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Analytica Chimica Acta 586 (2007) 35–42
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

Determination of anabolic steroids in dietary supplements by liquid chromatography–tandem mass spectrometry

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Received 19 June 2006; received in revised form 17 August 2006; accepted 18 September 2006
Available online 4 October 2006

Abstract

Nineteen different dietary supplements, ordered through the internet and intercepted by the Belgian pharmaceutical inspection at the post office, were analyzed by means of liquid chromatography–tandem mass spectrometry (LC–MS/MS) for the presence of anabolic steroids. After a methanolic extraction the samples were screened for the presence of 49 compounds. This resulted in almost 60% of the samples being suspected of containing one of these 49 anabolic compounds and being subjected to a confirmatory product ion scan. In all of these suspected samples we were able to confirm at least one anabolic steroid with concentrations between 0.01 and 2.5 mg unit^{-1} (unit: one capsule or tablet or for liquids: the prescribed dose). The anabolic steroids that was mostly encountered was testosterone (50%) followed by β-boldenone (25%). These results once more confirm the dubious reputation of over-the-counter dietary supplements.

Keywords: Dietary supplements; Prohormones; Anabolic steroids; Doping; Liquid chromatography–tandem mass spectrometry (LC–MS/MS)

1. Introduction

In order to improve their performance athletes are often tempted to use dietary supplements. A lot of these dietary supplements are freely available through numerous internet sites which cause an expansion in the use of these supplements. Annual sales in the US were estimated in 1999 at 12 billion dollars [1].

Several investigations on these over-the-counter supplements already pointed out that often the information on the label is misleading or incomplete and that they contain not listed prohormones [2–4]. Many of these so-called prohormones often elevate the testosterone/epitestosterone (T/E) ratio in urine above the threshold of 6/1 [2], which is considered indicative of suspected testosterone administration by many sport organizations. De Cock et al. [5] even showed that the single ingestion of a low dose (5 mg) of 19-nor-4-androstene-3,17-dione leads to a 48–144 h lasting increase of the norandrosterone level, a major urinary metabolite of nortestosterone, profile above the 2 ng mL^{-1} threshold.

Recently there have been reports that these dietary supplements are not only contaminated with prohormones but also with low concentrations of anabolic steroids such as methylboldenone [6,7], testosterone [2,6] and stanozolol [8].

In the light of these recent reports we decided to analyze some dietary supplements which were intercepted at the post office by the Belgian pharmaceutical inspection services for the presence of prohibited anabolic steroids.

2. Experimental

2.1. Reagents and chemicals

All steroid reference compounds were purchased from Sigma (Bornem, Belgium), RIVM (Bilthoven, The Netherlands), WIV-IPH (Brussels, Belgium) or Dr. Willems Instituut (Diepenbeek, Belgium).

Formic acid (pro analyse), ammonia (25%) and methanol for HPLC were purchased from Merck (Overijse, Belgium). High purity nitrogen and argon were supplied by Air Liquide (Belgium). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

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doi:10.1016/j.aca.2006.09.050

2.2. Standard solutions

Standard stock solutions (1 mg mL^{-1}) were prepared in methanol and were stable for at least 1 year when stored at 4 °C. Working standard solutions (1 ng μL^{-1}) were made by diluting the stock solutions in methanol. These solutions were stable for at least 3 months at 4 °C. Tuning solutions (1 ng μL^{-1}) were freshly prepared in methanol containing 0.3% formic acid for positive electrospray ionization (ESI+) and containing 0.3% ammonia for negative electrospray ionization (ESI−).

2.3. Extraction protocol

The content of one capsule or one grinded tablet (weight between 0.3 and 1 g) was extracted with 1 mL of methanol and subsequently centrifuged for 10 min at 2800 × *g*. The extract was transferred to a clean test tube. The sediment was resubmitted to the above described procedure and both methanol fractions were combined and evaporated to dryness at 40 °C under a mild stream of nitrogen. Finally the dry residue was dissolved in 2 mL methanol and a 100-fold dilution in methanol/water (65/35, v/v) was prepared for LC–MS/MS analysis.

2.4. Liquid chromatographic–tandem mass spectrometric conditions

The MS system used was a Quattro Micro mass spectrometer (Waters, Milford, MA, USA). High purity nitrogen was used as the drying and electrospray ionization (ESI) nebulizing gas and were set at 100 and 500 L h^{-1}, respectively. Argon

Table 1
Overview of the ESI+$_1$ MS/MS parameters and retention times

Compound	Retention time (min)	Precursor ion (*m*/*z*)	Product ions (*m*/*z*)	Cone voltage (V)	Collision energy (eV)
α-Boldenone	6.7	287.5	120.5[a]	25	15
			134.6		15
β-Boldenone	5.2	287.5	120.5[a]	25	15
			134.5		15
α-Nortestosterone	7.3	275.5	109[a]	34	22
			145		28
β-Nortestosterone	6.1	275.5	109[a]	34	22
			145		28
17α-Hydroxyprogesterone	8.6	331.5	314	40	25
			109[a]		15
Algeston acetophenide	49.7	449.5	330[a]	45	20
			296.5		17
Chloromadinon acetate	12.0	405.5	345.5[a]	35	35
			133		15
Clostebol acetate	32.7	365.5	305.5	35	25
			143[a]		15
Delmadinone acetate	9.3	403.5	205.5	40	40
			145[a]		20
Fluoxymesterone	5.9	337.5	281.5	46	28
			131[a]		22
Formebolone	2.9	345.5	309.5[a]	25	19
			175		12
Megestrol acetate	10.8	385.5	325.5[a]	35	20
			267.5		15
Melengestrol acetate	12.6	397.5	337.5[a]	35	20
			279.5		15
Methylboldenone	6.4	301	121[a]	28	18
			149		18
Methyltestosterone	9.7	303.5	97[a]	40	28
			121		26
Norethandrolone	14.5	303	109[a]	34	22
			267		22
Norethisterone	5.4	299.5	109[a]	40	30
			131		25
Norgestrel	8.8	313	109[a]	35	22
			245.5		22
Oxymetholone	32.7	333.5	133	45	35
			97[a]		35
Progesterone	16.5	315.5	97[a]	35	26
			123		24
Stanozolol	11.5	329.5	81[a]	70	35
			121		40
Trenbolone	5.3	271.5	253.5[a]	40	28
			199.5		22

[a] Most abundant fragment ion.