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The effect of the WADA list on the activity of the antidoping laboratories: reconsidering the "traditional" organization of the GC-MS screenings

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INTRODUCTION

The analytical activity of the antidoping laboratories strongly relies on GC-MS techniques, ensuring to cover, in principle, the screening of all the prohibited substances, with the exceptions of peptide hormones, of some drugs of abuse and, in some laboratories, of beta-adrenergic drugs (beta-blockers and beta agonists). The recent inclusion in the list of prohibited susbstances of new molecules, e.g. adrafinil and modafinil among the stimulants, gestrinone and tetrahydrogestrinone (THG) among the anabolic agents, and the complete class of glucocorticoids (to be searched for, like the cannabinoids, in all "in competition" samples), led the laboratories to adjust their internal procedures not to leave any vacancies among the screening methods. This is accomplished by trying both to "insert" new substances into existing methods and/or to set up newly designed, time consuming additional methods [1], that may also require the availability of additional, expensive instrumentation (e.g. LC-MS-MS) and/or of costly consumables (e.g. immunological kits and/or biochemical reagents).

In general, the new molecules recently added to the list are effectively screened by methods employing non-GC-MS techniques. The result is that the "traditional" organization of the GC-MS screening– known as screening I, II, IV (and its variations) and V – being no longer sufficient to ensure the proper detection of all the substances included in the list, is sided by a new series of LC-MS-MS and immunological screening methods.

This contribution considers the drastic reorganization of the structure of the screening methods consequent to the last upgrade of the list of prohibited substances and methods, focusing on the possibility to reorganize the internal workload of the laboratory keeping the overall number of internal procedures at a minimum.

Experimental Section

Instrumentation and reagents

All experiments were performed at the Antidoping Laboratory of Rome. All GC-MS systems, with autosampler, were from Agilent Technologies (GC 5890/MS 5973A). The microwave irradiation experiments were carried out on a programmable, technical microwave oven for organic synthesis (CEM MARS 5).

Certified reference standard were used for the preparation of methanolic stock solutions that, in turn, were used to prepare all the positive reference urine used in this work.

All reagents and solvents were of analytical grade.

GC/MS parameters of the new screening procedures

Screening IVb – steroids + beta blockers

Constant pressure; carrier gas He; 100% methylsilicone column (HP1), lenght 17m, internal diameter 0.2mm, film thickness 0.11 μm; injector T: 280°C; split ratio 1:10; solvent delay: 3.3 min; thermal program: 180°C (4.5 min), 3°C/min to 230°C (0 min), 20°C/min to 290°C (2 min), 30°C/min to 320°C (1.83 min); transfer line temperature: 280°C.

Screening IVb - glucocorticoids

Constant pressure; carrier gas He; 5% phenyl methyl silicone column (HP5), lenght 18m, internal diameter 0.2 mm, film thickness 0.33 μm; injector temperature: 280°C; split ratio 1:10; solvent delay: 12.0 min; thermal program: 180°C (6.5 min), 15°C/min to 300°C (11 min), 40°C/min to 320°C (1 min); transfer line temperature: 280°C.

Screening V

Constant pressure; carrier gas He; 5% phenyl methyl silicone column (HP5), lenght 18m, internal diameter 0.2 mm, film thickness 0.33 μm; injector temperature: 280°C; split ratio 1:10; solvent delay: 2.7 min; thermal 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.

Results and Discussion

Reorganization of the "traditional" screening I: from GC-NPD to GC-MS with SPME

Volatile nitrogen containing substances are effectively screened for by solid phase microextraction (SPME)/Gas Chromatography (see the contribution by Strano Rossi et al.,

this volume), allowing a drastic reduction of human resources. This choice imposes the use of mass spectrometry as the detection technique, since the GC-NPD chromatogram presents an unacceptably high background noise in the region of amphetamines and ephedrines. The newly setup technique also allows the detection of imidazoles and local anaesthetics (at present not forbidden by the WADA); the only drawback is that some compounds (strychnine, fenetylline, amiphenazole) are not detected and should therefore be included in other screening procedure (e.g. the new screening V, see below).

From an economical point of view, the choice of SPME, if aimed to the maximal automation of the procedure, imposes an *una tantum* extra cost (the dedicated SPME autosampler); however, for a workload not exceeding 200 samples/week, the cost of the consumables (basically the SPME fibres) is comparable (roughly 1 euro per assayed sample) to that of the traditional GC-NPD procedure (test tubes, buffers and extracting solvents, vials and caps) [2].

Elimination of the screening II

Phenolalkylamines and beta blockers, screened for in the laboratory of Rome by GC-MS with negative chemical ionization of the corresponding pentafluoropropionyl (PFP) derivatives, can be accommodated in other screening procedures, either already existing or newly upgraded; more specifically, beta blockers can relatively easily fit in the screening IVb (anabolic androgenic steroids and other substances excreted conjugated in urine and detectable by GC-MS as TMS-derivatives); while phenolalkylamines (and specifically their unconjugated fraction) can be searched for by either the newly redesigned screening I and/or the new, broader screening V (see below).

The elimination of the screening II allows the reallocation of instrumental and human resources (GC-MS spectrometers, technicians and analysts) that can be used for other screening procedures (primarily the GC-MS screening analysis of glucocorticoids).

The screening IVa: GC-MS-EI of the "steroid free fraction"

Although focused on a reduced number of target compounds (traditionally, anabolic steroids and beta-agonists detectable as TMS derivatives and excreted unconjugated in the urine), this screening is still very useful, also to double check the information possibly obtained by parallel screening procedures. Currently in progress are experiments aimed to automate the pretreatment process, and possibly to combine it with that of the screening V.

The new screening IVb: anabolics, drugs of abuse, betablockers (and glucocorticoids)

The implementation of the screening IVb considers both the pretreatment and the instrumental stages, thus making possible (a) the allocation of all beta-blockers left aside by the elimination of the screening II, and (b) the *ex novo* GC-MS screening of glucocorticoids. More specifically, while the allocation of beta blockers only requires a variation of the GC-MS conditions (see the Experimental Section for the upgraded gaschromatographic conditions and Figure 1 for the layout of the new windows), the screening of glucocorticoids requires the availability of dedicated GC-MS systems (reallocated from the old screening II) and at the same time imposes a reorganization of the pre-instrumental stage. Particularly, the extraction/hydrolysis of the glucocorticoid fraction can be combined with the traditional pretreatment procedure of the screening IVb, by using a double starting volume of urine (in our case 6 mL) that is processed as a whole up to the second extraction step, when the exract is splitted in two fractions that undergo the derivatization step independently: thermal derivatization (40 min, T = 70 °C) for the conjugated anabolic steroid fraction and thermal+microwave derivatization (40 min at 900 W and 90 min at T = 70 °C) for the glucocorticoid fraction [3-4].

The new screening V: not only diuretics

The upgrade of the screening I from GC-NPD to SPME/GC-MS, the elimination of the screening II and the basic goal to reduce the overall number of independent screening procedures, imposed a drastic reorganization of the screening V (GC-MS of methyl derivatives, no hydrolysis in the pretreatment process); the final objective was to make possible the detection of the maximum number of forbidden substances/metabolites not searched for by other screening procedures. This goal led us to reconsider the sample pre-treatment process of the screening V, and especially the extraction step, that was upgraded and structured in two subsequent stages (the former at pH 10 and the latter at pH 4) in order to embrace the widest possible range of compounds; while the extraction solvent, constituted by a mixture of chloroform:isopropanol:t-butyl methyl ether (TBME) 80:10:10, was selected after a series of dedicated experiments as the best compromise for the range of detectable analytes (data not shown). At the derivatization (methylation) stage, the use of microwave irradiation, at an emitted power of 1200 W, allowed more than satisfying derivatization yields also for apparently poorly reactive residues in very short times (≤ 10 min). The differences between the old and the new procedure are outlined in Table 1.

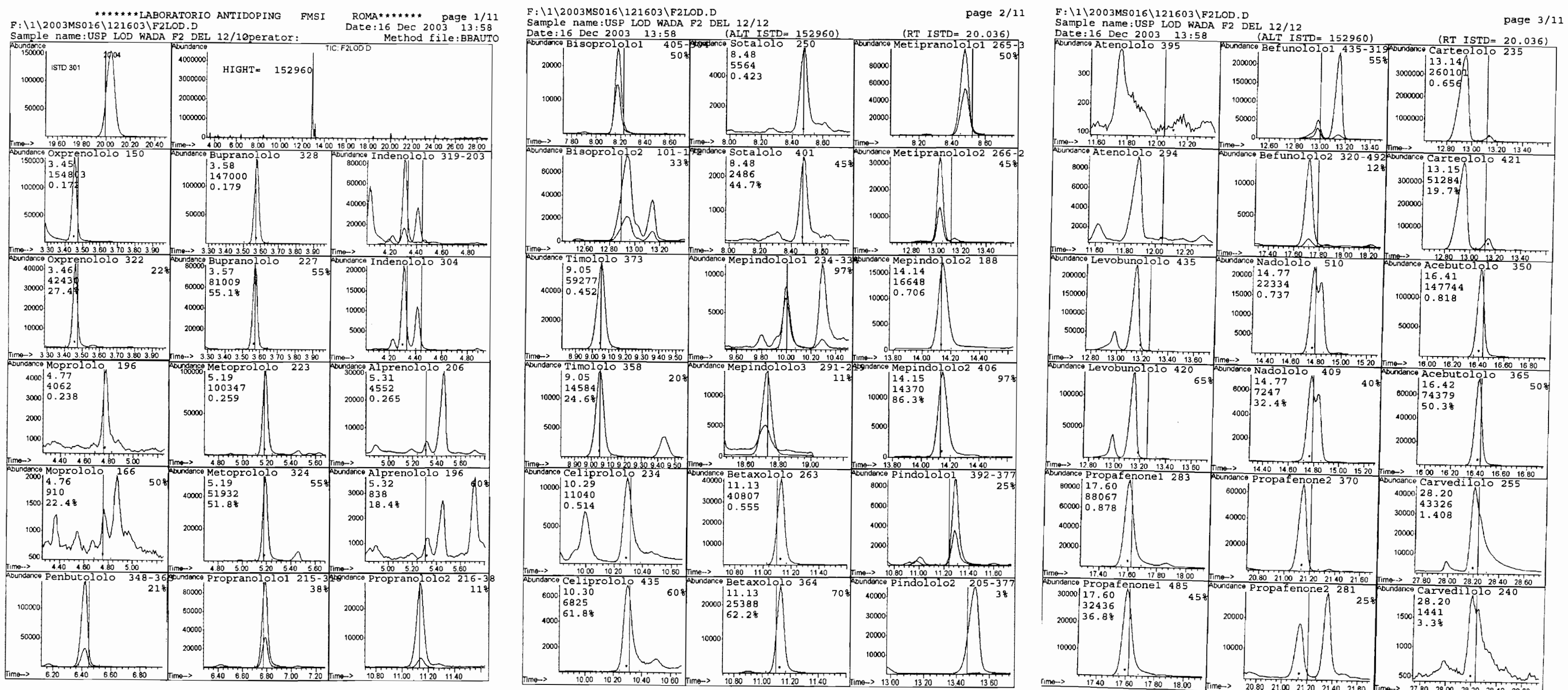
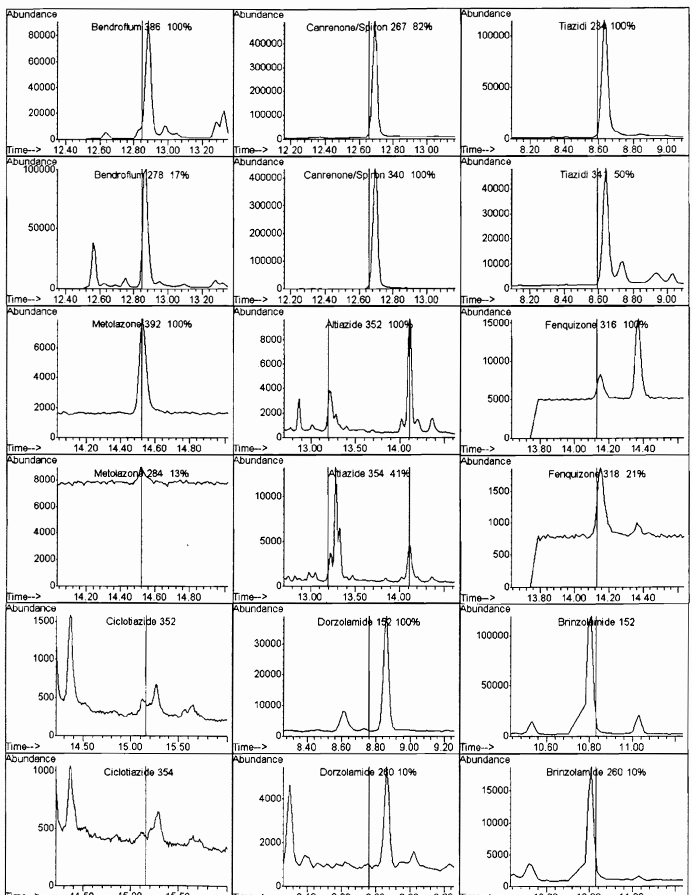


Figure 1. Representative pages of the new layout of the modified screening IVb, expanded to host the beta blockers and other compounds left out by the reorganization of other screening procedures. The above windows refer to a positive reference urine, i.e. a negative urine spiked with the compounds of interest at the WADA minimum required performance limit (MRPL).



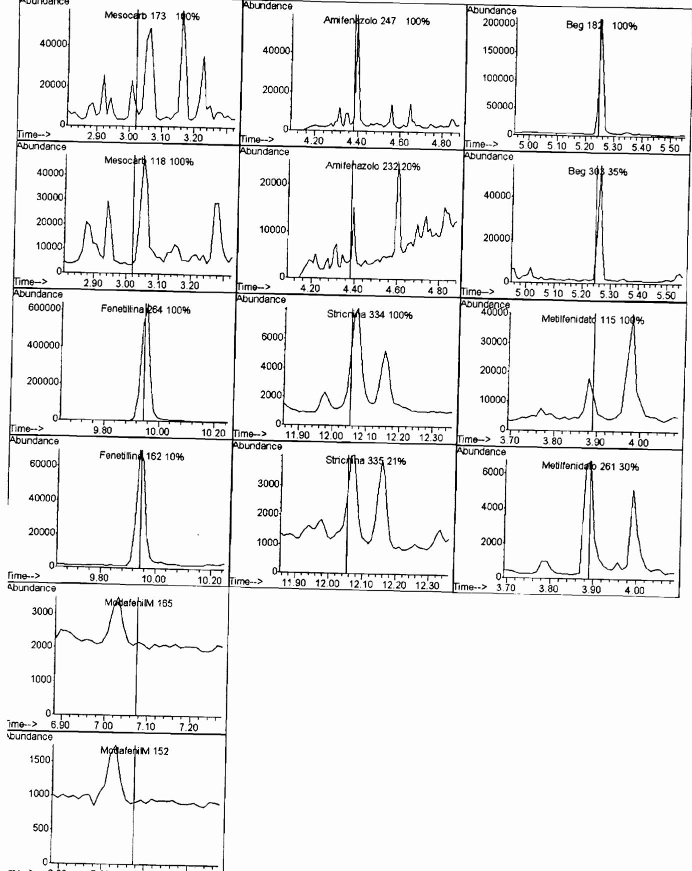


Figure 2. Layout of the last two pages of the modified screening V: the novel, topical carbonic anhydrase inhibitors brinzolamide and dorzolamide (left page), as well as several other "new entries" of the list and "new left out" by other screenings (mesocarb, phenetylline, amiphenazole, strychnine, methylphenidate, modafinil, and benzoyl ecgonine, all in the right page), are detectable by the new procedure. All the above windows refer to a spiked urine at the WADA MRPL.

The new pretreatment process allowed the detection of a broad range of different analytes, varying from the traditional diuretics to potentially related substances (the novel, topical carbonic anhydrase inhibitors brinzolamide and dorzolamide, [5]) to several other "new entries" of the list and "new left out" by other screenings (mesocarb, phenetylline, amiphenazole, strychnine, methylphenidate, modafinil, and benzoyl ecgonine) The LOD, RRT and diagnostic ions of the new compounds screened for by this procedure are summarized in Table 2.

Basically, apart from gestrinone and tetrahydrogestrinone (THG), all the outsiders left out by other old screenings are detected, with a LOD generally much lower than the WADA minimum required performance limit (MRPL); in some instances, the detection is also simpler (e.g. in the case of ritalinic acid, the main metabolite of methylphenidate, that, according to the suggested procedure, is successfully methylated back to its parent drug); finally, the double liquid/liquid extraction step, originally an apparent obstacle to upgrade the procedure, does not represent a major problem, but instead can be easily automated and reduces the cost for consumable (C18 cartridges). The most drastic cost reduction, however, comes from the elimination of immunological kits for cocaine/metabolites, given that benzoyl ecgonine (BEG) is now detectable (as methyl derivative) by GC-MS.

Table 1

Comparison among the old (left) and the new (right) urine pretreatment processes of the procedure V (detection of methyl-derivatives by GC-MS-EI)

	old [6]	common	new
Urine volume		5 mL	
Internal standard(s)		Indomethacine/mefruside	
1st Extraction	Sep-pak C18 cartridge		pH 10 (800 µL carbonate buffer 4M) extraction with 6 ml CHCl3:isopropanol:TBME (80:10:10)
Evaporation	-----		N_2 stream, 40 °C
2nd Extraction			pH 4 (100 µL formate buffer 5M) extraction with 6 ml CHCl3:isopropanol:TBME (80:10:10)
Evaporation		N_2 stream, 40 °C	
Derivatization		Acetone/CH_3I= 1/9, 200 µl Anhydrous K_2CO_3, 50 mg	
	MW 900W 10 min		MW 1200W 10 min
Injection volume		1 µL (split 1:10)	
Analysis		GC-MS EI SIM	

Table 2

Relative retention times, limits of detection and diagnostic ions of the new compounds screened for by the upgraded procedure V (detection of methyl-derivatives by GC-MS-EI)

Compound	RRT*	LOD (ng/mL)	Diagnostic ions (m/z) - screening	Other ions (m/z)
Dorzolamide	0.88	250	152, 260	324
Brinzolamide	1.08	250	152, 260	324
Mesocarb	0.30	150	118, 173	91, 65
Fenetylline	0.99	130	264, 162	119
Benzoylecgonine	0.53	400	182, 303	272
Modafinil	0.71	500**	165, 152	167
Strychnine	1.21	200	334, 335	319
Amiphenazole	0.44	300	247, 232	176
Methylphenidate	0.39	300	115, 261	84

*: Referred to Indomethacine, RT=10.0 min; **: estimated (certified standard missing)

CONCLUSIONS

The new organization of the screening procedures proposed in this work (the complete picture of the new series of screening procedures, compared to the previous one, is given in Table 3) presents some substantial advantages, especially if compared to the simple "upgrade" of the previous series of methods: firstly, despite the upgrade of the list, the overall number of GC-based screening procedures has been reduced from 5 to 4; among them, the recourse to SPME/GC-MS for the procedure I allows a further reduction of human resources at the pre-treatment stage. In addition to this, the upgrade of the old screening V allows to cover most of the outsiders left out by the new screening I and by the missing screening II. Finally, the upgrade from C18 cartridges to liquid/liquid extraction led to a substantial reduction of the costs for consumables, that, if considered in combination also to the reduced needs for costly immunological kits (cocaine and glucocorticoids are not assayed by immunoanalysis), make the new organization of the screening procedures extremely cost effective.

The proposed approach allowed to effectively optimize the internal workload of our laboratory: the screening of roughly 99% of the forbidden drugs can indeed be ensured by few

GC-MS based procedures, always satisfying the MPRL imposed by the WADA; immunoanalysis is used only for the screening of peptide hormones; while LC-MS-MS is selectively used for the screening for gestrinone and THG and for confirmation analyses of specific compounds.

The use of microwave (MW) irradiation at the derivatization stage presents several advantages if compared to the traditional thermal incubation: the MW-assisted derivatization procedures are indeed not simply faster, but, in some instances (e.g. for the sylylation of glucocorticoids [4]) also more effective as far as the overall yield is considered, allowing the derivatization of poorly reactive residues, possibly as a consequence of non-thermal effects [7-8] that may be responsible for the high efficacy of the technique; while in other cases (e.g. for the methylation of diuretics) the overall yields are comparable to those obtained by the traditional, thermal derivatizazion (R. Borrelli, C. Colamonici, F. Botrè, manuscript in preparation).

Table 3

Comparison among the traditional (above) and the upgraded (below) organization of the screening procedures in the antidoping laboratory of Rome (major modification in italics).

Traditional

- GC-NPD (Screening I: Volatile nitrogens-containing compounds)
- GC-MS-NCI (Screening II: Phenolakylamines & Beta Blockers, PFP derivatives)
- GC-MS-EI (Screening IVa: TMS-derivatives, free fraction)
- GC-MS-EI (Screening IVb: TMS-derivatives, total fraction)
- GC-HRMS-EI (High sensitivity: 5 anabolics, TMS derivatives)
- GC-MS-EI (Screening V: Diuretics & porbenecid, methyl derivatives)
- Immunoassays: cocaine, peptide hormones
- LC-MS-MS for glucocorticoids
- LC-MS-MS for gestrinone/THG
- EPO by isoelectric focusing/double blotting (with GC-MS for HES)

Upgraded

- *GC-MS with SPME (stimulants, local anaesthetics, imidazoles)*
- GC-MS-EI (Screening IVa: TMS-derivatives, free fraction)
- *GC-MS-EI (Screening IVb: TMS-derivatives, total fraction: double aliquot, splitted at the end, one half for the "anabolics" - β-2-agonists – and all beta blockers, THC, morphine; and one half for the glucocorticoids)*
- GC-HRMS-EI (High sensitivity: 5 anabolics, TMS derivatives)
- *GC-MS-EI (Screening V: methyl derivatives, double extraction for the "diuretics", now including also those compounds previously in screenings I-II, like amiphenazole, methylphenidate, fenetylline, and, among others, modafinil/adrafinil, mesocarb, and benzoylecgonine*

- *Immunoassays: only for peptide hormones*
- LC-MS-MS: only for gestrinone/THG
- EPO by isoelectric focusing/double blotting (with GC-MS for HES)

In conclusion, if the new substances are too many to be considered simply as "adds on", requiring the set up of additional, non-dedicated methods, a radical re-thinking of both the lot of the doping substances as a whole and of the series of screening procedures is mandatory. This approach is the most effective to set up the most appropriate combination of the screening methods, maximizing the benefits/costs ratio. A thoughtful planning of the experimental activity, still the key component of a correct and optimized management of an antidoping laboratory, requires an extensive evaluation of any potentially useful new method: in this way the need for costly instrumentation (especially LC-MS-MS) may be drastically reduced. Nonetheless, there might be in the near future the need to consider other techniques (i.e. capillary electrophoresis, surface plasmon resonance or electrochemical methods) for the screening analysis of non traditional doping substances and methods. In this sense, also given the fact that any potentially equivalent experimental choice has to be compliant with guidelines/specifications (mainly the compliance with the WADA MRPLs and with the ISO 17025 accreditation), additional time is almost as valuable as additional financial support.

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