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## **The Quantification of Prohormones in Nutritional Supplements**

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### **ABSTRACT**

The objective of the present study is the extension of our researches about the possible contamination of the nutritional supplements with doping agents. A number of 14 nutritional supplements merchandised on the Romanian market were tested for prohormones by GC/MS technique. The samples were extracted according to the Cologne Doping Laboratory procedure developed by Geyer et.al. High levels of DHEA and nandrolone precursor 19-nor-4-androstene-3,17-dione were found. Most frequently detected prohormone was 4-androstene-3,17-dione at considerable levels.

### **INTRODUCTION**

We considered necessary to continue the study on the contamination of the nutritional supplements distributed on the Romanian market we started during the last few years [1], by extending our researches with the prohormones analysis.

Both labeled and not-declared prohormones products were analyzed. These nutritional supplements were obtained from the Romanian market, being merchandised at pharmacies, most of them being produced by American and Canadian companies. A number of 14 nutritional supplements were tested by GC/MS for 9 anabolic steroids.

### **MATERIAL AND METHODS**

#### ***References substances***

The samples were screened for 9 different prohormones. The reference standards were purchased from Steraloids UK (19-nor-5-androstene-3 $\beta$ ,17 $\beta$ -diol, 19-nor-4-androstene-3 $\beta$ ,17 $\beta$ -diol, 4-androstene-3 $\beta$ ,17 $\beta$ -diol, 19-nor-4-androstene-3,17-dione, 5-androstene-3 $\beta$ ,17 $\beta$ -diol, 4-androsten-3,17-dione), from NARL Australia (d<sub>4</sub>-noretiocholanolone), from Sigma (DHEA, testosterone) and from Fluka (19-nortestosterone).

### ***Sample preparation***

The samples were prepared for the extraction of steroidal compounds according to the standard procedure [2, 3, 4], using 1g of each nutritional supplement.

The extraction was performed by liquid-liquid extraction of the dried methanolic extract with n-pentane (pH 12), followed by the re-extraction from the organic layer with methanol 95% and the GC/MS analysis of the bis-TMS derivatives of the steroids. D<sub>4</sub>-noretiocholanolone as internal standard (100µl of 1ppm solution) and 2µl of DIPA were added to each sample.

### ***GC/MS analysis***

System : Agilent GC 6890N / MS 5973 Network;

Column: ULTRA 1, 17m length, i.d. 0,25mm, film thickness 0,12µm;

Carrier gas: Helium 0,8 ml/min;

Injector temperature: 300°C, injection mode-split 1:10;

Oven: 160°C (2min), 5°C/min, 225°C, 30°C/min, 285°C (5min), 60°C/min, 300°C (2min);

Acquisition mode: SIM (retention times and monitored ions are presented in **Table 1**).

### ***Quantification***

*For small concentration ranges*

Method: internal standard (d<sub>4</sub>-noretiocholanolone) calibration curves;

Calibration solutions concentration: 10µg/ml, for each reference standard;

Working solution: 1µg/ml mixture of prohormones standards;

Calibration levels: 5-40ng (5,10,15,20,25,30,35,40µl working solution corresponding to 5,10,15,20,25,30,35,40ng);

Correlation coefficients: between 0,979-0,999.

In case of *larger prohormones amounts* in nutritional supplements a standard in a very similar concentration to that in product was used for a rough estimation of the concentration.

## **RESULTS AND DISCUSSION**

As already shown it is well known that the analysis of the nutritional supplements for anabolic steroids is rather difficult because of the strong influence of the matrix compounds [2] and that there are important variations between different capsules and tablets of the same lot number [3,4]. Therefore some precautions were taken in order to minimize the matrices effects, such as:

- careful homogenization of samples in a mortar and by stirring if necessary;
- DIPAs addition to the final extract;

- pure derivatisation reagent and blank of creatine injections included in GC/MS sequence;
- quantification with calibration curves for low concentrations and a rough estimation of concentration in case of larger amounts of prohormones.

The validation parameters, presented in **Table 1**, were determined in spiked pure creatine monohydrate powder and the recovery was determined at 200ng/g. The results of the analysis for the 14 nutritional supplements are presented in **Table 2**.

**Table 1.** Method parameters and validation parameters for the analyzed prohormones in nutritional supplements

Compound	Method parameters			Validation parameters		
	RT <sup>a</sup> (min)	RRT <sup>b</sup>	m/z (SIM) <sup>c</sup>	LOD <sup>d</sup> ng/g	RSD <sup>e</sup> (%)	Recovery (%)
d <sub>4</sub> -norethiocholanolone (ISTD)	11.88	1.000	<b>409, 424</b>	-	-	-
19-nor-5-androstene-3 $\beta$ ,17 $\beta$ -diol	12.67	1.066	225, <b>330</b>	1.8	2.52	48
19-nor-4-androstene-3 $\beta$ ,17 $\beta$ -diol	12.83	1.079	405, <b>420</b>	2.8	4.54	68
DHEA	13.33	1.122	417, <b>432</b>	2.5	2.29	75
4-androstene-3 $\beta$ ,17 $\beta$ -diol	13.37	1.125	419, 434	2.7	4.08	78
19-nor-4-androstene-3,17-dione	13.40	1.128	401, <b>416</b>	1.6	2.56	38
5-androstene-3 $\beta$ ,17 $\beta$ -diol	13.58	1.140	<b>239, 434</b>	3.8	2.60	46
19-nortestosterone	13.67	1.150	403, <b>418</b>	1.5	3.02	29
4-androsten-3,17-dione	14.03	1.181	169,415, <b>430</b>	2.0	3.93	60
testosterone	14.29	1.203	417, <b>432</b>	2.3	2.39	58

<sup>a</sup> Retention time

<sup>b</sup> Relative retention time to d<sub>4</sub>-norethiocholanolone

<sup>c</sup> m/z monitored in SIM acquisition mode, bolded ions for quantification

<sup>d</sup> Limit of detection

<sup>e</sup> Intra assay precision expressed as relative standard deviation

## CONCLUSIONS

The reason for the presence of the doping substances in nutritional supplements (contamination or intentional addition) being unknown and also the assumed concentration order, it is difficult to select a calibration range which should cover the analysis requirements.

We found that the nutritional supplements “Creatin Monohidrat”, “Max AminoVital”, “ProAnabolic” and “DietFuel” were contaminated with some prohormones, which were not declared on the label.

Some amounts of prohormones were also determined in products such as “Animal Stack”, “Androtech” and “Crea/Max”, according to their labeled composition.

4-androstene-3,17-dione was identified in five from seven contaminated nutritional supplements.

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**Table 2.** Analysis of nutritional supplements

No	Product	Labeled prohormones	Contaminated (Not-declared prohormones)	Prohormones detected amount (ng/g)				
				DHEA	4-androstene-3,17-dione	19-nor-4-androstene-3,17-dione	5- $\beta$ -androstene-3,17-diol	Testosterone
1.	Hyosport protein	no	no	-	-	-	-	-
2.	Amino 2000	no	no	-	-	-	-	-
3.	Crea/Max	yes	-	5895	-	-	-	-
4.	Liquid LCarnitine	no	no	-	-	-	-	-
5.	Creatinmonohidrat	no	yes	-	12.5	-	-	8.7
6.	Phosphocreatin Power	no	no	-	-	-	-	-
7.	Animal Stak	yes	-	4590	7850	5718	4850	6.5
8.	MaxAminoVital	no	yes	-	18.5	-	-	-
9.	Vitalitis Sport	no	no	-	-	-	-	-
10.	ProAnabolic	no	yes	25.8	-	-	-	-
11.	Power Blast	no	no	-	-	-	-	-
12.	Androtech	yes	-	-	8570	-	-	-
13.	Diet Fuel	no	yes	14.5	20.1	-	-	-
14.	Total Mass-4200	no	no	-	-	-	-	-