Validation of a GC–MS Screening Method for Anabolizing Agents in Aqueous Nutritional Supplements

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Abstract

A sensitive and selective method for the screening of anabolizing agents in aqueous nutritional supplements is described and validated. A total of 28 different anabolizing agents are screened for, including testosterone and prohormones, nandrolone and prohormones, stanozolol, and metandienone. The different analytes are extracted from the aqueous nutritional supplements by liquid–liquid extraction with a mixture of pentane and freshly distilled diethylether (1:1) after the supplements have been made alkaline with a $NaHCO_3$–K_2CO_3 (2:1) buffer. The anabolizing agents are derivatized with a mixture of MSTFA–NH_4I–ethanethiol (320:1:2) as routinely used for the screening of anabolic steroids extracted from urine. The derivatives are analyzed by gas chromatography (GC)–mass spectrometry (MS) in the selective ion monitoring mode. The limits of detection range from 1 to 10 ng/mL. One aqueous nutritional supplement (creatine serum) was analyzed with this screening method and was found to contain dehydroepiandrosterone (DHEA) at very low concentrations. The presence of DHEA could be confirmed with GC–MS–MS. Results of the application of this method and a similar method for solid nutritional supplements previously described are given.

Introduction

Nutritional supplements are food, supplied in one or more nutrients in a concentrated form such as minerals, vitamins, enzymes, etc., that are theoretically present in a normal balanced diet (1). Usually, they are offered in an atypical forms such as powder, tablets, or capsules. Also commercially available and very popular are sport and energy drinks and the recently commercialized creatine serums, which are aqueous solutions of creatine monohydrate. During the last decade, the use of nutritional supplements has increased tremendously (1–3).

Several factors have combined to cause the present situation in which the use of nutritional supplements by athletes has become a matter of concern. The ever increasing aim for success by athletes is stimulated by the high financial stakes of elite sport. On the other hand, since the adoption of the Dietary Supplement Health and Education Act in 1994 (4), prohormones became commercially available on the United States supplements market. According to the regulations of the International Olympic Committee (IOC), these prohormones belong to the prohibited class of anabolic steroids (5) because research has suggested a number of potential risks associated with prohormone use similar to those observed with use of anabolic steroids (6–8).

Evidence was recently found that several of these prohormones were present in "nonhormonal" nutritional supplements like vitamins, minerals, amino acids, etc. Prohormones were not declared on the label of these supplements (9–11). An international IOC study found 94 nutritional supplements out of 634 (14.8%) containing one or more prohormones not mentioned on the label (12). In two other studies, high doses of the anabolic steroid metandienone were found in supplements (13,14). In both cases the presence of this anabolic steroid was not mentioned on the label. The presence of these prohormones may lead to a positive doping test especially for the nandrolone metabolite norandrosterone (9–11). Because of the increasing use of nutritional supplements and the detection of several prohormones in nonhormonal supplements banned by international sport federations, methods for the detection of prohormones in nutritional supplements must be developed. Recently, an ISO 17025 validated method for the screening of anabolizing agents in solid nutritional supplements was presented (15). Here, a validated method for the screening of these forbidden substances in aqueous nutritional supplements is described. Also, an overview of the results obtained with both methods is given.

Experimental

Reagents

5α-Androstane-3α,17β-diol; 19-nor-4-androstene-3,17-dione; 5α-androstane-3β,17β-diol; 4-androstene-3,17-dione; boldenone; androsterone; and testosterone were obtained from Sigma (St. Louis, MO). 19-nor-4-Androstene-3β,17β-diol; 19-

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nor-5-androstene-3,17-dione; 4-androstene-3β,17β-diol; metandienone; 5-androstene-3β,17β-diol; 5-androstene-3,17-dione; 4-androstene-19-ol-3,17-dione; and 7-keto-dehydroepiandrosterone (7-keto-DHEA) were bought from Steraloids (Newport, RI). Dehydroepiandrosterone (DHEA) was from Serva (Heidelberg, Germany) and dihydrotestosterone (DHT) from Piette International Laboratories (Drogenbos, Belgium). Nandrolone and stanozolol were bought from NARL (Pymble, Australia). Clenbuterol was obtained from Boehringer Ingelheim (Ingelheim am Rhein, Germany). 17α-Methyltestosterone, testosterone propionate, testosterone isocaproate, testosterone decanoate, testosterone phenylpropionate, testosterone undecanoate, nandrolone decanoate, and nandrolone phenylpropionate were obtained from Organon (Oss, the Netherlands). Nandrolone laurate, Laurabolin, was from Intervet International (Boxmeer, the Netherlands). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Chem. Fabrik Karl Bucher (Waldstetten, Germany). All other chemicals were of analytical grade.

Nutritional supplement X (creatine serum) was of United States origin. The labelled content of this supplement was: 50 mg/mL creatine serum, 20 mg/mL inositol, 20 mg/mL D-glucose, 20 mg/mL glucosamine sulphate, 20 mg/mL magnesium ascorbate, 10 mg/mL calcium pyruvate, 10 mg/mL citrus-bioflavonoids, 10 mg/mL green tea extract, 10 mg/mL guarana extract, 10 mg/mL L-arginine, 10 mg/mL L-carnitine, 10 mg/mL L-glutamine, 10 mg/mL Siberian ginseng, 6 mg/mL royal jelly, 3.6 mg/mL vitamin B5, 2 mg/mL zinc gluconate, 2 µg/mL chromium gluconate, and 0.6 µg/mL vitamin B12. Other ingredients were water, glycerine, sorbitol, and aromas. The recommended dosage was 5 mL to be used 10 min before exertion, only on training days.

Gas chromatography–mass spectrometry conditions

The gas chromatography (GC)–mass spectrometry (MS) analysis was conducted in the selected-ion monitoring (SIM) mode on an HP 6890 GC directly coupled to an HP 5973 mass selective detector (HP, Palo Alto, CA). Three ions were monitored for each compound. The GC column was an HP-Ultra 1 (J&W, Folsom, CA), 100% methylsilicone column with a length of 17 m, an internal diameter of 0.2 mm, and a film thickness of 0.11 µm. Helium was used as the carrier gas (linear velocity of 41 cm/s). A total of 0.5 µL was injected splitless. The oven temperature program was as follows: 120°C (0 min), 70°C/min to 181°C (0 min), 4°C/min to 234°C (0.1 min), and 30°C/min to 300 °C (10 min). The electron energy was set at 70 eV, and the ion source temperature was set at 230°C.

Analysis of nutritional supplements

Five milliliters of the nutritional supplement was made alkaline with 1 g of a $NaHCO_3$–K_2CO_3 (2:1, w/w) buffer. After stirring, 50 µL of the internal standard androsterone (2 µg/mL in MeOH) was added, followed by 5 mL of a pentane–diethylether mixture (1:1). After extraction by rolling for 1 h and centrifugation, the organic layer was separated and dried under oxygen free nitrogen at 40°C ± 5°C. The residue was derivatized with 100 µL MSTFA–NH_4I–ethanethiol (320:1:2) at 80°C for 30 min and transferred to an autosampler microvial.

Analytical method validation

The analytical method validation for the screening of 28 compounds was performed according to Eurachem guidelines (16) on 10 different, randomly chosen aqueous nutritional supplements.

To determine the LODs, 10 different nutritional supplements were spiked with a reference mixture at different concentrations in the range 1–40 ng/mL (1, 2, 5, 10, 20, and 40 ng/mL). Selectivity was tested by the analysis of a reference mixture of 10 different other anabolizing agents in a concentration of 200 ng/mL. These compounds were: 19-noretiocholanolone, 17α-trenbolone, oxymesterone, 3'-OH-stanozolol, mesterolone, salbutamol, terbutaline, etiocholanolone, 5β-androst-1-ene-17β-ol-3-one, and oxandrolone. Specificity was tested by the analysis of 10 different nutritional supplements.

Results and Discussion

All of the compounds that were screened for are mentioned in Table I. These include prohormones of testosterone and nandrolone, the most commonly found in nutritional supplements (9–11), esters of both compounds, stanozolol, and metandienone. Under the chromatographic conditions described here, the internal standard androsterone-bis-

Table I. GC Relative Retention Times (RRT) and Monitored *m/z* Values for Trimethylsililated Compounds

Compound	RRT	*m/z*
Clenbuterol	0.46	335.1, 300.1, 86.1
Androsterone (IS)	1.00	434.3, 419.3, 329.2
5α-Androstane-3α,17β-diol	1.03	436.4, 331.2, 241.2
19-nor-4-Androstene-3,17-diol	1.06	420.3, 330.2, 240.2
DHEA	1.11	432.3, 417.3, 327.2
19-nor-4(5)-Androstene-3,17-dione	1.12	416.3, 401.2, 194.1
4-Androstene-3β-17β-diol	1.12	434.3, 405.3, 143.1
5-Androstene-3β-17β-diol	1.14	434.3, 344.3, 239.2
5α-Androstane-3β,17β-diol	1.15	436.4, 421.3, 241.2
Nandrolone	1.15	418.3, 403.3, 194.1
DHT	1.17	434.3, 405.3, 143.1
4(5)-Androstene-3,17-dione	1.19	430.3, 415.3, 234.1
Boldenone	1.19	325.2, 229.1, 206.1
Testosterone	1.22	432.3, 417.3, 209.0
Metandienone	1.33	444.3, 339.2, 206.1
17a-Methyltestosterone	1.35	446.3, 356.2, 301.2
4-Androstene-19-ol-3,17-dione	1.36	518.4, 428.3, 415.3
7-keto-DHEA	1.38	518.3, 429.2, 296.1
Testosterone propionate	1.41	416.3, 401.3, 343.2
Stanozolol	1.55	472.4, 457.3, 143.1
Testosterone isocaproate	1.56	458.4, 443.3, 343.2
Nandrolone decanoate	1.78	500.4, 485.4, 329.2
Nandrolone phenylpropionate	1.79	478.3, 463.3, 194.1
Testosterone decanoate	1.82	514.4, 499.4, 343.2
Testosterone phenylpropionate	1.83	492.4, 477.3, 105.0
Testosterone undecanoate	1.90	528.5, 513.4, 343.2
Nandrolone laurate	1.96	528.5, 513.4, 329.2

trimethylsilyl gave a sharp peak with a retention time of 10.81 min. The GC relative retention times and ions monitored (three per compound) are given in Table I.

For screening purposes, at least two ion traces were monitored for every substance. The presence of a substance is suspected, and the sample forwarded to confirmatory analysis, if the relative abundance of the ion traces is similar to the relative abundance of the ion traces in the reference (20% relative margin). As an extra criterion, a maximal deviation in relative retention time of 1% was used.

The validation was performed according to the Eurachem guidelines (16). According to these rules, the LOD is defined as the concentration in which an analyte can be detected with a certainty of 100% (cf., previously mentioned criteria) in all samples in the case of 10 spiked supplements analyzed. The resulting LODs are summarized in Table II. As can be seen, 12 analytes can be detected at a concentration of 1 ng/mL. The highest LOD obtained was 10 ng/mL, a concentration far below the LODs obtained with previous full-scan methods (11). Specificity and selectivity were tested according to the procedure described by Verwaal et al. (17). For a qualitative method, the analysis of the different negative matrices used to determine the limit of detection was sufficient to test for the specificity. No matrix interferences were found at the retention times of the 28 analytes, nor at the retention time of the internal standard androsterone. Selectivity was tested by the analysis of several related compounds. According to Verwaal et al. (17), the concentration of these compounds must be at least twice the LOD of the determined analytes. In this study, a concentration of 200 ng/mL was used. No interference of the related compounds was observed at the retention times of the different compounds and the internal standard, androsterone. Thus, this method seems to be specific and selective. In conclusion, it seems that this method is reliable and sensitive for the screening of anabolizing agents in aqueous nutritional supplements.

Previous analysis of nutritional supplement X with a full-scan method (11) did not result in the detection of one or more prohormones. Therefore, this matrix was used as a negative matrix during the validation procedure. Surprisingly, the test for specificity resulted in the detection of DHEA in very low

Table II. LODs for 28 Different Compounds

Compound	LOD (ng/mL)	Compound	LOD (ng/mL)
DHEA	1	Metandienone	2
19-nor-4(5)-Androstene-3,17-dione	1	Testosterone	2
Nandrolone	1	DHT	5
4(5)-Androstene-3,17-dione	1	5α-Androstane-3α,17β-diol	5
4-Androstene-3β-17β-diol	1	7-keto-DHEA	5
4-Androstene-19-ol-3,17-dione	1	Boldenone	5
Testosterone undecanoate	1	Clenbuterol	5
Testosterone decanoate	1	5α-Androstane-3β,17β-diol	5
Nandrolone phenylpropionate	1	testosterone Phenylpropionate	5
Nandrolone laurate	1	Testosterone isocaproate	5
17α-Methyltestosterone	1	5-Androstene-3β-17β-diol	5
Testosterone propionate	1	Nandrolone decanoate	5
19-nor-4-Androstene-3,17-diol	2	Stanozolol	10

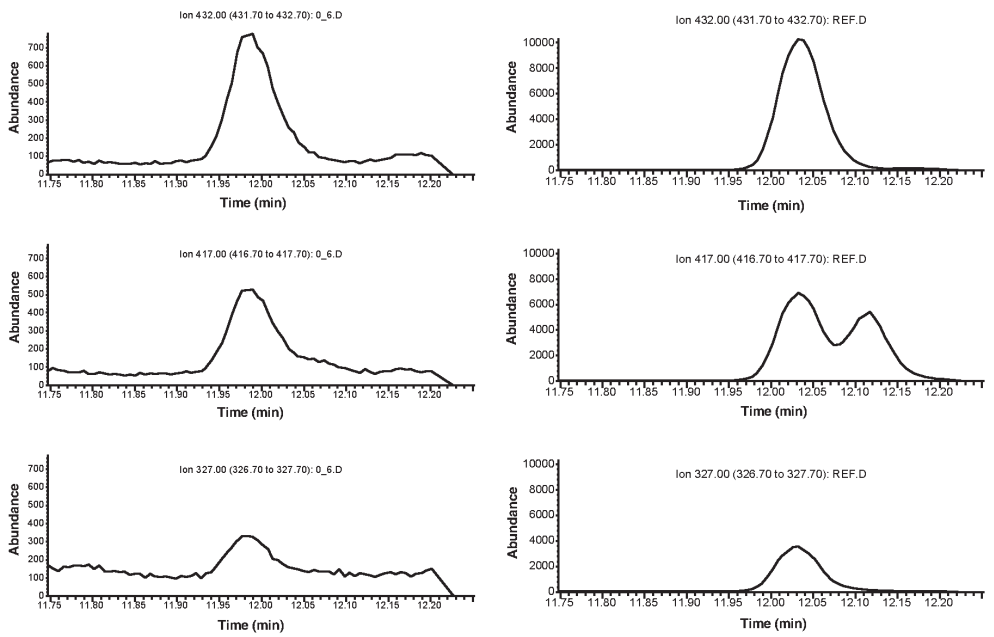

Figure 1. Positive screening results for DHEA in nutritional supplement X. (A) Nutritional supplement X and (B) DHEA.

concentrations. Screening results for DHEA in nutritional supplement X in comparison to a reference are shown in Figure 1. Confirmation of these results could be obtained with GC–MS–MS. The resulting daughter spectrum of DHEA in nutritional supplement X compared with a reference is given in Figure 2. Because DHEA could be confirmed in nutritional supplement X, the method was further validated by replacing this matrix by an additional negative nutritional supplement.

A total of 39 samples, both aqueous and solid nutritional supplements, were recently tested with the presented method and that for solid nutritional supplements previously described (15). Of those, 36 samples were from nutritional supplement selling companies, the other 3 were bought. For 8 samples (18.6 %), no reliable data could be obtained. The major reason for this lack of data was matrix effects. Most of these problems occurred with solid nutritional supplements or oily solutions. Further research will be needed to minimize matrix effects. Twelve (30.77%) nutritional supplements contained one or more anabolizing agents. The most commonly found were: DHEA (9 cases), testosterone (7 cases), and 4(5)-androstene-3,17-dion (6 cases).

Previous analysis of 133 nutritional supplements for anabolizing agents with a nonvalidated full-scan method slightly modified from De Cock et al. (11) resulted in only 7.52% positive cases. Most commonly found with this full-scan method were DHEA (8 samples) and 4(5)-androstene-3,17-dione (4 samples).

Conclusion

It can be concluded that the described method for the screening of anabolizing agents in aqueous nutritional supplements and the already published method for solid supplements (15) are more reliable and sensitive than the previous full-scan method (11).

The results presented here indicate that a large number of nutritional supplements are contaminated with prohormones. The development of a sensitive and ISO 17025 validated screening method for the detection of anabolizing agents in nutritional supplements could be helpful for manufacturers to avoid unintended contamination of their products.

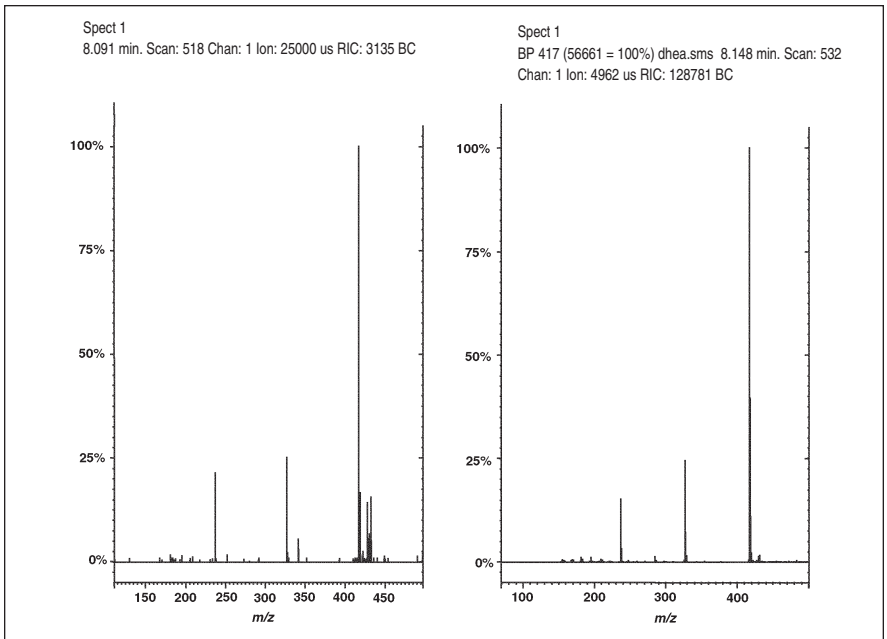

Figure 2. Positive screening results for DHEA in nutritional supplement X. (A) Nutritional supplement X and (B) DHEA.

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References

1. U. Schröder. "Health effects of nutritional supplements". In *Health and Doping Risks of Nutritional Supplements and Social Drugs*. W. Schänzer, F.T. Delbeke, A. Deligiannis, G. Gmeiner, R. Maughan, and J. Mester, Eds. Sport & Buch Strauss, Köln, Germany, 2002.
2. R.J. Maughan, D.S. King, and T. Lea. Dietary supplements. *J. Sports Sci.* **22:** 95–113 (2004).
3. R. Maughan. "The scope of nutritional supplements in sport". In *Health and Doping Risks of Nutritional Supplements and Social Drugs*. W. Schänzer, F.T. Delbeke, A. Deligiannis, G. Gmeiner, R. Maughan, and J. Mester, Eds. Sport & Buch Strauss, Köln, Germany, 2002.
4. United States Congress. Dietary Supplement Health and Education Act of 1994. Pub L No. 103–417, 103rd Congress, 2nd sess, S784. Washington, D.C., 1994.
5. International Olympic Committee. List of prohibited classes of substances and methods of doping. International Olympic Committee, Medical Commission, Lausanne, Switzerland, 2001.
6. C.E. Broeder, J. Quindry, K. Brittingham, L. Panton, J. Thomson, S. Appakondu, K. Breuel, R. Byrd, J. Douglas, C. Earnest, C. Mitchell, M. Olson, T. Roy, and C. Yarlagadda. The andro project: the physiological and hormonal influences of androstenedion supplementation in men 35 to 65 years old participating in high intensity resistance training program. *Arch. Intern. Med.* **160:** 3093–3104 (2000).
7. D.S. King, R.L. Sharp, M.D. Vukovich, G.A. Brown, T.A. Reifenrath, N.L. Uhl, and K.A. Parsons. Effect of oral androstenedione on serum testosterone and adaptations to resistance training in young men: a randomised controlled trial. *JAMA* **281(21):** 2020–28 (1999).
8. B.Z. Leder, C. Longcope, D.H. Catlin, B. Ahrens, D.A. Schoenfeld, and J.S. Finkelstein. Oral androstenedione administration and serum testosterone concentrations in young men. *JAMA* **283:** 779–82 (2000).

9. H. Geyer, U. Mareck-Engelke, U. Reinhart, M. Thevis, and W. Schänzer. Positive dopingfälle mit norandrosteron durch verunreinigte nahrungsergänzungsmittel. *Dtsch. Z. Sportmed.* **51(11):** 378–82 (2000).
10. H. Geyer, U. Mareck-Engelke, A. Wagner, and W. Schänzer. "The analysis of "non-hormonal" nutritional supplements for prohormones". In *Recent Advances in Doping Analysis (9)*. W. Schänzer, A. Gotzmann, and U. Mareck-Engelke, Eds. Sport & Buch Strauss, Koln, Germany, 2001, pp. 63–71.
11. K.J.S. De Cock, F.T. Delbeke, P. Van Eenoo, N. Desmet, K. Roels, and P. De Backer. Detection and determination of anabolic steroids in nutritional supplements. *J. Pharm. Biomed. Anal.* **25:** 843–52 (2001).
12. H. Geyer, M.K. Parr, U. Reinhart, Y. Schrader, U. Mareck, and W. Schänzer. "Analysis of non-hormonal nutritional supplements for anabolic androgenic steroids—an international study". In *Recent Advances in Doping Analysis* (10). W. Schänzer, H. Geyer, A. Gotzman, U. Mareck, Eds. Sport und Buch Strauss, Koln, Germany, 2002, pp. 83–85.
13. G. Gmeiner. Methandienon in sportnahrung. *Oster. J. Sportmed.* **2:** 33–34 (2002).
14. H. Geyer, M. Bredehöft, U. Mareck, M.K. Parr, and W. Schänzer. Hoge dosen des anabolikums metandienon in nahrungsergänzungsmittel. *Dtsch. Apoth. Ztg.* **142(29):** 50 (2002).
15. W. Van Thuyne and F.T. Delbeke. Validation of a GC-MS screening method for anabolizing agents in solid nutritional supplements. *Biomed. Chromatogr.* **18(3):** 155–59 (2004).
16. Eurachem. "The fitness for purpose of analytical methods". In *A Laboratory Guide to Method Validation and Related Topics*, Vol. 61. LGC Ltd., Teddington, U.K., 1998, pp. 1–61.
17. W. Verwaal, M. van Bavel, A. Boot, J. Bravenboer. F. de Goei, C. Maas, and A. van der Putten. Valideren van (fysisch-) chemische en (fysisch-) mechanische methoden op het niveau van de wettelijke eis. *De Ware(n) Chemicus* **26:** 25 (1996).

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