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A rapid screening LC-MS/MS method based on conventional HPLC pumps for the analysis of low molecular weight xenobiotics: application to doping control analysis

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This study presents a fast multi-analyte screening method specifically developed for the detection of xenobiotics in urine. The proposed method allows the screening of several classes of substance in a single chromatographic method with a run-time of 11 min, inclusive of post-run and reconditioning times. Chromatographic separation is achieved in 7.2 min using a reversed-phase 2.7 μm fused-core particle column, generating a back-pressure not exceeding 400 bar and therefore enabling the use of traditional high performance liquid chromatography (HPLC) instruments. The effectiveness of this approach was evaluated, by liquid-chromatography tandem mass spectrometry (LC-MS/MS) in positive electrospray ionization, using 20 blank urine samples spiked with 45 compounds prohibited in sport: 11 diuretics, 16 glucocorticoids, 9 stimulants, 5 anti-oestrogens, as well as formoterol, carboxy-finasteride (previously prohibited by the World Anti-Doping Agency (WADA) in 2008), gestrinone and tetrahydrogestrinone. Qualitative validation shows the proposed method to be specific with no significant interference. All of the analytes considered in this study were clearly distinguishable in urine, with limits of detection ranging from 5 ng/mL to 350 ng/mL, significantly below the Minimum Required Performance Levels (MRPL) set by WADA for the accredited sports anti-doping laboratories. All compounds of interest were separated, including synthetic and endogenous glucocorticoids with similar retention times and fragmentation patterns.

Keywords: fused-core particle columns; LC-MS/MS; anti-doping analysis; xenobiotics

Introduction

Gas chromatography-mass spectrometry (GC-MS) has long been valued for the screening and confirmation of xenobiotics and their metabolites in urine. However, with recent developments, liquid-chromatography tandem mass spectrometry (LC-MS/MS) has found increasing application in doping control analysis. LC-MS/MS allows the detection of compounds not amenable to GC-MS, often circumventing complex, time-consuming sample preparation procedures.[1–4] The advantages of simpler sample preparation, reduced time and cost of analysis, and simultaneous detection of different classes of substance have seen LC-MS/MS becoming widely accepted in most anti-doping laboratories accredited by the World Anti-Doping Agency (WADA) and also, in recent years, in toxicology laboratories. The reduction of the time and cost of analysis is becoming paramount in the development of modern, effective, and practically applicable methods of toxicological analysis, including the detection of specific compounds/classes of compound in biological matrices. In this context LC-MS/MS is now usually the tool of choice among reference laboratories. A number of screening methods based on LC-MS/MS for the detection of diuretics, glucocorticoids, stimulants, and beta-blockers have been published.[3,5–12] Several of these methods, however, still require relatively long run-times, generally not less than 15 min per sample.

Analysis time is often critical in doping analysis, limited to 24 or 48 h for negative and positive results, respectively, during major international sporting events such as the Olympic Games. Large numbers of samples to analyze within such restricted deadlines demand high-throughput, multi-component screening techniques to eliminate negative samples from additional investigation as early as possible.[13] Furthermore, shorter analysis times generally mean more cost-effective use of expensive instruments, reducing the number required.

Small particle size (less than 5 μm) stationary phases provide the most common means of increasing the speed of chromatographic separation.[14–18] Reduced particle size increases chromatographic performance, lowers and expands the range of optimal linear velocity. The resulting improved chromatographic efficiency permits greater resolution and sensitivity, or the use of higher

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flow rates to shorten analysis time without compromising the quality of a separation. The use of smaller and smaller particle sizes (less than 2 μm) and shorter columns (50–100 mm, 2.1 mm i.d.) to achieve sufficient resolution and faster chromatography suffers from an exponential increase in back-pressure, restricting their use with traditional high performance liquid chromatography (HPLC) instrumentation limited to maximum pressures of 400 bar (6000 psi). Recent advances in chromatographic hardware and column structure have made available instrumentation capable of operating at elevated pressure, enabling the use of sub-2 μm particulate columns at high flow rates, such as ultra performance liquid chromatography (UPLC®) from Waters, and Rapid Resolution Liquid Chromatography (RRLC®) from Agilent. Centred on the use of short columns packed with porous 1.7 μm particles, the UPLC® system can separate compounds under pressures as high as 1000 bar (15 000 psi). Although novel high-throughput UPLC methods[12–13] have the benefit of faster run times incorporating a wider range of doping agents, they necessitate expensive ultra high-pressure LC instrumentation (often not available in less well equipped anti-doping laboratories), coupled to a fast scanning mass spectrometer. More modern, low-cost mass spectrometers are capable of high-speed acquisitions, providing sufficient data points across the narrow peaks generated by fast LC, but conventional HPLC systems limited to 400 bar cannot be used to realize the productivity increases and analysis cost reductions of sub-2 μm particles.

The inherently high back-pressure generated by small particles may be compensated for via a compromise between temperature, column length, flow rate, mobile phase composition, or the use of monolithic columns or specifically designed materials. The high permeability of monoliths facilitates the use of high flow rates without the large back-pressures associated with particulate stationary phases, but their commercial availability is limited. Non-porous particulate materials demonstrate another alternative to sub-2 μm porous particles in achieving faster separations without the generation of such high back-pressures.[19–20] For example, Halo® columns (Advanced Materials Technology) used in this study feature 2.7 μm Fused-Core™ particles claimed to offer improvements in efficiency, resolution and sample throughput similar to sub-2 μm particles.[21–24] Comprising a thin, porous shell fused to a solid silica core, the small path for diffusion results in faster rates of mass transfer compared to totally porous particles, and hence more efficient columns. High flow rates can therefore be employed without sacrificing resolution to achieve fast separations. This is critical, as reduced analysis time must not compromise analytical performance or the ability to detect any target compound. The unambiguous identification of synthetic compounds, for example, betamethasone and dexamethasone which share the same mass spectra, is not required for a screening method since any sample in which the presence of a prohibited substance is suspected will undergo subsequent confirmation analyses. However, resolving power must be sufficient to separate target analytes from co-extracted matrix components such as endogenous compounds and synthetic substances with indistinguishable mass spectra and similar retention times.

Our aim was to develop a rapid method for the detection of a wide variety of low molecular weight xenobiotics in human urine using a conventional, low-cost LC pumping system. Although the method here presented is also applicable to other fields of analytical toxicology, including forensic investigation, its main use is for an anti-doping screening test for synthetic anabolic steroids, stimulants, glucocorticoids, anti-oestrogens and diuretics prohibited by WADA.[25] This study proposes a fast screening method based on fast LC-MS/MS for the simultaneous detection of 45 compounds (glucocorticoids, diuretics, stimulants, some anabolic steroids and anti-oestrogens; see Figure 1 for the molecular structures) included in WADA's Prohibited List which we considered to be amongst the most difficult to detect by existing methodologies. Particularly, the requirements for WADA accredited laboratories include an MRPL of 30 ng/mL for glucocorticoids, 10 ng/mL for the synthetic anabolic steroids included in this study, 50 ng/mL for anti-oestrogens, 500 ng/mL for stimulants (with an MRPL for strychnine of 200 ng/mL), and 250 ng/mL for diuretics.[26]

This paper presents the effective use of Fused-Core™ particle columns on a conventional (400 bar maximal pressure) HPLC system coupled to a triple quadrupole mass spectrometer for the rapid analysis of xenobiotics in a biological matrix.

Materials and Methods

Standards, Chemicals and Reagents

Purified standards of aminoglutethimide, beclomethasone, bendroflumethiazide, betamethasone, budesonide, bumetanide, canrenone, chlortalidone, clopamide, desonide, dexamethasone, famprofazone, flumethasone, flunisolide, fludrocortisone, indapamide, 6α-methylprednisolone, mefruside (used as internal standard for diuretics and stimulants) metolazone, pentetrazol, piretanide, prednisolone, prednisone, spironolactone, strychnine, torasemide, triamcinolone, triamcinolone acetonide, xipamide and 17α-methyltestosterone (used as internal standard for anti-oestrogenic drugs, anabolic steroids and glucocorticoids) were supplied by Sigma-Aldrich (Milan, Italy); fluocortolone, des*iso*butyryl-ciclesonide (ciclesonide metabolite) and 16α-hydroxyprednisolone (budesonide metabolite) were kindly supplied by the Belgian WADA-accredited Anti-Doping Laboratory (DoCoLab Ghent); isometheptene, tuaminoheptane, amiphenazole, raloxifene, tetrahydrogestrinone, methylphenidate, modafinil, 4-hydroxymesocarb (mesocarb metabolite) and carboxy-finasteride (finasteride metabolite) were purchased from NMI (National Measurement Institute, Pymble, Australia); anastrozole, letrozole and exemestane were purchased from Zhejiang Suntech Co Ltd (PR China); formoterol (as fumarate, 'Foradil') was purchased from Novartis Farma S.p.A. (Origgio Varese, Italy); gestrinone ('Dimetrose') was purchased from Searle (Pharmacia, Milan, Italy).

All chemicals (potassium carbonate, sodium phosphate, formic acid, *tert*-butylmethylether, methanol, acetonitrile, ethyl acetate) were of analytical or HPLC grade and provided by Carlo Erba (Milan, Italy); the enzyme β-glucuronidase (from *E. coli*), used for the enzymatic hydrolysis of glucuronide conjugates, was purchased from Roche (Monza, Italy). The distilled water used was of Milli-Q-grade (Waters, Milan, Italy).

Sample Preparation

A previously published sample preparation procedure was employed.[11] To 3 mL of urine, 1 mL of phosphate buffer (1 M, pH 7.4), 50 μL of β-glucuronidase from *E. coli* and 50 μL of the ISTD (17α-methyltestosterone, 12 μg/mL) were added and incubated for 1 h at 50 °C. After hydrolysis, 1 mL of carbonate/bicarbonate buffer (0.8 M, pH 9), to alkalinise the sample, was added and the extraction was carried out with 10 mL of *tert*-butylmethylether.

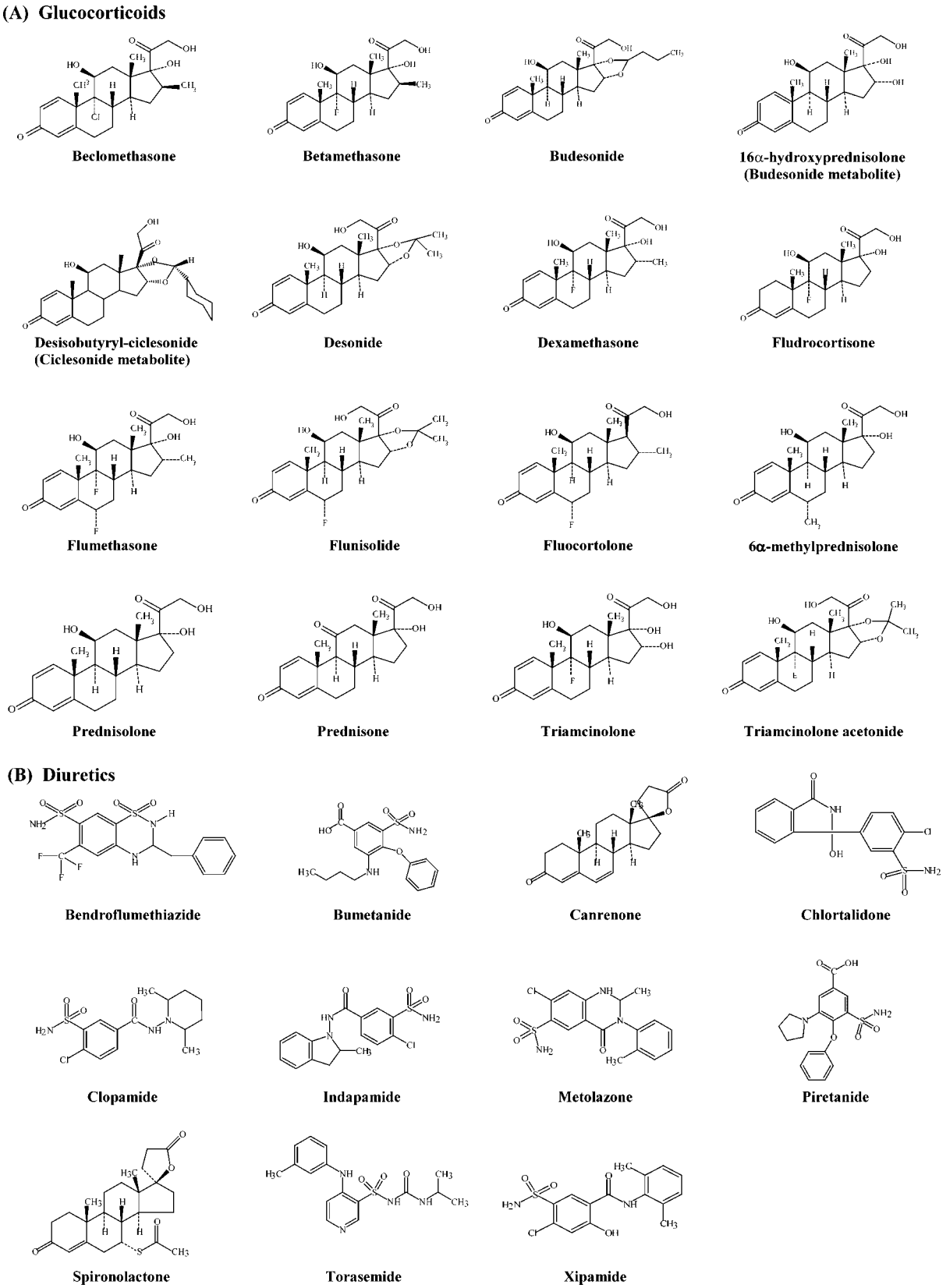

Figure 1. Chemical structures of the drugs and metabolites investigated in this study: (A: glucocorticoids; B: diuretics; C: stimulants; D: anti-oestrogens and other compounds).

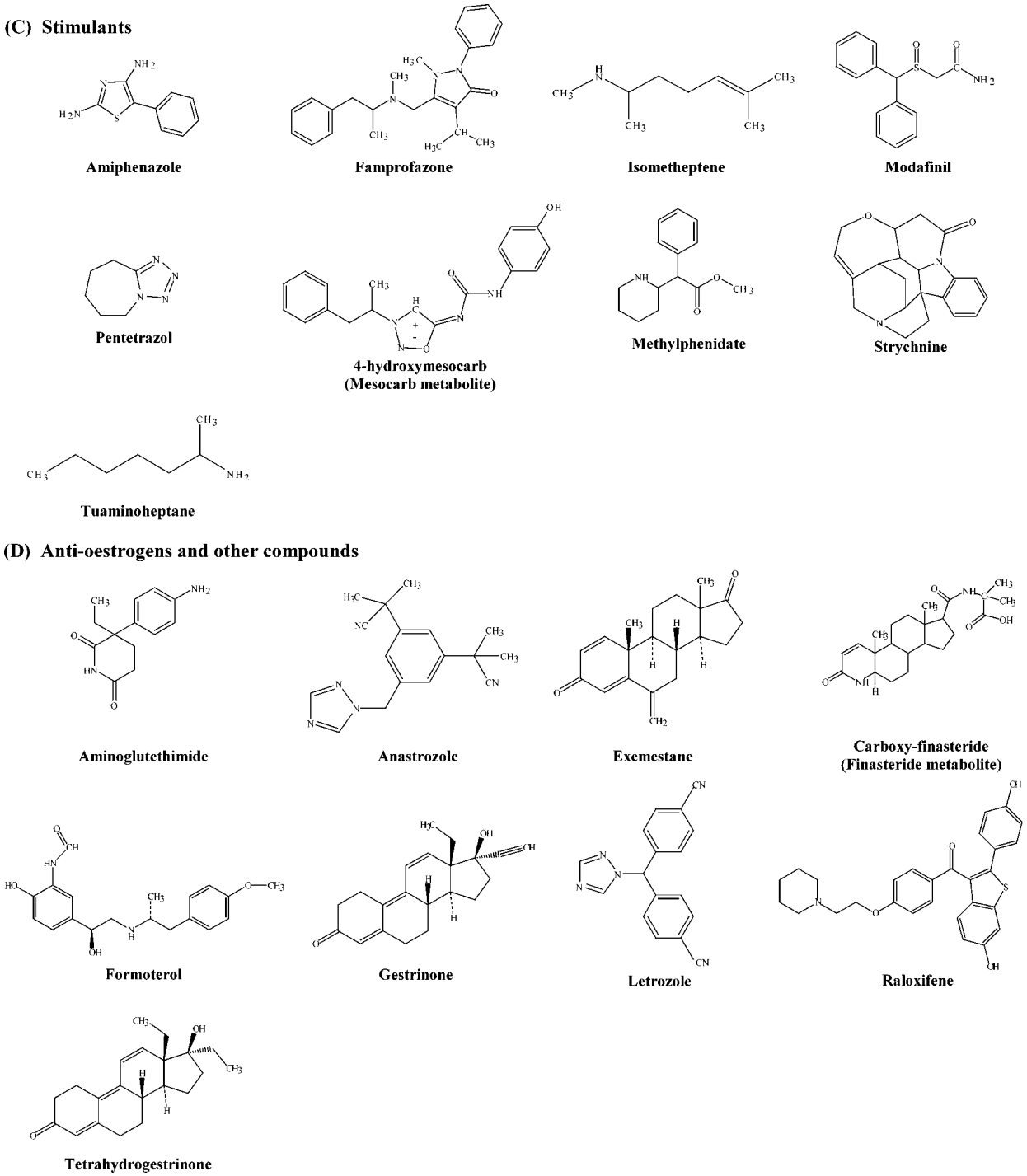

Figure 1. (*Continued*).

After centrifugation, the organic layer was transferred to a 10-mL tube and a second liquid/liquid extraction was carried out with 7 mL of ethyl acetate, after adjustment to pH 4–5 by adding 1 mL of formate buffer (2.7 M, pH 3.8), and addition of 50 µL of the second ISTD (mefruside, 12 µg/mL). After centrifugation, the organic layer was added to the first organic layer and evaporated to dryness. The residue was reconstituted in 50 µL of mobile phase and an aliquot of 10 µL was injected on the LC-MS/MS.

LC-MS/MS Conditions

All LC-MS/MS experiments were performed using an Agilent 1100 Series HPLC pump with binary gradient system and automatic injector (Agilent Technologies SpA, Cernusco sul Naviglio, Milan, Italy). Reversed-phase liquid chromatography was performed using a Supelco Discovery C18 column (150 × 2.1 mm, 5 µm) for the reference method and two different Halo® C18 columns (150 × 2.1 mm, 2.7 µm; and 100 × 2.1 mm, 2.7 µm) (Advanced Materials Technology, CPS analitica, Milan, Italy) for the

development of the rapid method here proposed. The solvents were: water containing 0.1% (v/v) formic acid (eluent A) and acetonitrile containing 0.1% (v/v) formic acid (eluent B). The reference gradient programme started at 15% B, increasing to 60% B in 7 min and then, after 6 min, to 100% B in 1 min. The column was flushed for 1 min at 100% B and finally re-equilibrated at 15% B for 4 min. The rapid gradient programme started at 10% B, increasing to 60% B in 3.5 min and then, after 1.5 min, to 100% B. The column was flushed for 2.2 min at 100% B and finally re-equilibrated at 10% B for 4 min. The flow rate was set at 250 µL/min for the reference method and at 400 µL/min for the rapid method using an oven temperature of 40 °C.

Data were acquired using an Applied Biosystems (Applied Biosystems Italia, Monza, Italy) API4000 triple-quadrupole instrument with positive electrospray ionization. The ion source was operated at 550 °C, the applied capillary voltage was 5500 V and selected reaction monitoring (SRM) experiments were performed employing collision-induced dissociation (CID) using nitrogen as collision gas at 5.8 mPa, obtained from a dedicated nitrogen generator system (Parker-Balston model 75-A74, gas purity 99.5%). The collision energies ranged from 20 to 70 eV, and the dwell time for each transition was 20 msec. Data acquisition was divided into three segments based on the expected retention times to improve the method sensitivity and to increase data point sampling across the chromatographic peaks. The acquisition segments for both methods, as well as the collision energies and the transitions used for the SRM methods, are presented in Table 1. The cycle times for the three SRM segments 1, 2 and 3 are 1.55, 0.80 and 0.885 s, respectively.

Validation Parameters

The method was validated according to WADA requirements for a qualitative screening procedure, with parameters including specificity, limits of detection (LODs), repeatability, carryover, and matrix interferences. This paper deals with chromatography and uses a previously validated sample preparation technique.[11] Each of the target analytes, at a concentration matching the specific WADA MRPL,[25] was added to 20 human urine samples from different individuals (10 male, 10 female) shown to be negative after routine doping analysis. Serial 1 : 2 dilutions were made with blank urine and the LOD was reported as the lowest concentration at which a compound could be identified in all 20 urines, with the least abundant diagnostic ion observed with a signal-to-noise (S/N) ratio greater than three. For the specificity and ion suppression validation, the same 20 blank urines were extracted as described above. Ion suppression due to matrix components was determined by comparing responses of the spiked extracts with a standard at the same concentration prepared in mobile phase.[11] Repeatability was evaluated from the between assay variation of the relative retention times (RRT) of the analytes and the relative ion abundances of the characteristic ions at the WADA MRPL.

Results and Discussion

This study presents a novel approach to reduce the time of the chromatographic run without employing expensive high-pressure pumps or sacrificing sensitivity, specificity, and resolution. Fused-Core™ columns packed with non-porous 2.7 µm particles have been used effectively with conventional HPLC instrumentation. Flow rate, temperature, and solvent gradient have been optimized to achieve a decrease in chromatographic run-time without sacrificing analytical performance. The back-pressure generated by each column, operating at a flow rate of 400 µL/min, did not exceed 400 bar (310 bar for the 150 mm Halo® column and 280 bar for the 100 mm Halo® column), enabling the use of traditional HPLC instruments. This contrasts with a typical back-pressure of 550 bar from a 100 × 2.1 mm column, or 800 bar from a 150 × 2.1 mm column, containing 1.7 µm particle packing material. Data from all the 20 spiked samples (i.e. negative urines fortified with one or more target analytes at the WADA MRPL) and several real samples (i.e. obtained from excretion studies and/or from the routine analysis) showed good signal-to-noise for all compounds.

Figure 2 compares the results obtained using a porous 5 µm particle size column (150 mm) with the same length 2.7 µm Halo® column and shorter (100 mm) Halo® column, showing the very large increase in speed (an acquisition time of 7.2 min instead of 14.0 min). Although an equilibration time of 4 min has been used, this can be reduced if one is prepared to have less consistent retention times, for example, slight retention changes can be tolerated where retention windows are not used. Also, shorter equilibration times can be achieved by using smaller dead volume pumps and system modification to reduce further the system dwell volume. The method has been shown to be capable of reliably detecting the suspected presence of the individual substances at the WADA MRPL (Figure 3). The SRM approach adopted was well within the capability of most instruments on the market.

Resolving power was evaluated by monitoring the separation of representative endogenous and synthetic target substances that show indistinguishable mass spectra and similar retention times. Although the acquisition time is almost 7 min shorter than with the reference comparative method, no loss of chromatographic performance was observed, as shown in Figure 4 where the synthetic glucocorticoid prednisolone is clearly separated from endogenous cortisone. In addition, enhanced resolution gained with the proposed method is illustrated by the separation of flunisolide and triamcinolone acetonide (Figure 3), not achievable with the comparative method.

The reliability of the method, in terms of specificity and repeatability of relative retention times (RRTs showed a CV% less than 1.2; see Table 1) and of relative abundances of characteristic ion transitions (CV% less than 10 for all target compounds), was confirmed. No significant interference was found at the expected retention time of each analyte, thus confirming the absence of false positive results. Ion suppression was not apparent with the 20 spiked urine samples but, as with all LC-MS methods, care must be taken. Carryover signal was not detected in blank urine samples that were injected in sequence after the analysis of the fortified urine samples at the highest concentration (three times the MRPL). In addition, the LODs for all target compounds were generally significantly less than the MRPL for laboratories set by WADA (Table 2). The suitability of the developed method for routine analysis was also checked by analyzing a real sample, previously found to be positive for the presence of betamethasone (Figure 5).

In summary, evaluation against the comparative LC-MS/MS method shows the following:

1. Our approach enables fast separations with pressures not exceeding the constraints of conventional instrumentation, offering an alternative to expensive, dedicated equipment in reducing analysis time.

Table 1. Acquisition segments, selective reaction monitoring (SRM) transitions and relative retention times (RRTs) of all the compounds considered in this study

	Acquisition segments (minutes)			RRT		
					Fast method	
Target Compound	Comparative method	Fast method	SRM transition (m/z)	Comparative method	Halo, 150 mm	Halo, 100 mm
Glucocorticoids						
Beclomethasone	9.5–11.0	5.2–5.8	409/337; 409/373	0.85	0.87	0.86
Betamethasone	9.5–11.0	5.2–5.8	393/337; 393/355	0.82	0.85	0.85
Budesonide	11.0–14.0	5.2–5.8	431/413; 431/341	0.98	0.98	0.98
16α-hydroxyprednisolone	0–9.5	0–5.2	377/359; 377/341	0.66	0.72	0.71
Des*iso*butyryl-ciclesonide	11.0–14.0	5.8-7.2	471/453; 471/323	1.13	1.08	1.09
Dexamethasone	9.5–11.0	5.2–5.8	393/337; 393/355	0.82	0.85	0.85
Desonide	9.5–11.0	5.2–5.8	417/399; 417/341	0.86	0.89	0.88
Fludrocortisone	0-9.5	0-5.2	381/343; 381/239	0.76	0.80	0.79
Flumethasone	9.5–11.0	5.2–5.8	411/253; 411/335	0.86	0.86	0.85
Flunisolide	9.5–11.0	5.2–5.8	435/397; 435/417	0.86	0.89	0.88
Fluocortolone	9.5–11.0	5.2–5.8	377/321; 377/303	0.89	0.91	0.87
6α-methylprednisolone	9.5–11.0	5.2–5.8	375/357; 375/339	0.81	0.85	0.83
Prednisolone	0-9.5	0-5.2	361/343; 361/325	0.75	0.79	0.78
Prednisone	0-9.5	0-5.2	359/323; 359/341	0.75	0.79	0.78
Triamcinolone	0-9.5	0-5.2	395/321; 395/357	0.66	0.72	0.71
Triamcinolone acetonide	9.5–11.0	5.2–5.8	435/397; 435/415	0.86	0.89	0.88
Diuretics						
Bendroflumethiazide	11.0–14.0	5.8-7.2	422/287; 422/271	0.95	0.95	0.92
Bumetanide	11.0–14.0	5.8-7.2	365/184; 365/240	0.99	0.97	0.97
Canrenone	11.0–14.0	5.8-7.2	341/157; 341/107	1.05	1.02	1.02
Chlortalidone	0-9.5	0-5.2	339/193; 339/135	0.66	0.70	0.68
Clopamide	0-9.5	0-5.2	346/169; 346/98	0.65	0.74	0.71
Indapamide	9.5–11.0	5.2–5.8	366/91; 366/116	0.87	0.87	0.86
Metolazone	9.5–11.0	5.2–5.8	366/259; 366/179	0.82	0.83	0.82
Piretanide	11.0–14.0	5.8-7.2	363/238; 363/196	0.96	0.95	0.95
Spironolactone	11.0–14.0	5.8-7.2	341/157; 341/107	1.05	1.02	1.02
Torasemide	0-9.5	0-5.2	349/290; 349/183	0.69	0.75	0.73
Xipamide	11.0–14.0	5.8-7.2	355/234; 355/274	0.97	0.93	0.92
Stimulants						
Amiphenazole	0-9.5	0-5.2	192/134; 192/150	0.16	0.17	0.11
Famprofazone	0-9.5	0-5.2	378/162; 378/119	0.74	0.82	0.79
Isometheptene	0-9.5	0-5.2	142/69; 142/41	0.42	0.59	0.53
Methylphenidate	0-9.5	0-5.2	234/174; 234/129	0.42	0.59	0.53
4-hydroxymesocarb	0-9.5	0-5.2	339/193; 339/135	0.74	0.82	0.79
Modafinil	0-9.5	0-5.2	274/165; 274/128	0.75	0.79	0.77
Pentetrazol	0-9.5	0-5.2	139/96; 139/69	0.29	0.40	0.28
Strychnine	0-9.5	0-5.2	335/156; 335/184	0.22	0.43	0.29
Tuaminoheptane	0-9.5	0-5.2	116/41; 116/57	0.28	0.49	0.30
Anti-oestrogens						
Aminoglutethimide	0-9.5	0-5.2	233/131; 233/203	0.20	0.31	0.19
Anastrozole	9.5–11.0	5.2–5.8	294/225; 294;210	0.86	0.87	0.86
Exemestane	11.0–14.0	5.8-7.2	297/279; 297/149	1.12	1.01	1.01
Letrozole	9.5–11.0	5.2–5.8	286/190; 286/176	0.87	0.87	0.86
Raloxifene	0-9.5	0-5.2	474/112; 474/269	0.72	0.74	0.73
Other compounds						
Gestrinone	11.0–14.0	5.8-7.2	309/291; 309/262	1.00	0.99	0.99
Tetrehydrogestrinone	11.0–14.0	5.8-7.2	313/295; 313/241	1.12	1.06	1.06
Carboxy-finasteride	0-9.5	0-5.2	403/175; 403/187	0.76	0.80	0.79
Formoterol	0-9.5	0-5.2	345/149; 345/121	0.53	0.62	0.59

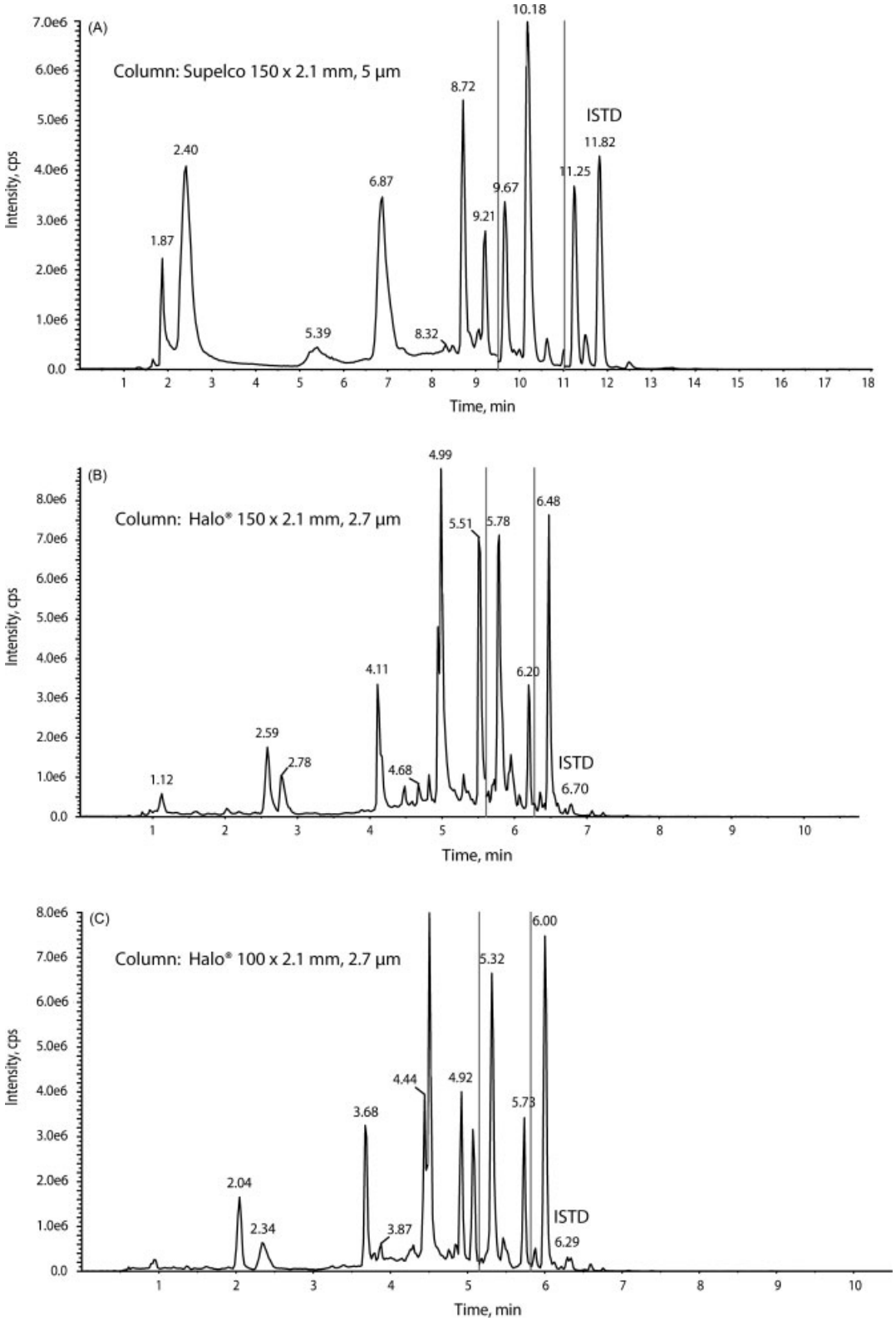

Figure 2. Chromatogram of blank urine sample spiked with all compounds studied at concentrations of 30 ng/mL for glucocorticoids, 100 ng/mL for diuretics and formoterol, 400 ng/mL for stimulants, 10 ng/mL for steroids, 50 ng/mL for anti-oestrogens and carboxy-finasteride: comparative method and column (150 × 2.1 mm, 5 µm) (A); fast method with a Halo® C18 column (150 × 2.1 mm, 2.7 µm) (B); fast method with a Halo® C18 column (100 × 2.1 mm, 2.7 µm) (C).

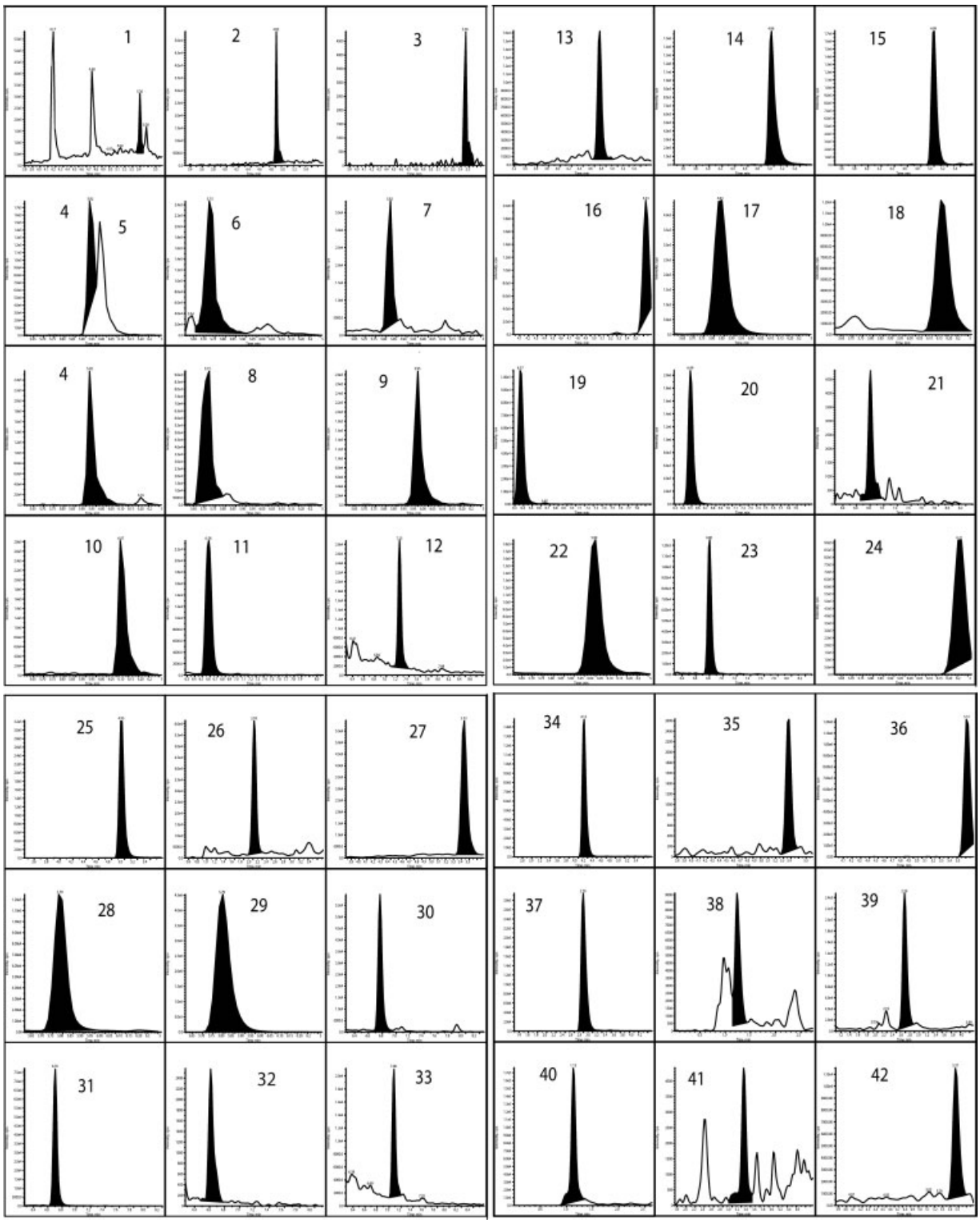

Figure 3. Extracted ion chromatogram of a blank urine sample spiked with most of the compounds studied at concentrations of 30 ng/mL for glucocorticoids, 100 ng/mL for diuretics and formoterol, 400 ng/mL for stimulants, 10 ng/mL for steroids, 50 ng/mL for anti-oestrogens and carboxy-finasteride) using the fast screening method and the Halo® C18 column (150 × 2.1 mm, 2.7 µm). Peak identities: 1. prednisolone; 2. triamcinolone; 3. fludrocortisone; 4. triamcinolone acetonide; 5. flunisolide; 6. flumethasone; 7. beclomethasone; 8. betamethasone dexamethasone; 9. desonide; 10. fluocortolone; 11. budesonide; 12 ciclesonide metabolite; 13. clorthalidone; 14. torasemide; 15. clopamide; 16. metolazone; 17. indapamide; 18. bendroflumethiazide; 19. piretanide; 20. bumetanide; 21. canrenone; 22. xipamide; 23. spironolactone; 24. diuretic internal standard (mefruside); 25. raloxifene; 26. aminogluthetimide; 27. finasteride metabolite; 28. letrozole; 29. anastrozole; 30. exemestane; 31. glucocorticoid internal standard (17α-methyltestosterone); 32. gestrinone; 33. tetrahydrogestrinone; 34. formoterol; 35. modafinil; 36. famprofazone; 37. strychnine; 38. isometheptene; 39. pentetrazol; 40. amiphenazole; 41. tuaminoheptane; 42. methylphenidate showing excellent signal to noise ratio for all compounds.

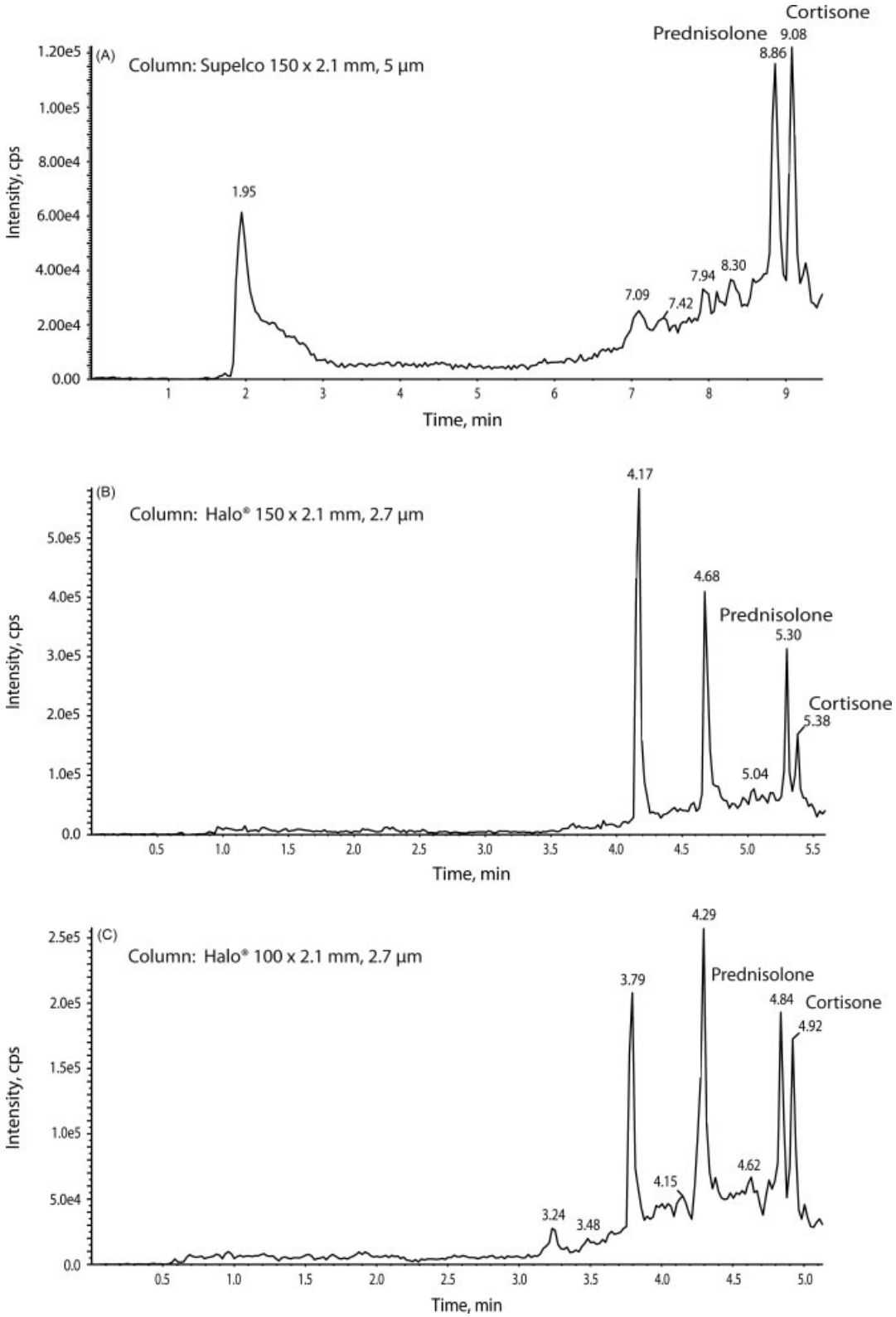

Figure 4. Extracted ion chromatograms of a spiked blank urine sample for prednisolone and cortisone at 30 ng/mL: comparative column (150 × 2.1 mm, 5 µm) (A); fast method with a Halo® C18 column (150 × 2.1 mm, 2.7 µm) (B); fast method with a Halo® C18 column (100 × 2.1 mm, 2.7 µm) (C).

Table 2. Limits of detection (LODs) for all compounds considered in this study using the fast screening method with the two Halo® C18 columns compared to the comparative method

Target Compound	LOD (ng/mL)		
	Comparative method	Fast method	
		150 mm	100 mm
Glucocorticoids			
Beclomethasone	20	15	15
Betamethasone	5	5	5
Budesonide	10	15	15
16α-hydroxyprednisolone	20	15	15
Des*iso*butyryl-ciclesonide	20	10	10
Dexamethasone	5	5	5
Desonide	5	10	10
Fludrocortisone	15	10	15
Flumethasone	10	5	5
Flunisolide	10	10	15
Fluocortolone	5	15	15
6α-methylprednisolone	20	15	15
Prednisolone	20	10	5
Prednisone	20	15	15
Triamcinolone	15	10	10
Triamcinolone acetonide	5	5	5
Diuretics			
Bendroflumethiazide	50	20	20
Bumetanide	50	20	20
Canrenone	50	55	50
Chlortalidone	100	125	150
Clopamide	50	50	50
Indapamide	50	50	50
Metolazone	50	50	50
Piretanide	50	50	50
Spironolactone	50	50	50
Torasemide	50	50	50
Xipamide	50	50	50
Stimulants			
Amiphenazole	350	250	300
Famprofazone	200	100	100
Isometheptene	300	150	150
Methylphenidate	300	150	150
4-hydroxymesocarb	400	350	350
Modafinil	300	300	300
Pentetrazol	200	100	90
Strychnine	100	70	70
Tuaminoheptane	300	155	150
Anti-oestrogens			
Aminoglutethimide	50	25	25
Anastrozole	30	25	25
Exemestane	30	15	25
Letrozole	30	15	25
Raloxifene	30	15	15
Other compounds			
Gestrinone	10	5	5
Tetrehydrogestrinone	5	5	5
Carboxy-finasteride	30	50	50
Formoterol	50	25	25

2. This qualitative method provides sufficient resolution between the endogenous glucocorticoid cortisone and the synthetic compound prednisolone.
3. For most compounds, the LODs obtained with these fast LC methods are improved compared with those of the traditional methods, due to the improved chromatography.
4. Although fewer data points per peak are obtained with our fast method, as a result of narrower peaks, these are still sufficient for an effective screening procedure.
5. No significant difference was detected between a 100 mm and a 150 mm Halo® column, in terms of retention time (slightly reduced with the 100 mm column), resolution (assessed by the separation between prednisolone and cortisone), data points per peak, and back-pressure (310 bar and 280 bar for the 100 mm and the 150 mm column, respectively).
6. Only positive ion SRM is tested in this study. Nevertheless, the number of data points and the good sensitivity and specificity suggest that it should be possible to increase the range of compounds that can be screened for by this approach, with the possibility of using both negative and positive modes in the same run, depending on the switching time of the mass spectrometer. If using a mass spectrometer with relatively slow switching time, such as was used in this study, then two runs would be needed to cover both polarities, however, this can also increase the number of analytes capable of being screened. Modern, low-cost mass spectrometers now have faster switching times, which permit combined analysis in positive and negative mode and the incorporation of a wider range of analytes without sacrificing the number of data points per peak.

In conclusion, the results of this study confirm that our approach permits a reduced analysis time without the need for expensive high-pressure pumps (which require more care in their operation because of potential leaks and dead volume) or sacrifice of sensitivity, specificity or resolution. The implementation of fast, universal screening methods of this kind is having a profound impact on doping control, offering higher throughput, quicker results and minimizing solvent consumption, reducing the time and cost of analysis. These features are extremely relevant whenever the time constraints are critical, such as during major international sport events, like the Olympic Games, when not only must the turnaround for reporting be within 24/48 h, but also the number of samples to be analyzed is increased.

Additional investigations are currently in progress to test other fused core columns, now available from several manufactures in a variety of chemistries, and to verify whether they are equally effective to the approach described herein; furthermore, the use of negative and positive modes is also being investigated. Judging from the data obtained in this study, we believe that it will be possible to use both positive and negative polarities in a single run, especially as the dwell time can be decreased with faster scanning mass spectrometers.

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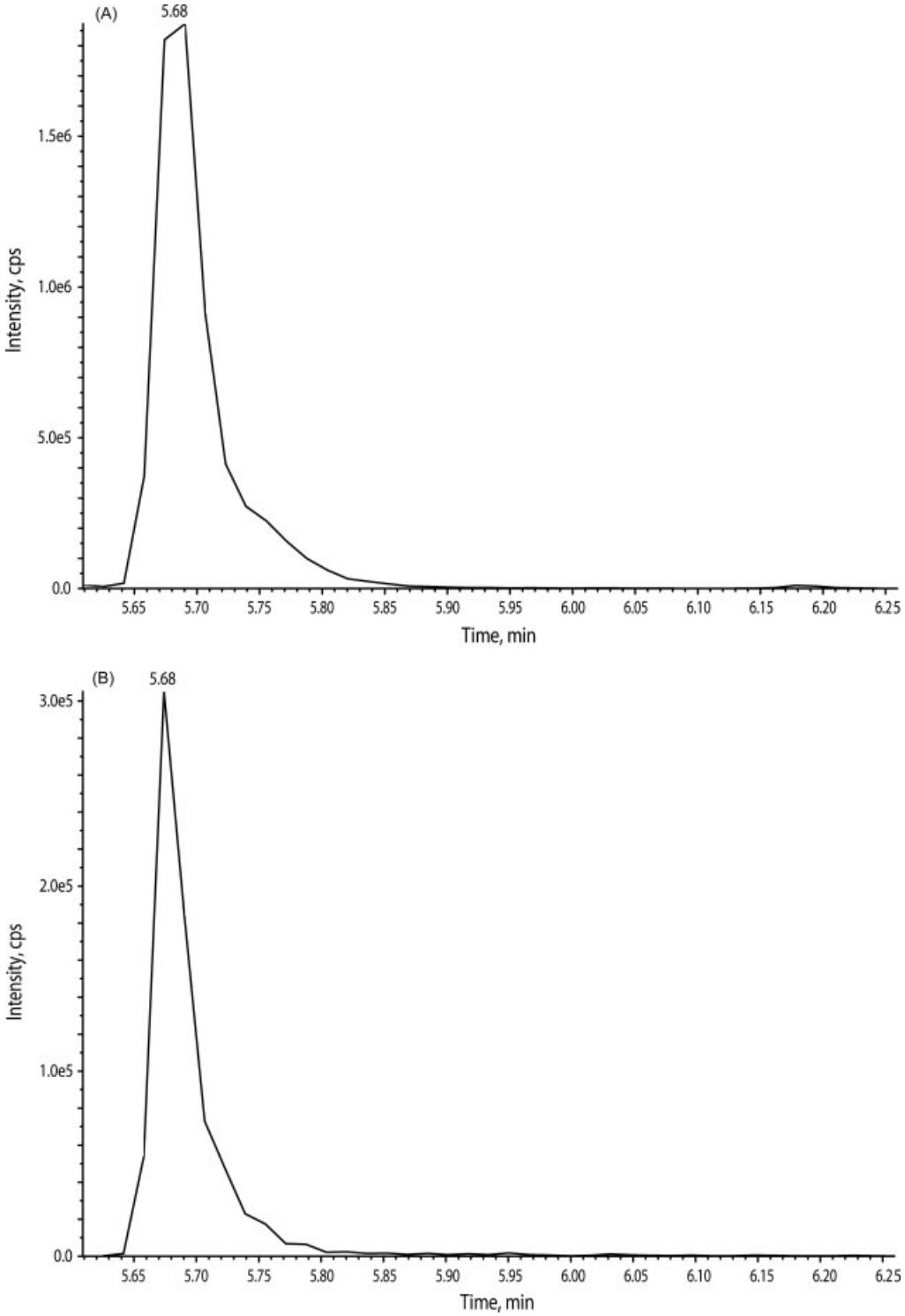

Figure 5. Extracted ion chromatogram of routine urine samples positive for betamethasone (A), and blank urine spiked with betamethasone at 30 ng/mL (B) using the fast method and Halo® C18 column (150 × 2.1 mm, 2.7 µm).

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