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Speeding up the rate of antidoping analysis: some perspectives on the use of microwave assisted extraction and fast chromatography

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INTRODUCTION

This contribution presents some proposals to improve the ratio samples/time for non peptide substances, focusing on the possibility to optimize the instrumental and human resources needed for every analytical line and, at the same time, to reduce the time and cost of the screening procedure. More specifically, we have considered both the sample pretreatment and the instrumental stage of a general anti-doping analytical procedure, focusing on the rate-determining steps of the overall process.

In the past years we have presented the possibility of using a microwave oven to speed up the derivatization process [1-4]; we have now evaluated the effect of microwave irradiation (varying time and the applied power) on the efficiency of the liquid/liquid extraction process as carried out in our laboratory for the following screening procedures: procedure IVa (drugs/metabolites excreted in non conjugated form and detected as TMS derivatives); procedure IVb (drugs/metabolites excreted as glucurono-and sulfo-conjugates and detected as TMS derivatives); procedure V (diuretics and other drugs/metabolites excreted in non conjugated form and detected as methyl-derivatives); procedure XII (glucocorticoids, gestrinone, tetrahydrogestrinone, formoterol, antiestrogens and mesocarb detected by LC/MS-MS). In the second part of the work we studied the possibility of reducing the time of the GC/MS and LC/MS-MS instrumental analysis using fast chromatography techniques: the recent introduction of narrow-bore columns, fast ovens, constant flow controller all interfaced with a fast chromatographic detector for gas-chromatography techniques [5-8] and the introduction of short HPLC column packed with 1.8 micrometers material and high-pressure LC instruments for liquid chromatography techniques offers unprecedented chromatographic resolution and speed, very useful especially when the

number of samples to analyse is very high and the time constraint critical as in the case of major International sport events.

EXPERIMENTAL SECTION

Instrumentation and reagents

All GC-MS systems, with autosampler, were from Agilent Technologies (GC 6890/MS 5973A). All LC/MS-MS experiments were performed using an Agilent 1100 Series (Agilent technologies SpA, Cernusco sul Naviglio, MI, Italy) liquid chromatograph which was interfaced by an electrospray ionisation (ESI) to an Applied Biosystems API4000 (Applera Europe B.V. Monza MI, Italy) triple quadrupole mass spectrometer.

The microwave irradiation experiments were carried out on a programmable, technical microwave oven for organic synthesis CEM MARS 5 from CEM corporation.

GC/MS and LC/MS parameters of the screening procedures

Screening IVb – steroids + beta blockers + narcotics + beta 2-agonists

Constant pressure; carrier gas He; 100% methylsilicone column (HP1), length 17 m, inner diameter 0.2 mm, film thickness 0.11 μm for traditional GC/MS; 100% methylsilicone column (Equity 1) length 5 m, inner diameter 0.10 mm, film thickness 0.10 μm for fast GC/MS. Injector T: 280 °C; split ratio 1:10 for both GC/MS techniques. Solvent delay: 3.3 min; temperature program: 180 °C (hold 4.5 min), 3 °C/min to 230 °C, 20 °C/min to 290 °C, 30 °C/min to 320 °C for traditional technique; while for fast GC/MS solvent delay: 2 min 180 °C (hold 1 min), 80 °C/min to 225 °C, 5 °C/min to 250 °C, 70 °C/min to 320 °C; for both procedures transfer line temperature: 280 °C.

Screening XII – glucocorticoids

Discovery-Supelco C18 column (2.1 × 150 mm, 5 μm) for classic LC/MS; Zorbax C18 column (2.1× 50 mm, 1.8 μm) for fast LC/MS. Chromatography was accomplished using 0.1% acetic acid (eluent A) and acetonitrile with 0.1% acetic acid (eluent B). A gradient was employed starting at 15% B, increasing to 60% B within 7 minutes and increasing to 100% B within 14 minutes for traditional method, while for fast screening method, the gradient started at 15% B, increasing to 60% B within 5 and increasing to 100% B within 7 minutes. For both procedure the column was flushed for one minute at 100% B and finally re-equilibrated at 15% B for two minutes. Different flows rates were used: 0.25 mL/min (pressure: 78 bar) for traditional method and 0.3 mL/min (pressure: 350 bar) for fast method. The ion source was

operated in the positive mode at 450 °C, and multiple reaction monitoring (MRM) experiments were performed employing collision-induced dissociation (CID) using nitrogen as collision gas at 5.8E-3 Pa (obtained from a nitrogen generator [Parker-Balston model 75-A74].

Screening V – diuretics

Constant pressure; carrier gas He; 5% phenyl methyl silicone column (HP5), lenght 18m, inner diameter 0.2 mm, film thickness 0.33 μm; injector temperature: 280 °C; split ratio 1:10; solvent delay: 2.7 min; temperature program: 140 °C (0 min), 20 °C/min to 190 °C (0 min), 30 °C/min to 260 °C (3 min), 12 °C/min to 320 °C (3.17 min); transfer line temperature: 280 °C.

Microwave oven conditions

Different powers applied (300 W, 600 W and 1200 W) and different times were studied (30 seconds, 1 and 2 minutes).

Sample preparation

Screening IVb. Screening XII

To 3 mL of urine 50 μL of internal standard (200 ng/mL of 17α-methyltestosterone), 1 mL of phosphate buffer (pH 7) and 30 μL of beta-glucuronidase from E. coli were added and hydrolysis was performed by heating for 1 h at 50 °C. After hydrolysis the buffered solution was alkalinised with 1 mL of carbonate buffer (pH 9) and the steroids were extracted with 10 mL of *tert*-butyl methyl ether on a mechanical shaker for 5 minutes. After centrifugation, the ethereal layer was transferred and evaporated to dryness under vacuum; the residue was derivatized by 50 μL of MSTFA:NH_4I:Dithioerythreitol (1000:2:4 v/w/w) and 1 μL for traditional GC or 0.2 μL for fast GC of the derivatized extract was injected directly into the injection port for screening IVb. For LC/MSMS analysis the dried extract was reconstituted in 50 μL of mobile phase and 15 μL for traditional LC or 5 μL for fast LC were injected into LC/MS-MS system for screening XII.

Screening V

To 3 mL of urine, 50 μL of internal standard (200 ng/mL of mefruside) and 300 μL of carbonate buffer pH 10, to alkalinize the sample, were added and the extraction was carried out by 6 mL of a solvents mix of cloroformio:tert-buthyl methylether:isoprapanolol/80:10:10. After centrifugation the organic layer was transferred and evaporated to dryness. Then 100 μL of formate buffer (pH 3.8) were added and a second liquid/liquid extraction was carried

out for 6 minutes by adding 5 mL of a solvent mix of cloroformio:*tert*-buthyl methylether:isoprapanolol/60:30:10. After centrifugation the organic layer was transferred together with the first organic layer and evaporated to dryness. The residue was derivatized by adding 50 mg of K_2CO_3 and 200 µL of acetone/CH_3I (9/1 v/v) and 1 µL of the derivatized extract was injected directly into the injection port for screening V.

RESULTS AND DISCUSSION

Liquid/liquid extraction

The use of microwave irradiation at the l/l extraction stage presents several advantages if compared to the traditional mechanical shaker. Figures 1-6 show that MW-assisted extraction is not simply faster (30 s - 1 min instead of 7-20 min), but, in some instances (e.g. for the beta-2-agonists, diuretics and glucocorticoids) also more effective, while in other cases (e.g. for steroids and narcotics) recoveries are comparable to those obtained by the traditional liquid/liquid extraction.

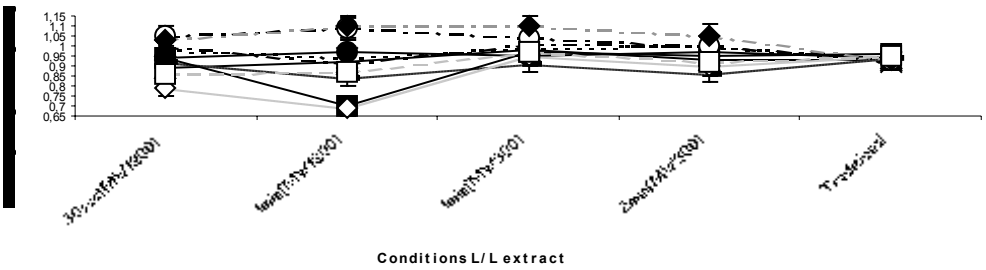

Figure 1. relative recovery data (MW vs mechanical shaker) of l/l extraction of steroids: □ 17α-ethyl-5α-estrane-3α,17β-diol; ■ 9α-fluoro-17α-methyl-androst-4-ene-3α6β11β17β-tetrol; ○ mibolerone; ● 4-cloro-3α-Hydroxyandrost-4-en-17-one; △ bolasterone; ▲ metenolone; ◊ epimetendiol; ♦ 19-norandrosterone; x 3β-androst-1-en-3α,17β-diol; - 4-cholro-6β-Hydroxymetandienone.

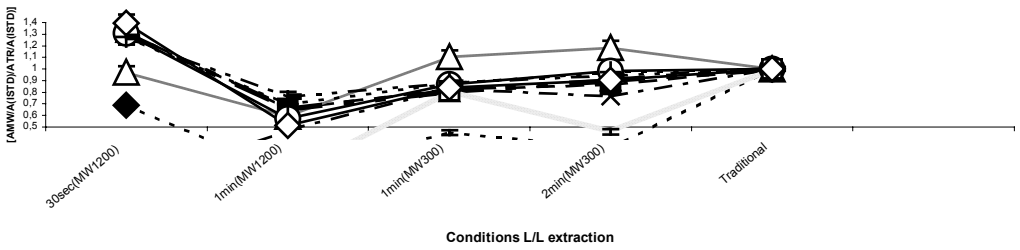

Figure 2. relative recovery data (MW vs mechanical shaker) of l/l extraction of glucocorticoids: □ beclometasone; ■ prednisolone; ○ methylprednisolone; ● triamcinolone acetonide, flunisolide; △ prednisone; ◊ flumetasone; ♦ triamcinolone; x fludrocortisone; - desonide; ▲ betamethasone, dexamethasone.

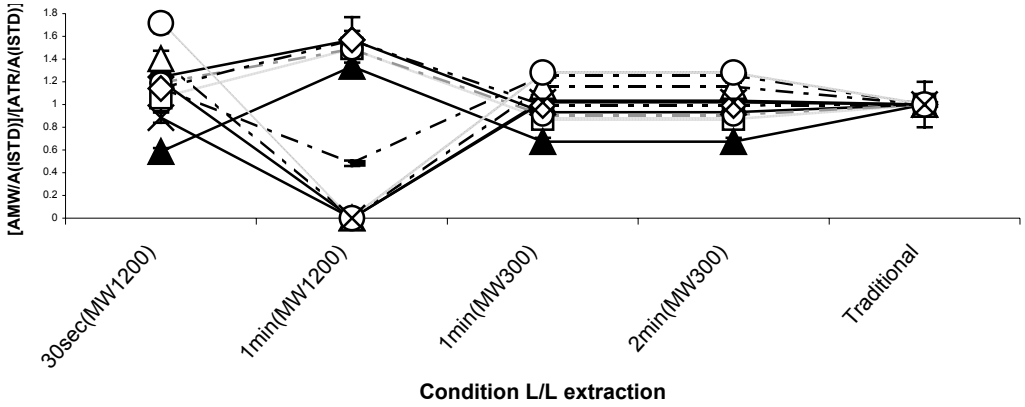

Figure 3. relative recovery data (MW vs mechanical shaker) of l/l extraction of diuretics: □ piretanide; ■ furosemide; ○ ethacrynic acid; ● bumetanide; △ acetazolamide; ◊ canrenone (spironolactone); ♦ clopamide; x diclofenamide; ▲ torasemide; - chlortalidone.

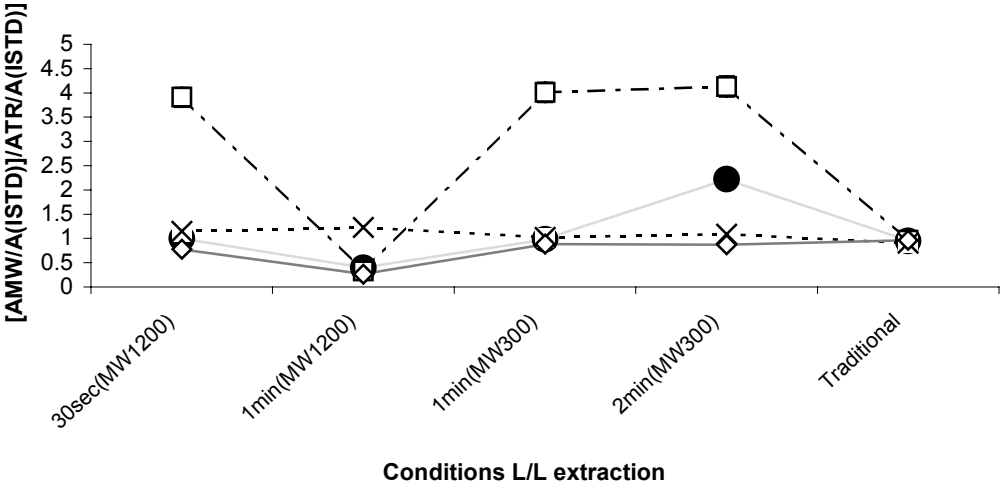

Figure 4. relative recovery data (MW vs mechanical shaker) of l/l extraction of beta2- agonists: □ salbutamol; ● terbutaline; ◊ zeranol; x clenbuterol.

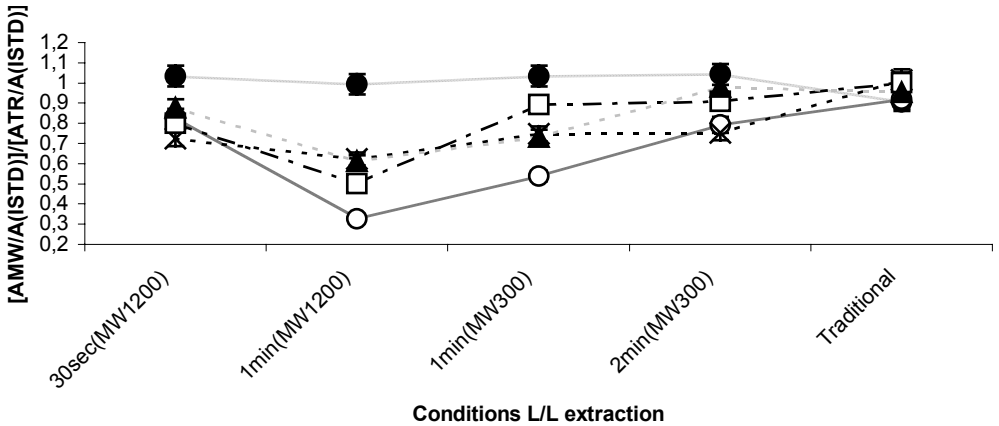

Figure 5. relative recovery data (MW vs mechanical shaker) of l/l extraction of beta-blokers: □ pindolol; ○ labetalol; ● levobunolol; ▲ propranolol; x carvedilol.

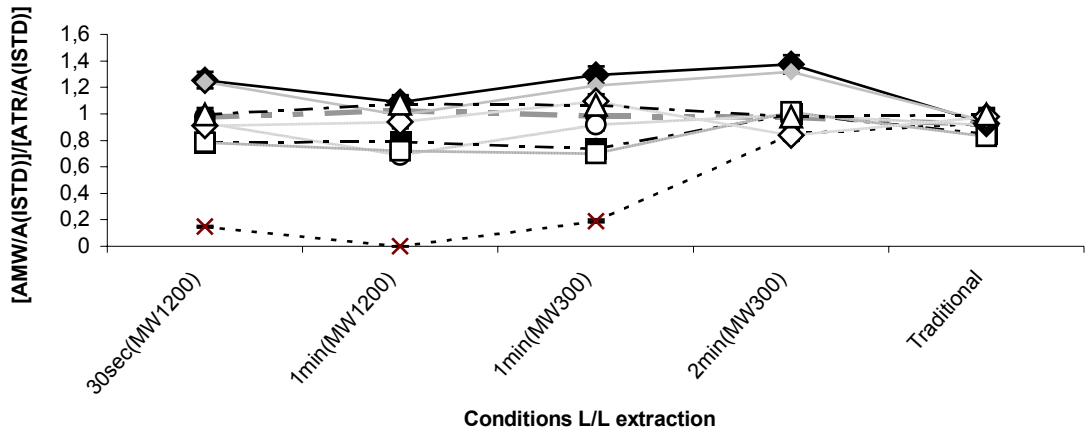

Figure 6. relative recovery data (MW vs mechanical shaker) of l/l extraction of narcotics/cannabinoids: □ oxymorphone; ■ oxycodone; ○ 9-carboxy-11-nor-Δ9-tetrahydrocannabinol; ● Hydroxybromantan; △ buprenorphine; ♦ morphine; x pemoline.

Fast techniques

The experimental data demonstrate that it is possible, using fast chromatography techniques, to carry out a rapid and simultaneous screening in human urine samples of a wide variety of drugs, some of them being also markedly different in terms of their chemical and pharmacological properties. The LODs for all target compounds are significantly lower than the minimum performance required limits (MRPL) for laboratories imposed by the WADA; in addition to this, the repeatability of both relative retention times and relative abundances are very satisfactory.

More specifically, Figures 7-8 show a fast LC/MS-MS analysis and a fast GC/MS analysis of a blank urine spiked with all compounds studied at a concentration close to the WADA MRPL. We can notice that, using a smaller size column, the chromatographic run (for both LC and GC analysis) lasts less than ten minutes, still with a good chromatographic resolution and repeatability of both relative retention times and relative abundances (see Tables 1 and 2).

In conclusion, our results indicate that the effect of microwave was very remarkable for all classes of substances: an efficient liquid/liquid extraction yield was achieved in 30 seconds-2 minutes (while the traditional extraction process gave the same yield in 5-7 minutes); furthermore, an increase in the absolute recovery value was recorded for specific compounds (diuretics, beta2-agonists and glucocorticoids). We also believe that the fast techniques are suitable for routine use in anti-doping laboratories, being particularly advantageous in those laboratories analyzing a considerable number of samples.

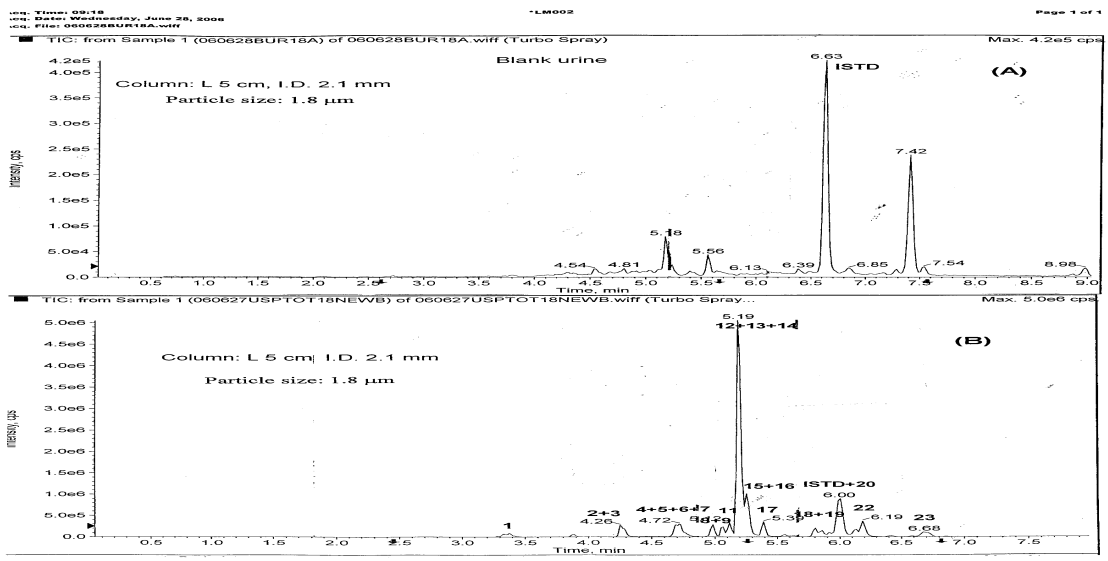

Figure 7. Chromatogram of a blank urine (A), and a blank urine sample spiked with all compounds studied (B) using fast LC. (Peaks identity: 1. formoterol, 2. 16α-Hydroxybudesonide, 3. triamcinolone, 4. modafinil, 5. prednisolone, 6. prednisone, 7. fludrocortisone, 8. methylprednisolone, 9. betamethasone/dexamethasone, 10. 3'Hydroxystanozolol 11. flumetasone, 12. beclometasone, 13. letrozole, 14. anastrozole, 15. desonide, 16. triamcinolone acetonide/flunisolide, 17. fluocortolone, 18. finasteride, 19. budesonide, 20. gestrinone, 21. mesocarb, 22. exemestane, 23. tetrahydrogestrinone).

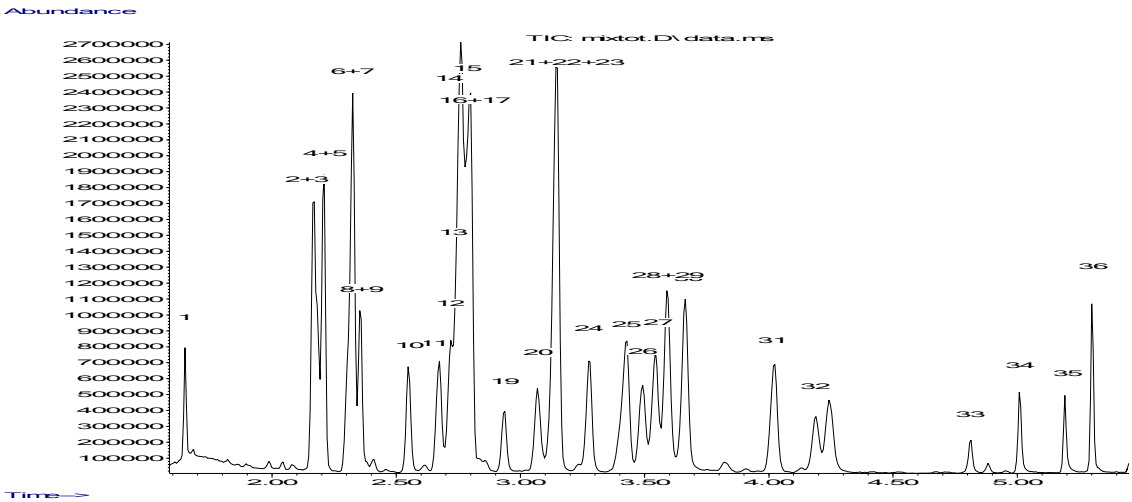

Figure 8: Chromatogram of a blank urine sample spiked with all compounds studied, using fast method. (Peaks identity: 2. 19-norandrosterone; 3. ethylestrenol; 4. 3β-androst-1-en-3α,17β-diol; 5. epimetendiol; 6. Hydromorphone; 7. 19-noretiocholanolone; 8. oxycodone; 9. morphine; 10. oxymorphone; 11. 3α-Hydroxy-1-methyl-en-5α-androstan-17-one; 12. Hydroxybromantan; 13. 9α-fluoro-17α-methyl-androst-4-ene-3α,6β,11β,17β-tetrol; 14. 1-testosterone; 15. 3α-Hydroxy-1α-methyl-5α-androstan-17-one; 16. 17α-methyl-5α-androstane-3α,17β-diol; 17. 17α-methyl-5β-androstane-3α,17β-diol; 18. epitrenbolone; 19. beta-boldenone; 20. epioxandrolone; 21. 4-chloro-3α-Hydroxyandrost-4-en-17-one; 22 17α-ethyl-5α-estrane-3α,17β-diol; 23. metenolone; 24. mibolerone; 25. norboletone metabolite 1; 26. bolasterone; 27. calusterone; 28. oxandrolone; 29. norboletone metabolite 2; 30. oxabolone; 31. 4-Hydroxytestosterone; 32. 6β-Hydroxymetandienone; 33. 4-chloro-6β-Hydroxymetandienone; 34. danazol; 35. 16β-Hydroxyfurazabol 36. 3'Hydroxystanozolol).

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For indeed, the GC/MS and LC/MS-MS runs last only 6 and 7 minutes, respectively, and the analysis cost (solvents and supplies) is lower than using traditional methods. These features are extremely significant whenever the ratio samples/instrument increases.

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Table 1. Limits of detection (LODs) and their variability, expressed as coefficient of variation % (CV %), for all target compounds considered in this study.

Target compound	Traditional LC				Fast LC			
	RRT	CV% (%)	LOD (ng/mL)	CV% (%)	RRT	CV% (%)	LOD (ng/mL)	CV% (%)
Beclometasone	0.84	0.52	20	8	0.86	0.62	20	8
Betamethasone/ Dexamethasone	0.83	0.89	1	2	0.83	0.87	1	4
Budesonide	0.98	0.25	15	6	0.98	0.33	15	5
16α-Hydroxyprednisolone	0.61	0.26	15	5	0.67	0.26	15	3
Desonide	0.86	0.90	5	4	0.88	0.95	5	4
Fludrocortisone	0.74	0.45	15	3	0.78	0.25	15	5
Flumetasone	0.74	0.22	10	3	0.86	0.25	10	3
Fluocortolone	0.89	0.21	5	4	0.90	0.45	5	2
Methylprednisolone	0.81	0.25	20	6	0.82	0.38	20	5
Prednisolone	0.73	0.33	15	5	0.77	0.45	15	5
Prednisone	0.73	0.35	15	5	0.77	0.48	15	6
Triamcinolone	0.61	0.45	15	7	0.68	0.68	15	7
Triamcinolone acetonide//flunisolide	0.86	0.46	5	4	0.88	0.67	5	4
Anastrozole	0.85	0.65	10	3	0.86	0.25	10	6
Exemestane	0.95	0.65	20	4	0.95	0.26	20	4
Formestane	0.85	0.25	30	5	0.86	0.80	30	7
Letrozole	0.85	0.24	10	5	0.86	0.75	10	3
Gestrinone	0.98	0.87	5	2	0.98	0.16	5	2
Tetrahydrogestrinone	1.12	0.80	5	2	1.11	0.25	5	2
Formoterol	0.17	0.11	100	6	0.29	0.98	100	6
Mesocarb	1.12	0.20	100	7	0.85	0.55	100	5

Table 2. Limits of detection (LODs) and their variability, expressed as coefficient of variation % (CV %), for all target compounds considered in this study.

	Traditional GC				Fast GC			
	RRT	**CV% (%)**	**LOD** (ng/mL)	**CV% (%)**	**RRT**	**CV% (%)**	**LOD** (ng/mL)	**CV% (%)**
19-norandrosterone	0.63	0.12	1	0.52	0.65	0.14	15	0.62
Ethylestrenol	0.62	0.22	10	0.89	0.65	0.10	5	0.87
3β-androst-1-en-3α,17β-diol	0.64	0.23	10	0.25	0.65	0.11	5	0.33
Epimetendiol	0.66	0.13	1	0.26	0.72	0.21	5	0.26
19-noretiocholanolone	0.69	0.31	1	0.90	0.72	0.22	5	0.95
3α-Hydroxy-1-methyl-en-5α-androstan-17-	0.83	0.11	10	0.45	0.78	0.16	10	0.45
9α-fluoro-17α-methyl-androst-4-ene-3α,6β,11β,17β-tetrol	0.85	0.11	10	0.22	0.80	0.13	5	0.25
1-testosterone	0.85	0.18	10	0.21	0.80	0.16	5	0.25
3α-Hydroxy-1α-methyl-5α-androstan-17-	0.86	0.21	10	0.25	0.80	0.12	5	0.45
17α-methyl-5(α)β-androstane-3α,17β-diol	0.86	0.34	2	0.33	0.80	0.11	10	0.38
Epitrenbolone	0.89	0.22	10	0.45	0.85	0.24	10	0.45
Beta-boldenone	0.91	0.22	10	0.46	0.85	0.22	5	0.48
Epioxandrolone	0.92	0.11	10	0.65	0.90	0.17	5	0.68
4-chloro-3α-Hydroxyandrost-4-en-17-one	0.95	0.14	10	0.65	0.90	0.14	10	0.67
17α-ethyl-5α-estrane-3α,17β-diol	0.96	0.67	10	0.25	0.92	0.11	10	0.25
Metenolone	0.96	0.22	10	0.24	0.92	0.22	5	0.26
Mibolerone	0.98	0.21	10	0.87	0.95	0.21	10	0.89
Norboletone metabolite1	1.00	0.11	10	0.80	1.00	0.55	10	0.80
Calusterone	1.01	0.23	10	0.11	1.02	0.14	5	0.16
Bolasterone	1.01	0.14	10	0.20	1.04	0.20	5	0.25
Norboletone metabolite2	1.02	0.11	10	0.65	1.05	0.15	10	0.65
Oxabolone	1.02	0.11	10	0.96	1.05	0.13	10	0.98
4-Hydroxytestosterone	1.05	0.11	10	0.55	1.18	0.12	5	0.55
6β-Hydroxymetandienone	1.06	0.24	10	0.43	1.21	0.18	10	0.45
4-cholro-6β-Hydroxymetandienone	1.09	0.16	10	0.33	1.43	0.15	10	0.30
Danazol	1.11	0.19	10	0.89	1.58	0.12	10	0.67
16β-Hydroxyfurazabol	1.13	0.54	10	0.23	1.80	0.33	10	0.55
3'Hydroxystanazolol	1.13	0.78	2	0.22	1.94	0.58	1	0.12
Hydroxybromantan	0.83	0.33	100	0.78	0.78	0.65	100	0.57
Oxycodone	0.69	0.46	100	0.45	0.73	0.44	100	0.48
Oxymorphone	0.78	0.46	100	0.34	0.75	0.55	100	0.37
Morphine	0.70	0.89	30	0.67	0.73	0.78	30	0.69
Hydromorphone	0.68	0.30	100	0.65	0.72	0.34	100	0.55

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