In: W Schänzer, H Geyer, A Gotzmann, U Mareck (eds.) Recent Advances In Doping Analysis (14). Sport und Buch Strauß - Köln 2006

Monica Mazzarino, Maria Cristina Braganò, Francesco Botrè

An LC/MS-MS screening method for synthetic glucocorticoids based on pharmacological structure-activity relationships

Laboratorio Antidoping, Federazione Medico-Sportiva Italiana, Roma, Italy

INTRODUCTION

The last few years have shown a remarkable evolution of the approach utilized at an international level to control the abuse of drugs in sport. A series of preventive actions have been put in place by the World Anti-Doping Agency (WADA) to deter the illicit use of performance-enhancing agents. These actions are complementary to the traditional activity of the anti-doping laboratories, which remains a key component of any effective anti-doping policy. At the same time, there has been recent discovery of "designer steroids" [1-2] and, more generally, other previously unknown substances endowed with pharmacologically properties, specifically designed and synthesized with the aim of improving sport performance without the risk of being caught through anti-doping control. This has resulted in a more or less pronounced revision of analytical screening protocols to allow also the detection of those novel "designer drugs". These compounds were not originally included in the WADA prohibited list, the international reference document detailing all the substances and methods forbidden for use by athletes [3].

This situation has led to the development of alternative analytical methods capable of detecting the presence of one or more drugs in human urine belonging to the same class of congeners, based on the recognition of common portions of the molecular structure. These tasks can be achieved primarily by biotests, i.e. effect-based assays capable of detecting the effects of, rather then the exposure to, forbidden substances [4], and/or by advanced chromatographic-spectrometric techniques, focused on the recognition of specific molecular portions which are common to the same pharmacological class of compounds [5-11].

This approach has already been successfully applied in the screening of different classes of pharmacologically active compounds, from beta-blockers [5] to metabolically

stable aryl-propianamide-derived selective androgen receptor modulators [11]. In principle, methods based on the study of structure-activity relationships of all the different classes of prohibited drugs can contribute to a tremendous reduction in time necessary to identify new doping substances and to include them in the anti-doping lists.

There are many approaches followed in synthesizing a new molecule, but usually the one most applied in drug development is the classical method, that of modifying the design of analogues of an active lead compound by selective structural modification (adding or changing the position of some functional groups may bring about changes in specificity and/or potency, alteration in absorption, protein binding, rate of metabolic transformation, rate of excretion, or membrane permeability). In general, as far as pharmacodynamic properties are concerned, the synthesis of a therapeutically successful drug takes into account: (a) receptor site (structure on the receptor), (b) pharmacophoric pattern (geometric arrangement), (c) pharmacophore (groups responsible for the effect) and (d) the correlation between biological activity and physicochemical characteristics. From an analytical point of view this means that since the pharmacological activity is a result of stereo-electronic interaction with a receptor, the recognition of groups responsible for the effect is more important than monitoring the residual structure of the entire drug molecule [12-13].

The goal of this work was to develop a general screening method for the detection of synthetic glucocorticoids and/or their metabolites in human urine. The method is based on LC/MS-MS using ESI in different acquisition modes, and is focused on the recognition of the common portion of the molecular structure of synthetic glucocorticoids (see Figure 1).

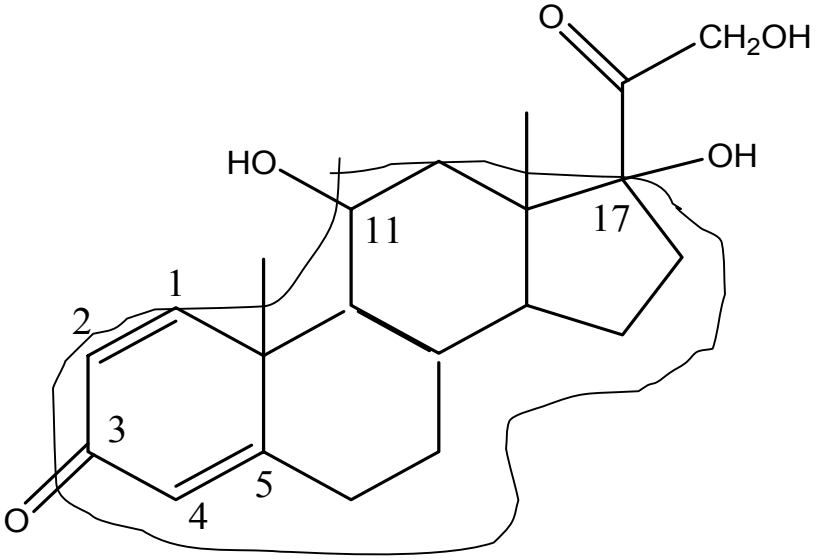


Figure 1: Basic molecular structure of glucocorticosteroids: selected fragment

MATERIAL AND METHOD

All LC/MS-MS experiments were performed using an Agilent 1100 Series liquid chromatograph which was interfaced by an ESI to an Applied Biosystems API4000 triple quadrupole mass spectrometer. The column was a Supelco Discovery C18 column (2.1 × 150 mm). Chromatography was accomplished using 0.1% acetic acid (eluent A) and acetonitrile with 0.1% acetic acid (eluent B). The gradient used began at 15% B, increasing to 60% B within 7 minutes and then increasing to 100% B within 14 minutes. The column was flushed for one minute at 100% B and finally re-equilibrated at 15% B for four minutes. The flow rate was set at 0.25mL/min.

The ion source was operated in the positive mode at 450 °C. Multiple reaction monitoring (MRM), precursor ion scan and neutral loss experiments were performed with collision-induced dissociation (CID) using nitrogen as collision gas at 5.8E-3 Pa, obtained from a dedicated nitrogen generator system (Parker-Balston model 75-A74). The collision energy was set at 30eV for all experiments, while the transitions used for the MRM method and the ions and mass ranges used for the precursor ion scan and neutral loss are reported in Table 1.

Table 1 Collision energy MRM/precursor ion scan/neutral loss; MRM transition, precursor ion scan and neutral loss ion selected.

Target compounds	Collision energy (eV)		MRM Transition *(m/z)*	Precursor ion scan *(m/z)*
	MRM	Precursor ion scan		
Beclometasone	20	30	409/373; 409/337	237
Betamethasone/Dexamethason	20	30	393/355; 393/337	237
Budesonide	20	30	431/413; 431/341	237
16α-Hydroxyprednisolone	20	30	377/359; 377/341	237
Desonide	20	30	417/399; 417/341	237
Fludrocortisone	38	30	381/343; 381/239	237
Flumetasone	20	30	411/335; 411/253	237
Fluocortolone	20	30	377/321; 377/303	237
Flunisolide	20	30	435/417; 435/397	237
Methylprednisolone	20	30	375/357; 375/339	237
Prednisolone	20	30	361/343; 361/325	237
Prednisone	20	30	359/34; 359/323	237
Triamcinolone	20	30	395/357; 395/321	237
Triamcinolone acetonide	20	30	435/415; 435/397	237

Sample preparation

To 3 mL of urine, 1 mL of phosphate buffer (K_3PO_4/KH_2PO_4 (1M), pH 7.4), 50 µL of β-glucuronidase and 50 µL internal standard (100 ng/mL of deflazacort) were added and incubated for one hour at 50 ºC. After hydrolysis 1 mL of carbonate buffer ($K_2CO_3/KHCO_3$ (1:1), pH 9) was added to alkalinize the hydrolyzed solution. Extraction was carried out using 10 mL of *tert*-butyl methyl ether for 6 minutes; after centrifugation the organic layer was transferred and evaporated to dryness. The dried extract was reconstituted in 50 µL of mobile phase and 15 µL were injected.

RESULTS AND DISCUSSION

The experimental data shown here demonstrate that it is possible to carry out a rapid and specific screening for glucocorticoids in human urine using precursor ion scans as the acquisition mode. Specifically, as shown in Table 2, the LOD's for all target compounds are lower than the WADA minimum required performance levels (MRPL). Similarly, due to the good selectivity of the method for the substances examined, it is unlikely that the number of false positives would be higher than those obtained using the traditional MRM method.

Table 2 Limits of detection (LOD's) and their variability, expressed as coefficient of variation % (CV %), for all target compounds in this study, evaluated using the two different LC/MS-MS methods.

	MRM		**Precursor ion scan**	
Target compounds	**LOD (ng/mL**	**CV%**	**LOD (ng/mL)**	**CV%**
Beclometasone	10	8	10	6
Betamethasone	1	2	1	2
Budesonide	15	6	20	7
16α-Hydroxyprednisolone	10	5	15	5
Desonide	5	4	3	4
Dexamethasone	1	3	1	3
Fludrocortisone	15	3	30	8
Flumetasone	5	3	5	3
Fluocortolone	5	4	5	4
Flunisolide	3	5	3	6
Methylprednisolone	15	6	15	6
Prednisolone	15	5	10	5
Prednisone	15	5	10	5
Triamcinolone	15	7	10	5
Triamcinolone acetonide	3	4	3	4

Figures 2 and 3 show respectively the chromatograms of a blank urine and a blank urine spiked with all the glucocorticoids on the WADA list at a concentration of 30 ng/mL. The acquisition modes were as follows: Figure 2—MRM and Figure 3—precursor ion scan. For both the MRM and precursor ion scan analyses all glucocorticoids are detected at the MRPL.

Figure 4 shows the precursor ion scan screening analysis, performed under the same conditions as in Figure 3, of an athlete sample found to be positive for betamethasone at a concentration near the MPRL (35 ng/mL). Additional confirmation of the validity of this method is seen in Figure 5. It shows the precursor ion scan screening analysis of a blank urine (A) and a deflazacort excretion urine containing the metabolite 21-desacetyl-deflazacort (B).

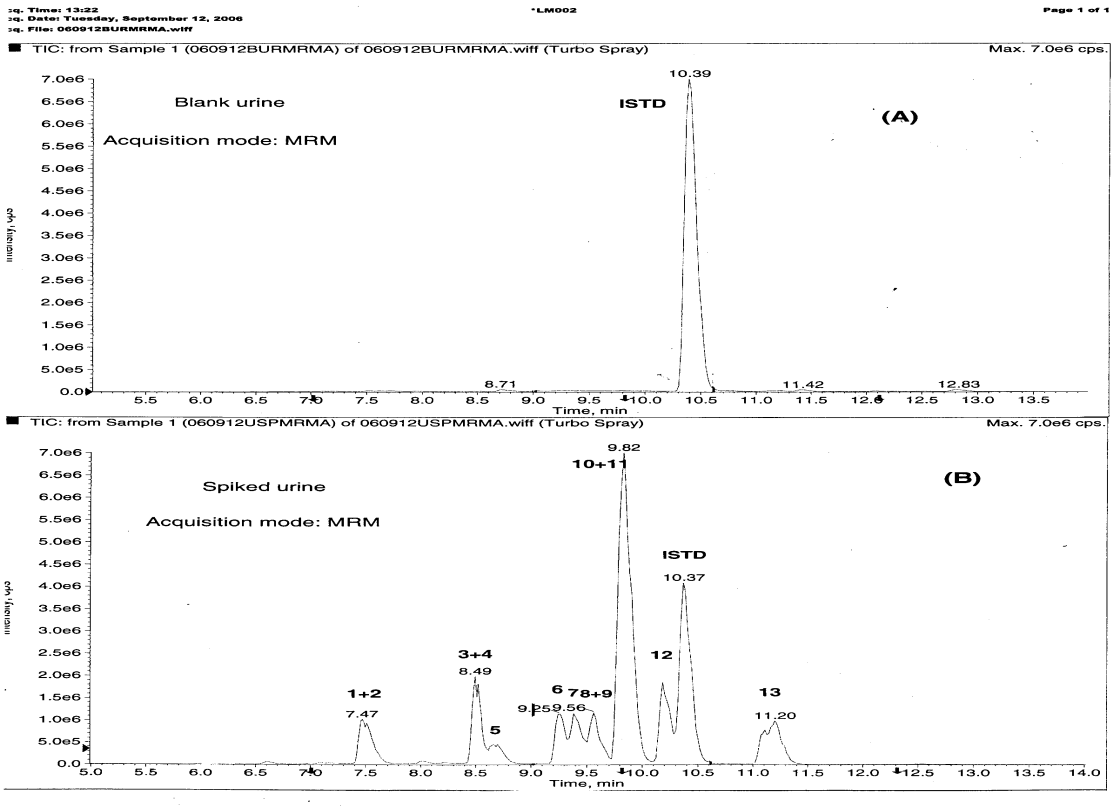


Figure 2: Chromatograms of a blank urine (A) and of a spiked urine (B) using MRM. Peak identity: 1. 16α-Hydroxyprednisolone, 2. triamcinolone, 3. prednisolone, 4. prednisone, 5. fludrocortisone, 6. methylprednisolone, 7. betamethasone/dexamethasone, 8. flumetasone, 9. beclometasone, 10. desonide, 11. triamcinolone acetonide/flunisolide, 12. fluocortolone, 13. budesonide.

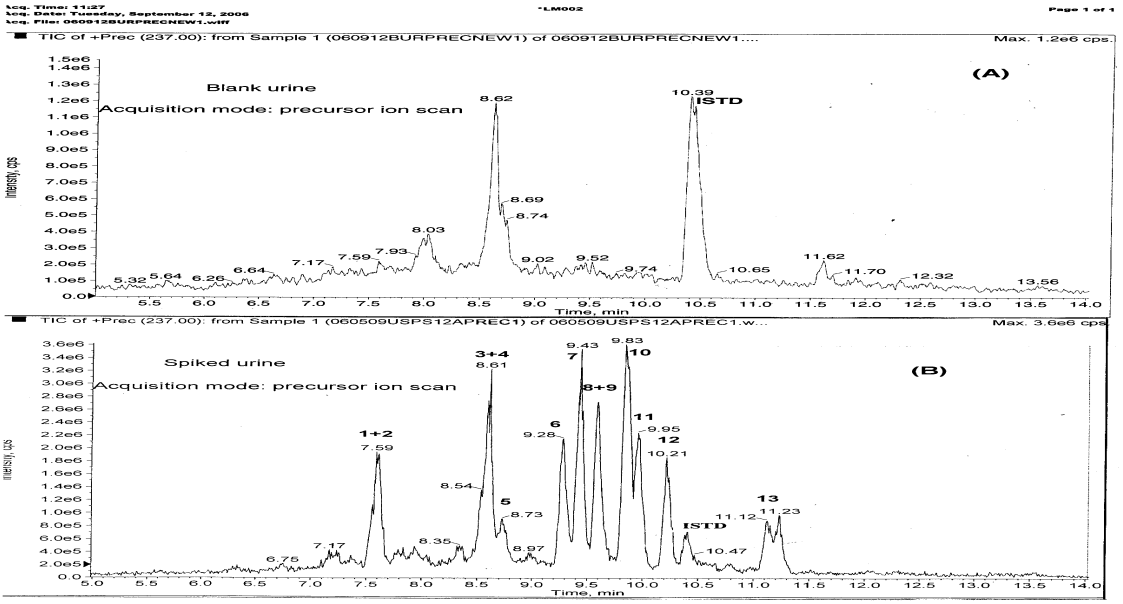


Figure 3: Chromatograms of a blank urine (A) and of a spiked urine (B) using precursor ion scan. Peak identity: 1. 16α-Hydroxyprednisolone, 2. triamcinolone, 3. prednisolone, 4. prednisone, 5. fludrocortisone, 6. methylprednisolone, 7. betamethasone/dexamethasone, 8. flumetasone, 9. beclometasone, 10. desonide, 11. triamcinolone acetonide/flunisolide, 12. fluocortolone, 13. budesonide.

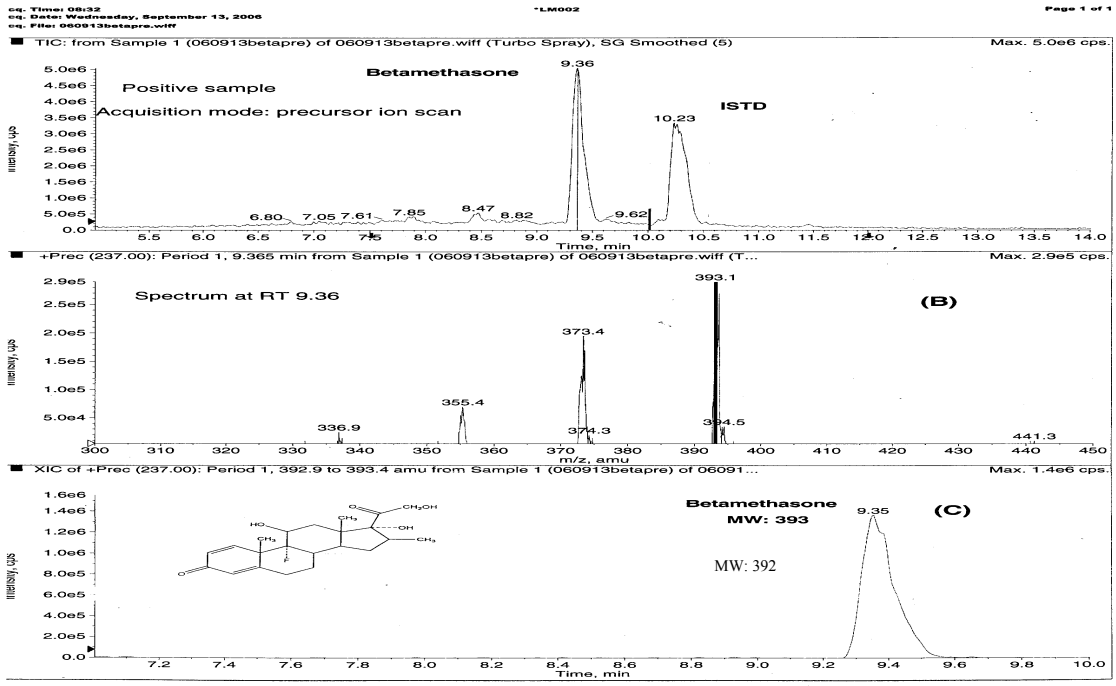
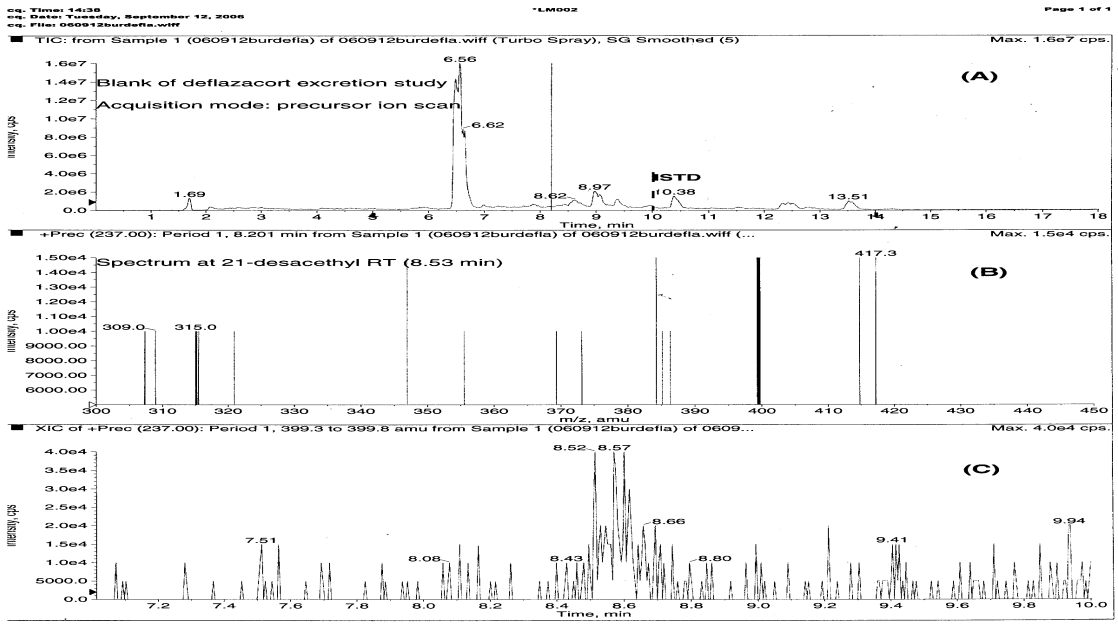


Figure 4: Chromatograms and spectrum of an athlete's urine positive for betamethasone using precursor ion scan.

(A)



(B)

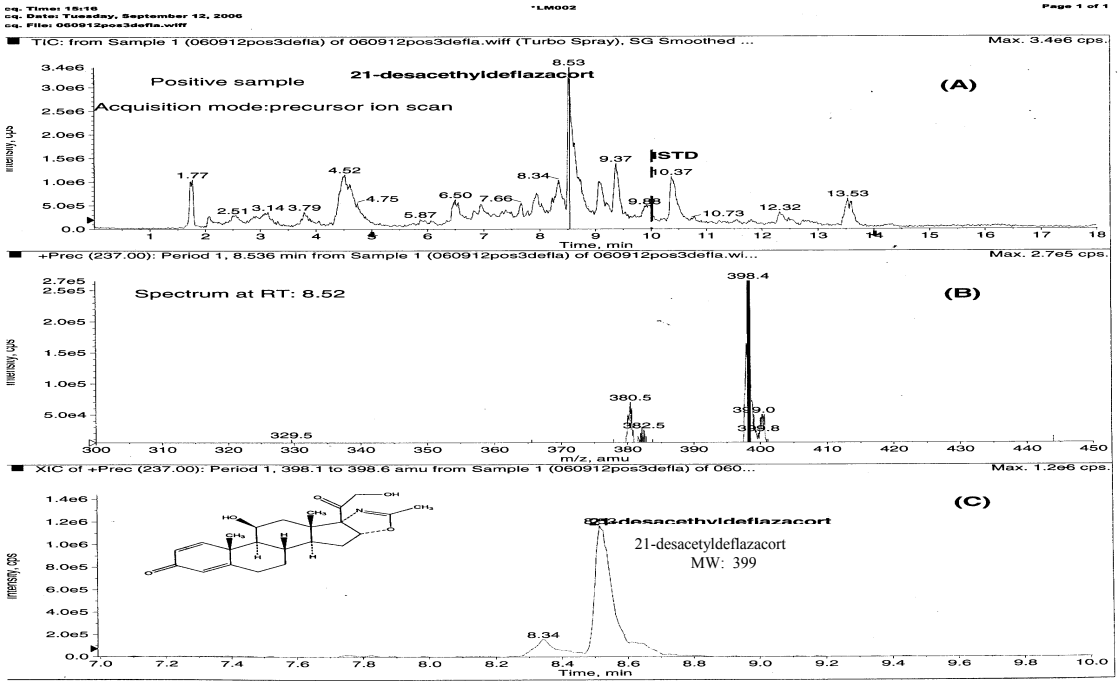


Figure 5: Chromatograms and spectrum of a blank urine (A) and a deflazacort excretion study (B) using precursor ion scan.

Table 3 Limits of detection (LODs) and their variability, expressed as coefficient of variation % (CV %), for all target compounds in this study using MRM plus precursor ion scan method.

	MRM		Precursor ion scan plus MRM	
Target compounds	**LOD (ng/mL**	**CV%**	**LOD (ng/mL)**	**CV%**
Beclometasone	10	8	10	6
Betamethasone/Dexamethasone	1	2	1	2
Budesonide	15	6	20	7
16α-Hydroxyprednisolone	10	5	15	5
Desonide	5	4	3	4
Fludrocortisone	15	3	30	8
Flumetasone	5	3	5	3
Fluocortolone	5	4	5	4
Methylprednisolone	15	6	15	6
Prednisolone	15	5	10	5
Prednisone	15	5	10	5
Triamcinolone	15	7	10	5
Triamcinolone acetonide/Flunisolide	3	4	3	4
Gestrinone	10	5	10	5
Tetrahydrogestrinone	10	5	10	5
Stanozolol metabolite	2	6	2	6
Anastrozole	10	9	15	9
Exemestane	30	7	30	7
Letrozole	10	5	15	5
Formoterol	100	8	100	8
Modafinil	100	7	150	7

CONCLUSIONS

- Screening of glucocorticoids using precursor ion scan as the acquisition mode is sufficient to detect all synthetic glucocorticoids on the WADA list, at a limit of detection satisfying the MRPL. Using precursor ion scan as acquisition mode is also possible to detect synthetic glucocorticoids and their metabolites not included on the WADA list, at a limit of detection satisfying the MRPL.
- Using both precursor ion scan and MRM as acquisition mode it is possible to carry out screening XII (gestrinone, tetrahydrogestrinone, glucocorticoids, formoterol, anti-oestrogens, mesocarb, modafinil) at a limit of detection satisfying the MRPL; also to detect glucocorticoids not included in the WADA list (see Table 3).

ACKNOWLEDGEMENT

This work has been supported in part by a Research Grant of the Italian Department of Health ("Ministero della Salute, Commissione per la vigilanza sul doping e sulla tutela sanitaria delle attività sportive"). The Authors also wish to thank Mr. Roberto Romani for the technical assistance.

REFERENCES

1. Thevis M., Kamber M., Schanzer W., *Rapid Commun. Mass Spectrom.*; 2006; 20: 870-876
2. Nielen MWF, Bovee TFH.. In: Schänzer W, Geyer H, Gotzmann A, Mareck U (eds.) *Recent advances in doping analysis, Sport und Büch Strauß*, 2001;157-8.
3. The World Anti-Doping Code. The 2006 Prohibited List International Standard. World Anti-Doping Agency, Montreal (Canada), 2006 (also available on-line, at the website www.wada-ama.org).
4. Amendola L., Molaioni F., Botrè F., *J. Pharmaceut. Biomed. Anal.*, 2000; 23: 211-221.
5. WADA Technical Document TD2004MRPL (Minimum required performance limits for detection of prohibited substances), World Anti-Doping Agency: Montreal, Canada, 2004. Available: www.wada-ama.org
6. Nogrady T. Medicinal chemistry. A biomedical approach; 2nd ed.. New York, Oxford: Oxford University Press 1988; 448-474.
7. Goodman L. and Gilman A., The Pharmacological Basis of Therapeutics, 10th Ed., Mc Graw-Hill 1996; 1459-1486.
8. Norman AW, Litwack G. Hormones; 2nd ed. San Diego: Academic Press 1991; 283-317.
9. Rodchenkov G., Sobolevsky T. In: Schänzer W, Geyer H, Gotzmann A, Mareck U (eds.) *Recent advances in doping analysis, Sport und Büch Strauß,* 2006; in press.
10. Malvey T.C., Armsey T.D., 2nd Curr. Sport Med. Rep. 2005, 4: 227-230
11. Thevis M., Geyer H., Mareck U., Schanzer W., *J. Mass Spectrom*. 2005; 40: 955
12. Thevis M., Schanzer W., *De Gruyter Berlin -New York* 2006; 30: 26-32
13. Bohets H., Annaert P., Mannens G. *Curr. Topics in Med. Chemistry* 2001; 1:367-383.