Validation of a LC-MS-MS method for anabolic steroids in nutritional supplements

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A survey of nutritional supplements for selected illegal anabolic steroids and ephedrine using LC/MS/MS and GC/MS methods respectively

Abstract

Several studies have highlighted that nutritional supplements may contain undeclared substances that are banned by the International Olympic Committee/World Anti-Doping Agency. This paper describes a qualitative LC-MS-MS method to detect anabolic androgenic steroids (4-androsten-3,17-dion, 4-estren-3,17-dion, 5 α -androsten-17 β -ol-3-one, boldenone, nandrolone, nandrolone decanoate, testosterone, testosterone decanoate) and ephedrine in food supplements. The products are dissolved in methanol and analysed by GC/MS. The methanolic solution was added with testosterone-d3, evaporated to dryness, mixed with NaOH and extracted with n-pentane:diethylether (9:1). LC/MS/MS analyses were performed in SRM on an ion trap equipped with atmospheric pressure chemical ionisation (APCI) probe operating in positive ion mode. The method was applied to 64 nutritional supplements. 12.5% of the nutritonal supplements analysed contained banned substances not declared on the label (anabolic steroids and ephedrine). Detection limits were in the range 1-25 ng/g.

Keywords: Nutritional supplements; Anabolic steroids; Anabolic steroids esters; Prohormones; LC-MS-MS analysis

Introduction

Several doping cases have been reported in official doping tests taken during sports competitions worldwide. In some cases athletes denied the use of doping agents and claimed to have inadvertently or passively absorbed the drug, for example by the ingestion of food or products sold as nutritional supplements that contained prohibited substances [?? *et al.* 2004]. Nutritional supplements seem to modify the body appearance and to promote more consistent and intensive training for athletes by allowing a speedy recovery between training sessions and by enhancing competitive performance. Most of these products do not contain doping agents, but studies have demonstrated that some dietary supplements may contain substances that are not declared on the label, and have been prohibited by the International Olympic Committee/World Anti-Doping Agency [Parr *et al.* 2004, De Cock *et al.* 2001, Delbeke *et al.* 2003., Pipe, Ayotte 2002, Ayotte *et al.* 2001]. Banned substances such as ephedrine, caffeine, steroids and prohormones were found in some “non-hormonal” nutritional supplements [Geyer *et al.* 2004]. It is not known whether the reason is faulty production control or intentional addition [Maughan *et al.* 2004, Ziegenfuss *et al.* 2002, Broeder 2003, Millman, Ross 2003].

In spite of the widespread use of nutritional supplements among sportsmen, the European Community promulgated the first Council Directive on dietary supplements (2002/46/CE), but supplements commonly used by sportsmen (BCCA, amino acids, proteins, etc) are not mentioned. In doping control, present testing methods for anabolic androgenic steroids in nutritional supplements rely on GC/MS [Parr *et al.* 2004, De Cock *et al.* 2001, Delbeke *et al.* 2003] or tandem mass spectrometry [De Cock *et al.* 2001, Van Thuyne, Delbeke 2004]. The methods developed using LC-MS [Joos, Van Ryckeghem 1999] and LC-MS-MS [Leinonen *et al.* 2002, Ho *et al.* 2006, Guan *et al.* 2005, Devebter *et al.* 2006, Politi *et al.* 2006] have not been applied to nutritional supplements. Only Reilly and Crouch report a LC-MS-MS method applied to 5 α -androst-1-en-3,17-

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dione and/or 5 α -androst-1-en-3 β ,17 β -diol (1AD), used as nutritional supplements and analysed in plasma and urine [Reilly and Crouch 2004].

This study describes the validation of a qualitative LC-MS-MS method for the determination of 8 doping substances. The anabolic steroids selected were those most commonly found in nutritional supplements, as shown by litterature data [De Cock *et al.* 2001, Geyer *et al.* 2004]. Moreover in nutritional supplements ephedrine was detected by GC/MS and was confirmed by LC/MS/MS. The use of LC-MS-MS provides several advantages over current methods because this technique allows the direct separation and identification without the need for derivatization, reducing the time of analysis, eliminating predictable sources of error and decreasing the use of hazardous and expensive reagents [Leinonen *et al.* 2002].

Materials and Methods

Nutritional supplements

64 nutritional supplements were purchased from shops and judical proceeding. The ingredients contained in the products has been classified as following: 4 vitamins/minerals, 7 glutamine/creatine, 9 amino acids, 12 proteins, 8 banned substances, 12 herbal extracts, 4 other. The manufacturer's recommended doses vary according to the supplement.

Chemicals and reagents

The following reference standards were used: ephedrine, testosterone, testosterone decanoate, nandrolone, nandrolone decanoate, testosterone-d₃ from Sigma (Saint Louis, Missouri USA); boldenone, 4-androsten-3,17-dione, 4-estren-3,17-dione and 5-androsten-3 β -ol-17-one (DHEA), methyl-testosterone, metandienone, oxandrolone, stanozolol, trenbolone, testosterone propionate, testosterone acetate, testosterone enanthate, 4-androsten-3 β ,17 β -diol, 5 α -androstan-3 β ,17 β -diol, 5 α -androstan-17 β -ol-3-one from Steraloids Inc. (Newport R.I. USA). The stock standard solutions

(1mg/ml) were prepared by dissolving 5 mg of each standard in 5 ml of methanol. Working standard solutions were made by dilution with methanol to the appropriate concentration (10 µg/ml and 1 µg/ml).

All solvents were HPLC grade and were obtained from Merck (Darmstadt, Germany); formic acid (extra pure) was obtained from Sigma-Aldrich-Riedel-de Haen (Seelze, Germany).

Materials and apparatus

The GC-MS analyses were performed on a Thermo electron gas chromatography coupled to a DSQ quadrupole mass spectrometer. Each sample was run through the following temperature program: initial temperature 70° C for 1 minute, then 10° C/min increments to 280° C, followed by a final isotherm of 5 minutes. The inject port and the source temperature were set at 250° C.

About LC-MS-MS parameters, the analytes were separated on a Discovery HS C₁₈ column (150 × 4.6 mm, 5 µm, Supelco, Bellefonte PA). A Discovery HS C₁₈ guard column (20 × 4.6 mm, 5 µm) was used prior to the analytical column. The mobile phase was a mixture of methanol (A) and acetic acid 0.03% (B) with a flow of 1.2 ml/min. The gradient program of the mobile phase started with A:B (60:40, v/v), was maintained for 3 minutes, then changed to A:B (95:5, v/v) over 12 min. Then the column was set back to the initial mobile phase conditions in 10 min. A sample loop of 20 µl was used.

A Spectra System P4000 pump coupled with LCQ Advantage ion trap mass spectrometer (Thermoelectron Corp., San José, CA, USA) was used for the LC-MS-MS analyses. The mass spectrometer was equipped with an atmospheric pressure chemical ionisation (APCI) probe operating in positive ion mode. The source parameters were as follows: nitrogen gas used as sheath and auxiliary gas at flow 1.2 L/min and 6 L/min respectively; vaporizer temperature 450° C; spray voltage 4500 V; capillary voltage and capillary temperature 5 V and 220° C respectively; and tube lens offset of 50 V. The mass spectra of the standard compounds were first acquired in full scan mode (150-460 *m/z*) by infusion of a reference solution of 1 µg/ml. From these spectra the

precursor ions were selected and fragmented acquiring the full scan MS-MS spectra. At last the higher product ions were chosen and each compound was analysed in SRM mode to obtain the best sensitivity and specificity. Fragmentation of the selected precursor ions was performed by collision-induced dissociation (CID) with helium, which fills the ion trap. The helium pressure in mass analyzer cavity was maintained at approximately 0.1 Pa.

Extraction procedure of nutritional supplements

The extraction procedure followed the validated method of Van Thuyne et al. (2004), modified for liquid chromatography detection [Van Thuyne and Delbeke, 2004]. Nutritional supplement (1 g) was added with the internal standard (testosterone-d3 100 ng/g). The residue was reconstituted with 5 ml MeOH and shaken by the vortex. 1 µl of the methanolic solution was injected into the GC-MS to detect the presence of ephedrine. Then the methanol was evaporated to dryness, mixed with 2 ml NaOH (1 M) and shaken with 5 ml n-pentane:diethylether (9:1) for 1 hr. After centrifugation the organic layer was separated and evaporated under nitrogen flow at 40°C. The residue was reconstituted with 50 µl of mobile phase (methanol:water with acetic acid 0.03%, 50:50) and injected into the LC-MS-MS system.

Validation Method

The method was validated using a blank nutritional supplement containing branched chain amino acids. This nutritional supplement was chosen to represent the spectrum of nutritional supplements because it is commonly used by body builders. After the validation, the method was applied to 64 unknown nutritional supplements. The analytical method for the determination of 8 anabolic compounds was validated in accordance to the Eurachem Guide suggestions [Eurachem.1998, 61]. According to Van Thuyne and Delbeke [2004], the method has only been just validated qualitatively as anabolic androgenic steroids and their esters in nutritional supplements are absolutely prohibited, with no limits of concentration allowed.

The Limit of Detection (LoD) was calculated by qualitative measurements spiking a blank nutritional supplement with a reference mixture at concentrations of 1, 5, 10, 25 and 50 ng/g. Each level was performed on five different replicates of the blank supplement. Repeatability (intra-assay precision) for LoDs level was in the range 8-14%. The selected compounds of the reference mixture were: 4-androsten-3,17-dione, 4-estren-3,17-dione, 5-androsten-3 β -ol-17-one (DHEA), boldenone, nandrolone, nandrolone decanoate, testosterone and testosterone decanoate. The specificity was tested during the validation procedure by extracting 10 aliquots of the blank nutritional supplement, to look for possible matrix interferences.

Results and Discussion

The relative retention time, the precursor ion and the product ions for each standard compound are given in Table I. The LC-MS-MS chromatograms of a blank nutritional supplement and a nutritional supplement spiked at 50 ng/g, extracted as above, are shown in Figure 1 and 2, respectively.

In order to test the specificity 10 aliquots of the blank supplement were analysed and no matrix interference was found at the retention time of the 8 analytes and the internal standard. The relative retention time of the analyte should correspond to that of the standard analyte, from a spiked sample, with a tolerance of $\pm 2\%$. The relative intensities of the detected ions, expressed as a percentage of the intensity of the most intense transition, must correspond to those of the standard analyte, from a spiked sample with the relative tolerance of $\pm 25\%$, as suggested by the World Anti-Doping Agency [The WADA Technical Document – TD2003IDCR]. All these criteria were applied to all the spiked samples for the LoD determination.

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2 According to the Eurachem guidelines [Eurachem 1998, 61] the LoD for qualitative measurements
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4 is a concentration threshold below which specificity becomes unreliable. It was defined as the
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6 concentration level where a compound could be detected in all five of the tested samples. Table II
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8 shows how this threshold concentration was determined for each standard compounds and figure 3
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10 reports the relative LODs chromatograms According to our experimental data six compounds can
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12 be detected at or below 10 ng/g, two at 25 ng/g.
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18 The validated method was applied to 64 nutritional supplements, containing ingredients as
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20 described above. 12.5% of the nutritional supplements tested contained substances not declared on
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22 the label. The positive nutritional supplements are reported in table III, in which we reported: the
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24 producer, the ingredients declared in the label and the banned substances. Sample 1, 2, 4, 7, 8, 9 and
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26 12 contain banned substances not declared; sample 5 and 6 confirm the presence of ephedrine and
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28 in sample 3 we found ephedrine but the label reports only the plant source. Finally for sample 17 we
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30 could not confirm the presence of ephedrine as reported on the label. In figure 4 a nutritional
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32 supplement positive to 4-estren-3,17-dione and 4-androsten-3,17-dione is shown.
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40 **Conclusions**

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42 The method reported here is sufficiently sensitive, specific and selective for the detection and
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44 confirmation of 4-androsten-3,17-dione, 4-estren-3,17-dione, 5-androsten-3 β -ol-17-one (DHEA),
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46 boldenone, nandrolone, nandrolone decanoate, testosterone and testosterone decanoate in nutritional
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48 supplements, in accordance to the validation criteria suggested by Eurachem guidelines. This LC-
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50 MS-MS method was applied to 64 nutritional supplements and 12.5% of them were found positive.
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52 The low levels of the compounds found in the samples (in the range of ng/g) may indicate an
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54 accidental contamination and not an intentional admixture. However, athletes should consider only
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56 purchasing from companies performing quality tests on prohormones and testing for possible
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58 contamination during production.
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Figure 1

Figure 2

Figure 3

Figure 4

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Table I: Relative retention times, precursor ions and product ions of each compound of the standard mixture.

Compound	RRT	Parent Ion (<i>m/z</i>)	Product Ions (<i>m/z</i>)
4-estren-3,17-dione	0.761	273	255, 237, 197
Boldenone	0.828	287	269, 173, 135
4-androsten-3,17-dione	0.884	287	269, 251
Nandrolone	0.900	275	257, 239
Testosterone-d3	1.000	292	274, 256
Testosterone	1.003	289	271, 253
5-androsten-3β-ol-17-one	1.014	271	271, 253
Nandrolone decanoate	2.226	429	257, 239, 275
Testosterone decanoate	2.387	443	271, 253

	Number of positive and negative findings in spiked samples at different analyte concentrations							
Concentration (ng/g)	4-estren- 3,17-dione	Boldenone	4-androsten- 3,17-dione	Nandrolone	Testosteron e	DHEA	Nandrolone decanoate	Testosterone decanoate
50	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0
25	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0
10	5/0	5/0	5/0	5/0	5/0	2/3	5/0	3/2
5	5/0	2/3	5/0	5/0	5/0	0/5	3/2	1/4
1	2/3	1/4	2/3	3/2	5/0	0/5	1/4	0/5
LOD	5 ng/g	10 ng/g	5 ng/g	5 ng/g	1 ng/g	25 ng/g	10 ng/g	25 ng/g

Table II

N.	Producer	Ingredients declared on the label	Banned substances
1	A	L-glutamine	4-estren-3,17-dion 4-androsten-3,17-dion
2	A	Proteins, carbohydrates	4-estren-3,17-dion
3	B	Ephedra sinica/Ma Huang; Guaranà; White willow bark; Chromium picolinate	ephedrine
4	B	L-carnitine; Garcinia Cambogia; Vitamin B6; Chromium	ephedrine
5	C	Ma Huang (ephedrine alkaloids); Guaranà extract; Green tea extract; L-phenylalanine; L-tyrosine; White willow bark; Citrus aurantium (sinephrine alkaloids); Herbal blend (ginger, passion flower, yohimbe)	ephedrine
6	C	Ephedrine alkaloids; Guaranà extract; Yerba mate extract; citrimax extract; L-carnitine; L-tyrosine	ephedrine
7	D	Branched-Chain Amino Acids (BCCA)	nandrolone decanoate
8	D	L-carnitine	testosterone decanoate
9	D	Amino acids	testosterone
10	E	Branched-Chain Amino Acids (BCCA)	4-androstendion
11	F	Ephedrine; guaifenesin	ephedrine
12	G	Dehydroepiandrosteron (DHEA)	DHEA testosterone
13	G	Dehydroepiandrosteron (DHEA)	DHEA
14	H	Ephedrine; guaifenesin	ephedrine
15	I	Testosterone decanoate	Testosterone decanoate
16	I	prohormones	4-androstendion 4-estrendion
17	C	Ephedrine (from herbal extract); guarana extract; yerba mate extract; citrimax; white willow bark; yohimbe; bioperine	ephedrine declared on the label not found

Table III