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Extension of an existing screening method for diuretics with beta-blocking agents

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1. Introduction

At the beginning of 2004 WADA took over the fight against doping from the IOC.
The existing list of prohibited compounds was re-evaluated thouroughly. New compounds were added to the list[1] . One group of compounds which was extended are the beta-blocking agents. It was evident to include these new beta-blockers in the existing GC/MS-screening method for beta-blocking agents[2-3] (screening II) However problems with derivatization occured and therefore the detection by LC/MS has been investigated resulting in a highly sensitive method [4].
The aim of this study was to include the beta-blockers in the existing LC/MS screening method for diuretics[5].

2. Experimental

2.1. Chemicals and reagents

The origin and suppliers of the diuretics have been previously described[5].
Beta-blockers obtained as reference substances were: acebutolol from Rhone-Poulenc (Brussels), alprenolol from Astra Chemicals (Holstein, Germany), atenolol and propranolol from ICI (Kortenberg, Belgium), betaxolol from Synthelabo (Brussels), labetolol from Glaxo (Brussels),

metoprolol from Ciba-Geigy (Groot-Bijgaarden), nadolol from Squibb (Braine l'Alleud, Belgium), oxprenolol from CIBA (Dilbeek, Belgium), pindolol from Sandoz (Vilvoorde, Belgium), sotalol from Pfizer (Brussels), timolol from MSD (Brussels), penbutolol from Thomson (London, United Kingdom), bisoprolol from Merck, mepindolol from Schering (Machelen, Belgium) and carvedilol from Roche (Mannheim, Germany).
levobunolol (*l*-bunolol) and esmolol were a kind gift from the South African doping control laboratory. Carteolol was a gift from the doping control laboratory from Portugal.
The following products were extracted from therapeutic preparations: celiprolol (Selectol®, Pharmacia, Brussels) and metipranolol (Beta-Ophtiole®, Tramedic, Sint-Niklaas, Belgium)..

2.2. Sample preparation

An internal standard solution (50 µL mefruside, 20 µg/mL) was added to 2 mL of urine, followed by addition of 1 mL of sodium acetate buffer (pH 5.2; 1.5 M). Liquid-liquid extraction was performed by rolling for 20 min with 4 mL ethyl acetate. After centrifugation the organic layer was transferred into a new tube. To the remaining urine 250 mg of potassium carbonate was added and a second liquid-liquid extraction was performed with 4 mL ethyl acetate. After centrifugation, both organic layers were combined and evaporated until dryness under oxygen free nitrogen (OFN) at 40 ºC. The remaining residue was dissolved in 200 µL of the initial mobile phase.

2.3. Validation

The validation was carried out following Eurachem validation guidelines[6].
Ten human urine samples, declared negative after routine doping analysis, were spiked at 9 different levels ranging from 1 up to 500 ng/mL.
The detection limit was defined as the lowest level at which a compound could be identified in all 10 urines, with diagnostic ions present with a signal to noise (S/N) ratio greater than 3
Both selectivity and sensitivity were tested as well.

2.5. Instrument parameters

A Thermo Separation Products (TSP) Model P4000 quaternary pump equipped with a TSP Model AS 3000 autosampler with a 100 µL sample-loop and connected to a Thermo Electron LCQ-Deca® mass spectrometer was used.
A Nucleosil C18 column 3 mm x 100 mm, 5 µm (Chrompack, Antwerp, Belgium) and a guard column (Chromsep, SS 10 x 2 mm, Chrompack), was used for the separation.
The mobile phase consisted of 1% acetic acid (solvent A) and acetonitrile (B). Gradient elution at a flow rate of 0.3 mL/min was as follows: 85% A for 2 min, linear to 45% in 10 min, linear to 35% in 8 min followed by an increase to 85% with 10 min equilibration time before the next injection. Total run time: 30 minutes. Ionization of analytes was carried out using electrospray ionisation. The capillary temperature was maintained at 300 ºC, the ion source voltage was set at 5000 V and the nebulizer gas (nitrogen) was set at 80 units. The make up gas (nitrogen) was set to a value of 30. The capillary voltage was set at 10 V in positive mode and -4 V in negative ionisation mode, respectively. When MS/MS was applied the isolation width was set at 3.0, the activation q at 0.250 and the activation time at 30 ms. An exception was made for acetazolamide for which a q value of 0.3 and an activation time of 70 ms was applied.

3. Results and Discussion

3.1. Mass spectrometry

Optimization was limited since our aim was to add the new compounds to the existing screening method for the diuretics.
Flow injection analysis was performed to determine the presence of diagnostic ions for the newly added beta-blockers. For each compound a solution (50/50 A/B) of 5 µg/mL was infused at a flow rate of 10 µL/min. Beta-blockers contain a basic group which can be easily protonated. Very abundant protonated molecular ions $[M+H]^+$ were observed after ESI using MS. No deprotonated molecular ions were detected in negative ionisation mode.

3.2. Scan to scan polarity changing

Due to the acidic nature of most diuretics, negative ionisation is generally prefered (Table 3). For the basic diuretics (e.g.: amiloride, triamterene) and beta-blockers however positively charged ions are formed. Hence, positive and negative scan events are necessary to cover all compounds included in the screening method. In addition coelution of positively and negatively charged ions makes scan to scan polarity switching unavoidable. Before the introduction of robust instruments scan to scan polarity switching was technically difficult to perform and two consecutive runs in both ionisation modes were necessary[7]. Soon after the introduction of reliable and fast polarity switching instruments reproducible scan to scan polarity switching was reported[8]. Nevertheless loss in sensitivity resulting from the polarity switching was observed as a reduction of scans by a factor of 4 when both alternating positive and negative ionisation is used.

3.2. Validation

All diuretics and beta-blockers could be detected at least at a level of 100 ng/mL and 500 ng/mL respectively. The limits of detection for all compounds are given in Table 1.
The described method seems to be very selective as no interferences were detected when other doping substances including narcotics, corticosteroids, stimulants and anabolic steroids were analysed.

Table 1: Urinary detection limits for diuretics and beta-blockers

Beta-blockers			
Substance	(ng/mL)	Substance	(ng/mL)
Mepindolol	500	Pindolol	25
Propranolol	10	Bisoprolol	10
Alprenolol	10	Acebutolol	25
Oxprenolol	10	Betaxolol	25
Penbutolol	5	Carvedilol	10
Labetolol	10	Carteolol	10
Metoprolol	10	Levobunolol	5
Timolol	25	Metipranolol	25
Atenolol	50	Celiprolol	5
Sotalol	50	Esmolol	5
Nadolol	10		
Diuretics			
Substance	(ng/mL)	Substance	(ng/mL)
Acetazolamide	50	Xipamide	25
Hydrochlorothiazide	100	Bumetanide	25
Amiloride	50	Etachrynic acid	10
Triamterene	10	Spironolactone	10
Diclofenamide	50	Canrenone	5
Chlortalidone	25	Hydroflumethiazide	50
Clopamide	10	Polythiazide	10
Bemithizide	25	Mebutizide	100
Epitizide	10	Cyclopenthiazide	10
Trichlormethiazide	25	Althiazide	50
Furosemide	50	Spironolactone metabolite	100
Torasemide	25	Piretanide	50
Indapamide	25	Probenecid	25
Bendroflumethiazide	10	Mefruside*	-

*Internal Standard

Specificity was satisfactory as no interfering substances at the appropriate retention times were found when 10 blank urine samples were analysed. Retention times and diagnostic ions are presented in Table 2.

Table 2: Retention time, ionisation mode and diagnostic ions for beta-blockers

Substance	RT	MW	Ionization mode	MS	Diagnostic ions
Acebutolol	9.27	336	+	MS	337
Alprenolol	14.23	249	+	MS	250
Atenolol	2.69	266	+	MS	267
Betaxolol	14.75	307	+	MS	308
Bisoprolol	12.55	325	+	MS	326
Carteolol	5.63	292	+	MS	293
Carvedilol	19.30	406	+	MS	407
Celiprolol	11.32	379	+	MS	380
Esmolol	11.01	295	+	MS	296
Labetolol	12.21	328	+	MS	329
Levobunolol	10.27	291	+	MS	292
Mepindolol	8.97	262	+	MS	263
Metipranolol	13.54	309	+	MS	310
Metoprolol	9.80	267	+	MS	268
Nadolol	5.50	309	+	MS	310
Oxprenolol	11.89	265	+	MS	266
Penbutolol	22.1	291	+	MS	292
Pindolol	7.04	248	+	MS	249
Propranolol	13.87	259	+	MS	260
Sotalol	3.01	272	+	MS	273
Timolol	8.97	316	+	MS	317

4. Conclusion

The present study demostrates that 27 diuretics, probenecid and 21 beta-blockers in urine samples can be analysed in a single HPLC run, based on LC/ESI/$MS^{(n)}$ with scan to scan polarity change.

Detection limits were at least 100 ng/mL for diuretics and 500 ng/mL for beta-blockers respectively.

Nevertheless full scan mass spectrometry does not provide any structural information using protonated molecules without CID. Hence, for confirmation of suspicious samples in doping analysis[9], tandem mass spectrometry should be used in order to obtain additional structural information

Moreover, to enhance sensitivity, analysis should be performed in a single polarisation mode.

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