (4) chlorthalidone; (5) clopamide; (6) furosemide; (7) piretanide; (8) bumetanide; (9) spironolactone; (10) amiloride; (IS) caffeine.

Relative retention time (RRT) of the ten diuretics estimated by the ratio of retention time of the diuretic and retention time of internal standard are described in Table 2.

The values of limits of detection (LOD) estimated for the ten diuretic compounds varied from 0.09 to 0.75 μg/mL and are showed in Table 3.

Recovery results of each diuretic submitted to acid and basic extraction procedures are shown in Table 4.

Table 2. Mean of retention time, coefficient of variation and relative retention time (RRT) of the diuretics studied and caffeine (IS) (n = 6)

Diuretics	Mean ± SD	CV	RRT
Acetazolamide	1.79 ± 0.015	0.838	0.52
Amiloride	2.75 ± 0.15	0.545	0.80
Bumetanide	7.90 ± 0.06	0.076	2.30
Caffeine (IS)	3.44 ± 0.021	0.610	1.00
Clopamide	5.76 ± 0.010	0.174	1.66
Chlorthalidone	5.05 ± 0.016	0.317	1.47
Furosemide	6.39 ± 0.015	0.235	1.86
Spironolactone	8.83 ± 0.017	0.193	2.57
Hydrochlorothiazide	2.41 ± 0.015	0.622	0.70
Piretanide	7.46 ± 0.015	0.201	2.18
Triamterene	4.70 ± 0.010	0.213	1.37

SD = Standard deviation.
CV = Coefficient of variation.
RRT = Retention time relative to caffeine.
IS = Internal standard.

Intra and inter-assay precision by analysing urine samples spiked with the drugs in two different concentrations (high and low) are expressed in Table 5.

DISCUSSION

Due to the different chemical structures (functional groups) and physico-chemical properties such as p*K*a values, volatility and polarity, the develop-

Table 3. Limit of detection values in the screening of the ten diuretic compounds

Diuretics	LOD (μg/mL)
Acetazolamide	0.09
Amiloride	0.75
Bumetanide	0.35
Clopamide	0.25
Chlorthalidone	0.25
Spironolactone	0.25
Furosemide	0.125
Hydrochlorothiazida	0.09
Piretanide	0.50
Triamterene	0.09

Table 4. Extraction recovery values of the diuretics submitted to the screening procedure in acidic and basic media

		% Recovery	
Diuretics	Conc. (μg/mL)	Acidic extraction	Basic extraction
Acetazolamide	40	62.48	—
	0.18	51.74	—
Amiloride	2.5	—	43.44
	1.5	—	55.26
Bumetanide	1.0	88.12	50.22
	0.7	85.63	75.12
Clopamide	1.0	83.83	91.28
	0.5	80.24	88.04
Chlorthalidone	2.0	94.82	79.72
	0.5	72.63	81.43
Spironolactone	8.80	87.19	50.22
	0.5	39.16	75.12
Furosemide	6.0	62.54	33.25
	0.25	66.34	31.24
Hydrochlorothiazide	4.0	82.56	—
	0.18	69.30	—
Piretanide	2.88	66.32	23.72
	1.0	44.59	18.54
Triamterene	2.5	5.76	72.05
	0.18	—	41.41

ment of common screening procedures for diuretics and metabolites is not an easy task. Methods to detect diuretics in pharmaceutical dosage forms or individually in biological samples have been reported in the literature. Screening methods for some groups of compounds using different chromatographic techniques (paper in combination with spectrophotometry, TLC) were proposed many decades ago, and are now totally abandoned due to lack of sensitivity and specificity.

Gas-liquid chromatography/mass spectrometry (GC/MS) and HPLC are the techniques of choice to determine diuretics in urine for doping control (confirmation and screening respectively).

GC/MS presents some major difficulties in separating diuretics without prior derivatization of compounds with functional polar groups. Besides it is time consuming, and for some compounds, the formation of suitable derivatives are not possible.

A great variety of HPLC methods have been proposed to detect diuretics after a single or double (acidic, basic or neutral) extraction procedures. In this work, a HPLC separation system equipped with a diode-array detector was

Table 5. Intra- and inter assay precision, expressed by peak areas by analysing samples of urine spiked with the diuretics in six replicates at the same day and during six days respectively

Diuretics	Conc. (μg/mL)	Mean ± SD		Extraction	Wavelength (nm)
		Intra-assay	Inter-assay		
Acetazolamide	40.00	5567.20 ± 437.20	6157.59 ± 543.3	Acidic	270
	0.18	31.37 ± 2.60	32.35 ± 1.43		
Amiloride	2.5	293.22 ± 17.27	277.58 ± 50.00	Basic	360
	1.5	85.39 ± 15.80	89.37 ± 12.42		
Bumetanide	1.0	92.76 ± 6.03	89.50 ± 6.77	Acidic	260
	0.70	44.59 ± 6.59	50.33 ± 5.89		
Clopamide	1.5	111.86 ± 6.90	118.88 ± 6.09	Acidic	260
	0.5	42.34 ± 7.42	46.60 ± 3.69		

(*continued*)

Table 5. Continued

Diuretics	Conc. (μg/mL)	Mean ± SD		Extraction	Wavelength (nm)
		Intra-assay	Inter-assay		
Chlorthalidone	2.00	73.01 ± 4.06	75.54 ± 2.19	Acidic	260
	0.50	17.58 ± 1.80	18.62 ± 1.17		
Spironolactone	8.80	143.27 ± 16.35	167.10 ± 23.27	Acidic	260
	0.50	25.91 ± 1.79	26.73 ± 1.21		
Furosemide	6.0	816.46 ± 35.31	846.23 ± 104.40	Acidic	270
	0.25	20.86 ± 2.0	26.37 ± 4.90		
Hydrochlorotiazine	4.0	1147.32 ± 85.55	1140.51 ± 24.41	Acidic	270
	0.18	49.79 ± 3.01	52.25 ± 6.46		
Piretanide	2.88	179.49 ± 6.63	194.21 ± 13.74	Acidic	270
	1.0	40.54 ± 6.30	45.92 ± 7.32		
Triamterene	2.5	378.44 ± 66.79	461.29 ± 94.76	Basic	360
	0.18	56.08 ± 5.11	58.05 ± 4.17		

optimized for either ten organic acid or organic base diuretics of doping interest in order to afford a unified and rapid separation method.

Diode array detection improves selectivity by means of UV spectra of peaks in a certain region of the chromatogram. Reversed phase separations and gradient elutions using acidic mobile phases have been proposed[8] due to the different physico-chemical properties (acidic-basic behaviour and lipid solubility) of the diuretics.

The choice of a chromatographic column is a very important step to achieve good separation and resolution of the peaks in chromatographic techniques. Octadecyl columns (C_{18}) are the most commonly used for HPLC separation. In this study a Spherisorb OGS2 column was used. In a reversed phase separation besides an acidic mobile phase, it is necessary to use a basic modifier such as propilamine, triethylamine, or even ammonium salts, to minimize the interaction of basic diuretics with sylanol groups (Si-OH) of the stationary phase of the column.[10] In order to promote good simultaneous resolution of the compounds and reduction of analysis time, a buffer solution of ammonium acetate 0.05 M, pH 3.00 and acetonitrile was used in this work. Caffeine was chosen as internal standard due to the fact that it was observed previously that it does not co-elute with any of the studied diuretics. Besides, it is a neutral compound, which can be extract either in acidic or in basic conditions of pH.

The HPLC system employed in this study achieved the unequivocal separation of the ten compounds studied as shown in Figure 1. As the diode-array detection permits the simultaneous determination of compounds in up to five different wavelengths, the most adequate ones were selected in a range from 200 to 400 nm, UV-visible,(Figure 2). The wavelength of choice was 270 nm in which all the compounds could be absorbed, except amiloride that absorbed in 360 (Figure 1).

Liquid-liquid is the extraction procedure most frequently found in the literature with some minor advantages over a solid-phase extraction.[6,10–12]

Interfering compounds present in urine samples must be removed with proton acceptor solvents like ethyl acetate, diethyl ether, or methyl isobuthyl ketone.[11] Due to its higher polarity, extraction performed with ethyl acetate yielded good recovery of weak-basic diuretics when compared with diethyl ether extraction.

The method proposed by Cooper et al.[5] proved to be the best option among all described in the literature, since it has permitted the liquid-liquid extraction of the ten diuretic in this study. At the end of the extraction procedures (in acidic and basic media) the extracts, contrarily to what it is proposed in the original method, were not pooled. They were evaporated, reconstituted, and analysed separately. This modification was necessary in order to avoid the co elution of hydrochlorothiazide with an interfering urinary compound extracted in the acid medium. Besides, the diuretic drugs acetazolamide and amiloride would be very diluted if the extracts were reconstituted together; the former is not extracted in basic medium and the latter is not extracted in acidic conditions.

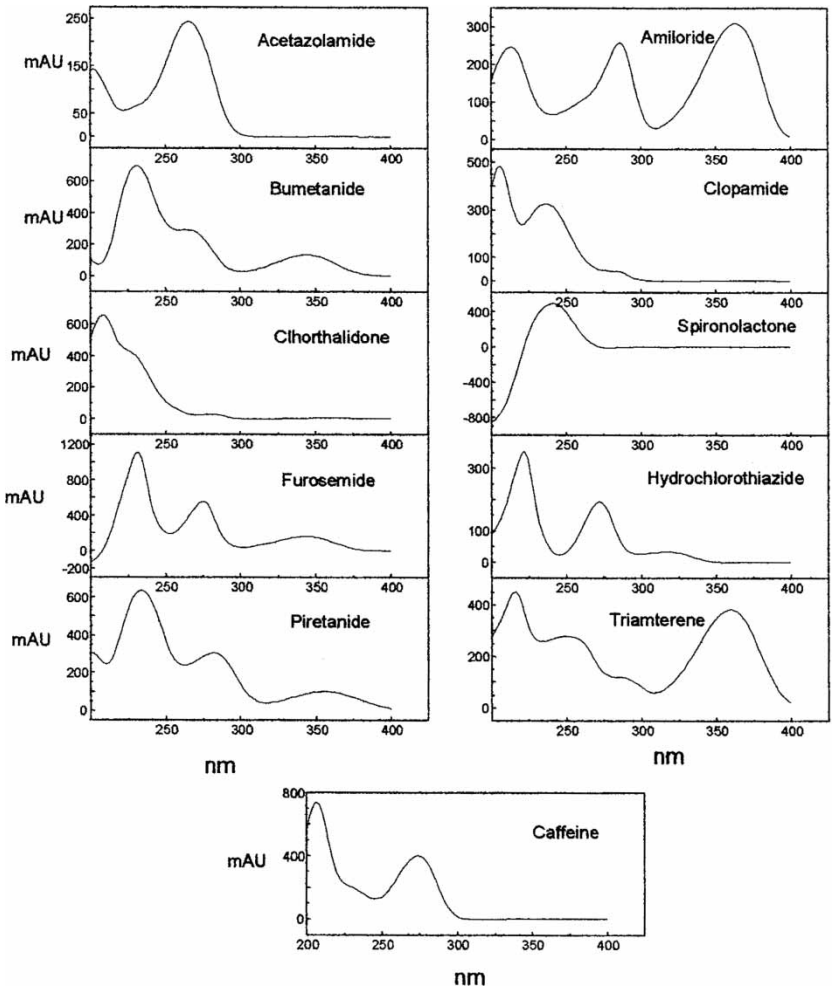

Figure 2. UV spectra of the ten compounds listed in the legend of Table 2.

Aliquots of blank urine samples were repeatedly extracted in both media, submitted to chromatography in the three different wavelengths, and no significant interfering compounds were observed in the chromatograms (Figures 3 and 4).

Aliquots of a pool of blank urine samples ($n = 5$) spiked with the ten diuretic drugs and caffeine as internal standard, were submitted to the extraction procedures and to the HPLC technique as cited above. The chromatograms obtained showed good separation and resolution of the peaks (Figures 5 and 6).

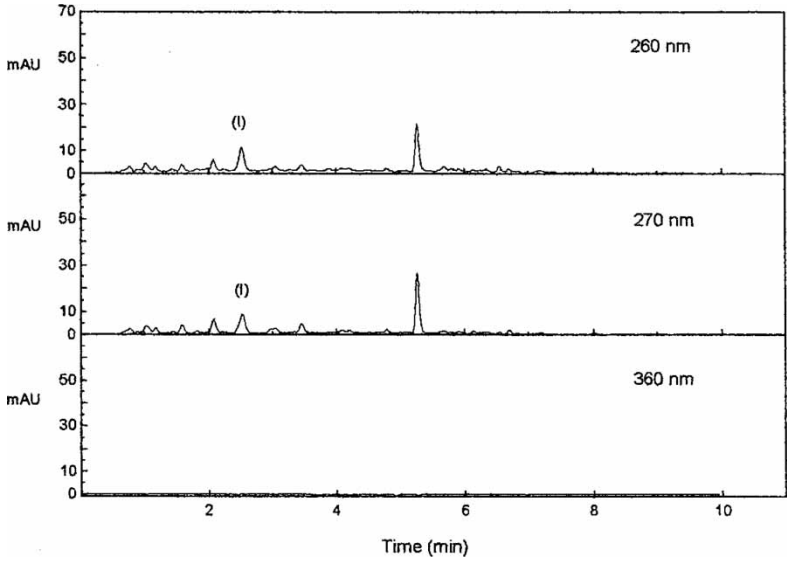

Figure 3. Chromatographic profile (HPLC) obtained by the analyses of aliquots of blank urine for diuretics and caffeine in acid medium (pH 5.0–5.5).

To validate the method optimised, the following parameters were evaluated: limits of detection, recoveries, inter- and intra assay precision.

The limit of detection (LOD) obtained (Table 3) using this described procedure, varied from 0.09 to 0.75 μg/mL and are lower than those

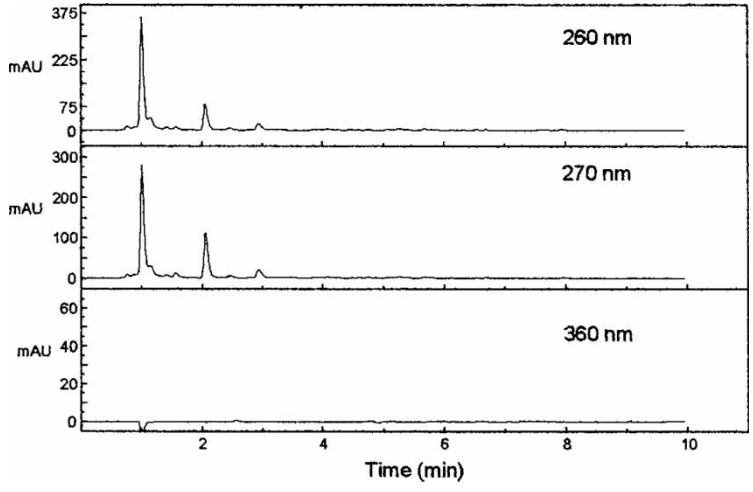

Figure 4. Chromatographic profile (HPLC) obtained by the analyses of aliquots of blank urine for diuretics and caffeine in basic medium (pH 9.0–9.5).

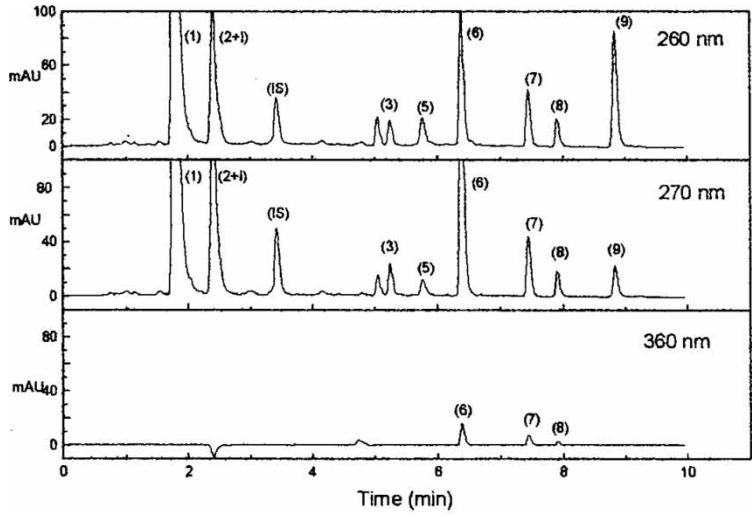

Figure 5. Chromatograms of diuretics extracted in acidic medium from spiked urine blank and analysed at 260, 270, and 360 nm wavelength: (1) acetazolamide (20 μg/mL); (2 + I) hydrochlorthiazide co-eluted with interfering substance; (3) triamterene (1.25 μg/mL); (4) chlorthalidone (1.0 μg/mL); (5) clopamide (0.75 μg/mL); (6) furosemide (3.0 μg/mL); (7) piretanide (1.44 μg/mL); (8) bumetanide (0.5 μg/mL); (9) spironolactone (0.3 μg/mL); (10) amiloride (1.25 μg/mL); caffeine IS (2 μg/mL).

presented by Guchelaar et al.[6] for the same diuretic drugs: clopamide, chlorthalidone, and spironolactone. Lower values for LOD were also obtained when compared with those described by Cooper et al.[5] However, results for LOD presented by Ventura et al.[8] with a one-step basic extraction were lower than the ones presented in this paper.

Extraction recovery studies at two concentrations (low corresponding to two times the LOD and high corresponding to two times the urinary concentration after a therapeutic administration (Table 1) of the diuretics submitted to the screening procedure) provided acceptable values for the acid extraction (Table 4) with an exception: triamterene and amiloride which were recovered only in basic extraction. Triamterene was recovered at 72.05% and amiloride at 55.22% only in this type of extraction. This screening procedure presents good inter- and intra assay precision for both concentrations with coefficient of variation lower than 20%.

Reproducibility and specificity of the validated screening method were performed under the authorization of the College of Pharmaceutical Ethical Committee, by analysing current urine samples of healthy volunteers (athletes of different sports activities) who declared to have used diuretics some days or hours prior to the sample collection, as well as urine samples

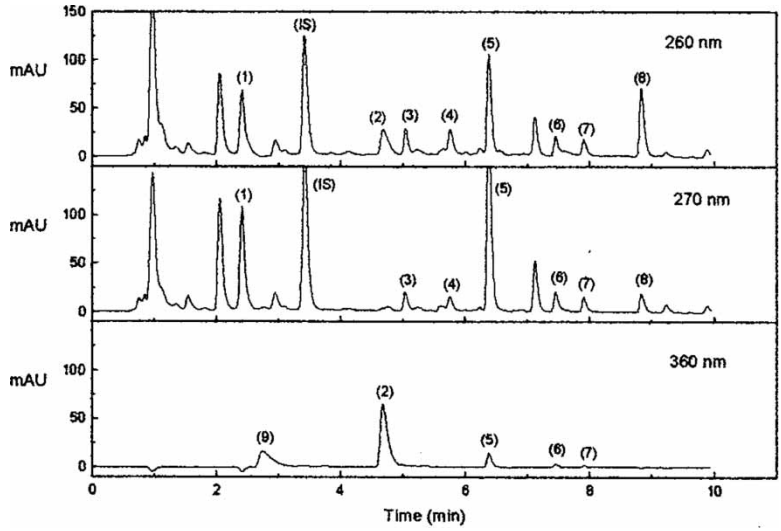

Figure 6. HPLC chromatograms obtained from basic extraction (pH 9.0–9.5) of urine blank spiked with the diuretics and detected at 260, 270, and 360 nm wavelength: (1) hydrochlorotiazide (20 μg/mL); (2) triamterene (1.25 μg/mL); (3) chlortalidone (1.0 μg/mL); (4) clopamide (0.75 μg/mL); (5) furosemide (3.0 μg/mL); (6) piretanide (1.44 μg/mL); (7) bumetanide (0.5 μg/mL); (8) spironolactone (0.3 μg/mL); (9) amiloride (1.25 μg/mL); (IS) caffeine (2 μg/mL).

of athletes submitted to Doping Control at the Laboratories of Toxicological Analysis, College of Pharmacy, University of S. Paulo.

The application of a simple screening method is a factor that contributes to the success of the analysis in doping control. Thus, the method optimized and validated in HPLC coupled diode array, in this study, proved to adjust for detection of ten diuretics in human urine, with the objective of doping control of this class of prohibited drugs in sports.

REFERENCES

1. Segura, J. Doping control in sports medicine. Ther. Drug Monit. **1996**, *18*, 471–476.
2. Verroken, M. Drug use and abuse in sport. Best Pract. Res. Cl. En. **2000**, *14* (1), 1–23.
3. Eichner, E.R. Ergolytic drugs in medicine and sports. Amer. J. Med. **1993**, *94*, 205–211.
4. The World Anti-Doping Agency (WADA). The World Anti-Doping Code. The 2005 prohibited list. 2005; 1–9.
5. Cooper, F.S.; Massé, R.; Dugal, R.J. Comprehensive screening procedure for diuretics in urine by high-performance liquid chromatography. J. Chromatogr. **1989**, *489*, 65–88.

6. Guchelaar, H.J.; Chandi, L.; Schouten, O. A high performance liquid chromatographic method for the screening of 17 diuretics in human urine. Fresen. J. Anal. Chem. **1999**, *363*, 700–705.
7. Campins–Falcó, P.; Herráez-Hernández, R.; Pastor-Navarro, M.D. Analysis of diuretics in urine by column-switching chromatography and fluoresence detection. J. Liq. Chrom. & Rel. Techonol. **1997**, *20* (12), 1867–1885.
8. Ventura, R.; Nadal, T.; Alcalde, P.; Pascual, J.A.; Segura, J.J. Chromatogr. **1993**, *655*, 233–242.
9. Chasin, A.A.M.; Nascimento, E.; Ribeiro-Neto, L.M.; Siquiera, M.E.P.B.; Andraus, M.H.; Salvadori, M.C.; Fernícola, N.A.G.; Gorni, R.; Salcedo, S. Validação de métodos em análises toxicológicas: uma abordagem peral. Rev. Bras. Toxicol. **1998**, *11* (1), 1–6.
10. Ventura, R.; Segura, J. Detection of diuretics agents in doping control. J. Chromatogr. **1996**, *687*, 127–144.
11. Tsai, F.Y.; Lui, L.; Chang, B. Análysis of diuretic doping agents by HPLC screening and GC-MSD confirmation. J. Pharmaceut. Biomed. Anal. **1991**, *9*, 1069–1076.
12. Fullinfaw, R.O.; Bury, R.W.; Moulds, R.F.W. Liquid chromatographic screening of diuretics in urine. J. Chromatogr. **1987**, *415*, 347–356.

Received April 8, 2005
Accepted April 30, 2005
Manuscript 6624