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Optimization and validation of analytical method for anabolic steroids in nutritional supplements by GC/MS

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Introduction

The widespread use of nutritional supplements may be problematic for athletes worldwide.
Several studies demonstrated the occurrence of anabolic steroids in some nutritional supplements [1].
The consumption of such products by athletes may constitute a serious risk of positive response in anti-doping controls [2].
The aim of this study is to develop a new method for the detection of 20 anabolic steroids in some energy drinks available in the Tunisian markets.

Experimental

Supplements

Ten aqueous nutritional supplement were commercialized in Tunisian markets and imported from different countries most of them from Italy and Austria.

Sample preparation

The optimization was realized firstly by using several solvents (MTBE, n-Pentane and DEE) to extract anabolic steroids from these aqueous nutritional supplements at fixed pH=11, according to the screening procedure for anabolic steroids in free fraction; secondly we study the effect of the variation of the pH values (9, 10, 11, 12, 14) with fixed solvent (MTBE).
This protocol of extraction was applied to 5 ml of the liquid supplements, 20µl of 17α-methyltestosterone used as internal standard were added, the pH was adjusted to 10. The sample was extracted with 5 ml of a mixture of Diethylether/n-pentane (50/50) for one hour, followed by centrifugation 2500 rpm for 5 min to separate the organic phase from the aqueous. The aqueous phase was discarded and the organic phase was then evaporated to dryness under a nitrogen stream. The dry residue was derivatized with 50 µl MSTFA/NH_4I/dithioerythritol (5:2:1 v/w/w) and heated for 30 min at 65°C. 3 µl of the solution were injected into the gas chromatography/mass spectrometry (GC/MS) system.

Instrumentation

GC: HP6890 MS: HP5973

HP Ultra-1 column, L = 25 m, i.d. = 0.2 mm, ef = 0.11 µm

Injection: 3µl, split ratio 1/20

Temp. program: 190°C, 2°C per min. until 230°C, 18°C per min, 320°C

MS Parameters: SIM acquisition

Results and discussion

The results (table 1) from the extraction experiments at different pH-values show that at pH = 10 the extraction proceeds with better yield than at others values. The extractions performed with a mixture of diethylether and n-pentane gave higher yields of extraction than the other investigated solvents. The extraction recovery for all the compounds in the optimum conditions is acceptable at fixed concentration of 100 ng/ml.

Table 1: Effect of pH and organic solvents on the recovery

Compound	solvent effect on pH= 11					pH effect with MTBE as extraction's solvent				
	MTBE	n-pentane	DEE	MeOH/ n-pentane 50/50	DEE/ n-pentane 50/50	pH= 9	pH= 10	pH= 11	pH= 12	pH= 14
19Norandrostenedione	73.65	63.31	58.13	18.20	87.36	80.76	97.85	98.79	90.56	92.21
Androstenedione	66.41	71.50	77.71	28.11	95.56	93.21	99.46	81.10	82.99	95.79
Bolasterone	78.56	82.67	92.14	33.84	87.42	92.39	93.92	78.64	94.15	90.30
Boldenone	67.75	44.48	52.10	3.24	90.17	43.89	94.54	84.63	93.83	97.35
Clenbuterol	68.91	51.24	67.68	15.16	70.27	97.11	93.64	99.27	91.67	96.66
Danazol	30.91	80.20	34.10	24.89	83.32	82.33	93.15	90.15	96.19	52.12
DHEA	72.43	68.34	104.11	28.78	84.50	85.57	97.41	99.93	93.36	91.83
Epitestosterone	69.57	65.61	106.88	14.36	85.00	92.96	94.81	92.08	92.18	94.57
Fluoxymesterone	66.73	96.43	51.29	40.77	33.03	66.53	80.75	78.41	84.83	71.94
Furazabol	81.10	83.27	96.75	13.33	61.76	59.58	96.76	85.87	99.24	89.34
Mesterolone	84.02	70.67	94.61	41.18	87.95	51.16	99.77	51.04	90.09	71.19
Methandienone	63.30	63.80	84.54	6.67	93.99	54.21	98.22	96.54	96.75	94.86
Methenolone	67.35	76.08	99.36	16.37	84.44	97.19	98.08	105.79	98.51	95.38
Mibolerone	66.46	67.83	89.80	19.55	91.74	90.52	97.57	94.43	95.89	89.23
Nandrolone deca.	45.80	23.85	57.49	98.79	50.67	45.76	48.88	43.68	58.67	37.20
Oxandrolone	89.90	6.02	16.75	2.63	71.15	80.22	90.67	51.90	0.05	0.05
Stanozolol	88.13	65.93	20.24	73.88	78.74	67.57	63.07	103.52	52.05	26.75
Testostérone	72.63	60.02	108.04	9.89	83.82	93.29	97.44	103.30	95.92	93.67
Zeranol	79.83	1.68	57.40	2.84	81.07	101.77	97.42	97.21	16.24	0.25

Validation parameters

Limit of detection

The limits of detection (table 2) at signal to noise ratio (S/N >= 3) obtained were between 1ng/ml and 20 ng/ml.

Specificity

No interference was found.

The reproducibility of the method was determined by analyzing three different spiked juices at three levels (1.5LOD, 3LOD, 6LOD) for three days. The RSD ranged from 0.37% to 19.34% fulfilled the following acceptance criteria; no more than 20% [3].

Recovery

Recovery was calculated at three different levels (1.5LOD, 3LOD, 6LOD) using spiked juice with three replications per concentration during three days. The recovery is between 52.6 and 98.4 % for the different compounds.

Table 2: Method parameters and validation parameters for anabolic steroids in nutritional supplements

Compound	**Method parameters**			**Validation parameters**		
	RT [a] (min)	RRT [b]	*m/z* (SIM)[c]	LOD[d] (ng/ml)	Intra assay precision (CV%)	Recovery (%)
19-norandrostenedione	20.25	0.830	416, 401, 194	1	13,5	96
Androstenedione	21.87	0.890	430, 415, 234	1	3,0	91
Bolasterone	24.40	1.011	445, 460, 355	5	1,1	94,3
Boldenone	21.64	0.890	206, 430, 415	5	3,1	93
Clenbuterol	7.64	0.317	86, 335, 300	1	2,4	93
Danazol	27.79	1.150	466, 481, 346	10	8,8	94
DHEA	20.00	0.820	432, 417, 327	1	1,5	100,1
DHT	21.17	0.870	434, 405, 143	2	9,9	101
Epitestosterone	20.77	0.857	432, 417, 327	1	3,8	95
Fluoxymesterone	26.60	1.099	552, 462, 407	20	10,8	60
Furazabol	26.18	1.081	143, 387, 402	5	12,1	95
Mesterolone	21.80	0.895	433, 448, 157	10	4,1	109
Methandienone	24.01	0.988	206, 444, 339	2	8,0	95
Methenolone	23.07	0.948	195, 431, 446	5	1,7	88,2
$Methyltestosterone_{ISTD}$	24.24	1.000	301, 446	-[e]	-[e]	-[e]
Mibolerone	23.60	0.976	431, 446, 341	5	2,3	93
Nandrolone decanoate	31.99	1.289	500, 485, 329	2	8,8	90
Oxandrolone	24.70	1.017	143, 308, 321	10	2,6	89
Stanozolol	28.00	1.154	143, 472, 457	10	5,7	70
Testosterone	22.12	0.911	432, 417, 209	1	11,6	98
Zeranol	25.20	1.048	433, 538, 523	2	0,3	93,8

[a] Retention time
[b] Relative retention time to Methyltestosterone
[c] m/z monitored in SIM acquisition mode
[d] Limit of detection
[e] Not validated

Method application

According to the validated analytical method previously described five energy drinks were tested for anabolic steroids. As expected no prohibited substance was detected.

Conclusion

A GC/MS method for the detection of several anabolic steroids present in some nutritional supplements was developed and validated. The results showed that the developed method is suitable to detect simultaneously and various substances with concentrations as low as 1 ng/ml. The application of the proposed method show that no prohibited substances were detected in such category of supplements present in the Tunisian market, nevertheless it could be better to extend this study to other forms.

References

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