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Research Article

Development and validation of RP-HPLC method for analytical characterization of the anabolic steroid Methenolone acetate in food supplements

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Abstract

Nutritional supplements are concentrated sources of nutrients, vitamins and minerals with a nutritional or physiological effect, the purpose of which is to supplement the normal nutritional balance. A necessity of the health risk associated with their unregulated usage requires development of suitable fast and precise methods for their evaluation. As an answer to this a novel RP-HPLC method for analytical characterization of the anabolic steroid Methenolone acetate in food supplement was developed. The method is based on separation and evaluation performed on an analytical column Kintex 5μm EVO C18 (100 × 4.6 mm) with mobile phase acetonitrile:water = 60:40, v/v; flow rate of 1.0 mL/min and UV-detection at 240 nm and column temperature, at 25 °C. The method has proved to be specific, linear, accurate and precise. The method identified a presence of both Methenolone and Methenolone acetate in the evaluated sample.

Keywords

anabolic steroid, Methenolone, RP-HPLC

Introduction

In recent years, there has been increased interest in food supplements. Nutritional supplements are concentrated sources of nutrients, vitamins and minerals with a nutritional or physiological effect, the purpose of which is to supplement the normal nutritional balance (Odoardi et al. 2015). Due to the liberal legislative framework worldwide, there are often undeclared ingredients in food supplements or a discrepancy between the actual qualitative and quantitative content and the one announced by the manufacturer. All this requires an assessment of the risk associated with the health, well-being and safety of food supplements users and the development of appropriate methods for analysis related to unregulated ingredients in certain food supplements (Walters et al. 1990).

There are nutritional supplements with a steroid structure (Mellion 1984; Kibble and Ross 1987; Penn 1988). Steroids are cyclopentanoperhydrophenanthrenes with lipid properties. Structurally, they are tetracyclic compounds. Steroids differ in their functional groups associated with these rings (Birdsong 1986; Moore 1988). Many anabolic steroids are used by athletes to improve their performance. For pharmacological purposes, anabolic steroids are used for hormonal problems, such as delayed puberty and reduced secondary sexual characteristics. In addition, steroids

are used to combat muscle loss (cachexia). Anabolic abuse can have negative effects on mental health, such as paranoid and extreme feelings of jealousy, extreme irritability and aggression, misconceptions and perceptions, blurred judgment, mania. In addition, a common effect is acne and swelling of parts of the body, often the limbs. Long-term effects such as kidney, liver problems, high blood pressure, problems with cholesterol levels, higher risk of clots are also possible (De Cock et al. 2001).

Methenolone also known as 1-methyl-5α-androst-1-en-17β-ol-3-one is an androgen and anabolic steroid

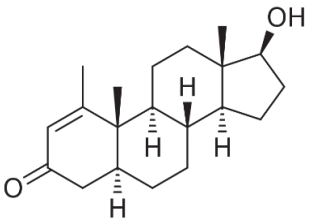

Figure 1. Structure of Methenolone.

(AAS) (Figure 1) (Taylor and Francis 2000; Llewellyn 2011; Morton and Hall 2012; Elks 2014). It is applied as esters like Methenolone acetate and Methenolone enanthate. Methenolone esters are used in the treatment of anemia (Jameson JL and De Groot LJ, 2015), osteoporosis and sarcopenia, to inhibit the natural loss of muscle mass with aging, and to promote weight gain in underweight premature infants and children (Llewellyn 2011). Methenolone acetate is widely used in athletes to increase muscle mass and strength (Sandra et al.1989; Noggle et al. 1990; Apffel and Perry 1991). It has good anabolic and very weak androgenic properties, which makes it preferred by both men and women. Moderate or low physical dependence or high psychological dependence may occur with Methenolone acetate abuse.

This study is focused on the development and validation of an easy, selective and accurate RP-HPLC method for identification of Methenolone and its acetate ester in nutritional supplements used to build muscle mass.

Materials and methods

Chemicals and reagents

All the necessary reagents for preparation of the mobile phase and solutions were obtained from Sigma-Aldrich (Steinheim, Germany).

Instrumentation and chromatographic conditions

UltiMateDionex 3000 SD liquid chromatograph, Chromeleon 7.2 SR3 Systems, Thermo Fisher Scientific Inc. was used to analyze Methenolone in a food supplement. The chromatographic system was equipped with an analytical column Kintex 5µm EVO C18, 100 × 4.6 mm, with a column temperature of 25 ˚C and injection volume of 20 µl. The mixture of an acetonitrile and water in a ratio 60:40 (v/v) was used as a mobile phase. The flow rate was set to 1.0 mL/min. Detection was performed by measurement of the absorption at 240 nm.

Mobile phase composition

The mobile phase that was prepared and implemented consisted of CH_3CN:H_2O = 60:40 (v/v).

Preparation of the standard solutions

A 0.001 g of Methenolone and Methenolone acetate were weighed and dissolved in methanol in two 10 ml volumetric flasks. The obtained solutions were injected into the HPLC system.

Preparation of the test solution

Five tablets of the tested food supplement were weighed on an analytical balance and powdered using mortar and pestle. The resulting mixture was transferred to a 500 ml volumetric flask and dissolved in methanol to the mark. The resulting solution was centrifuged for 5 min. at 2000 rpm. The supernatant was filtered first through a 0.45 µm filter and then through 0.22 µm. A sample of 20 µl of the obtained solution was injected into the HPLC system and analyzed.

Results and discussion

The main purpose of this study was to develop an easy, selective and accurate RP-HPLC method for the identification of Methenolone in the food supplement. An analytical column Kintex 5µm EVO C18, 100 × 4.6 mm was selected. During the method development, several mobile phases were investigated. The chemical structure of the analyte determined the following reagents and mixtures as most suitable for evaluation: methanol, acetonitrile and phosphate buffers with different pH. The best selectivity and resolution were achieved in the mobile phase consisting of acetonitrile: water (60:40 v/v). The final chromatographic conditions are shown in Table 1.

Validation of the developed RP-HPLC method

The developed RP-HPLC method is validated with respect to following parameters: specificity, linearity, accuracy, precision, as required by ICH (International Council for Harmonisation).

Specificity

No significant interfering peaks (peak area > 0.1%) were observed during the retention time of the analyzed Methenolone and mixtures in blank solution. In addition, no

Table 1. The final chromatographic conditions of the developed RP-HPLC method.

RP-HPLC column	Kintex 5µm EVO C4.6 × 100 ,18 mm
Mobile phase	$CH_3CN:H_2O$ = 60:40 (v/v)
Wavelength	240 nm
Flow rate	1.0 mL/min
Injection volume	20 µl
Temperature	25 °C

evidence of co-elution was noted using peak purity analysis for the tested Methenolone.

Linearity

The linearity of the developed method was observed in the concentration range from 0.0125 µg / mL to 0.1 µg / mL. The correlation coefficient of the linearity R^2 is 0.9926, which shows a linear relationship between the range of concentrations studied and the peak area as shown in Figure 2.

Accuracy

The accuracy of the analytical procedure is defined as the degree of coincidence between the measurement result and the true value of the measured quantity. Evaluation of the accuracy of the method developed in this study was within the range of 99.8% to 100.4% recovery. These results show that the developed method was accurate within the acceptable limits as presented in Table 2.

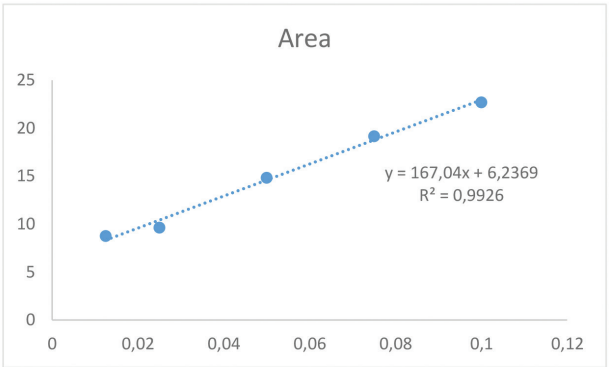

Figure 2. Linearity of the developed RP-HPLC method.

Precision

The precision of the method was assessed by analysis of six Methenolone solutions at 100% area. The calculated value of RSD % for precision assessment is 0.182, thus meeting the RSD ≤ 2% criteria, which confirms that the method is precise within acceptable limits. The precision of the method is presented in Table 3.

Due to the insolubility and poor pharmacokinetic of Methenolone, as most appropriate for therapeutic application is found to be the corresponding Methenolone acetate ester. In order to be able to identify both the Methenolone and its acetate ester, standard solutions of both samples were analyzed with the developed and validated RP-HPLC procedure. The obtained chromatograms are presented on Figure 3 and Figure 4 for the evaluated stan-

Table 2. Evaluation of the accuracy of the developed method.

Level	Replicate number	Area	Recovery
50%	0,025	9,625	99,9
	0,025	9,653	100,2
	0,025	9,634	100,0
100%	0,05	14,873	100,1
	0,05	14,835	99,8
	0,05	14,868	100,0
150%	0,075	19,141	100,1
	0,075	19,113	99,9
	0,075	19,134	100,0

Table 3. Evaluation of the precision of the developed method.

Replicate number	Area
1	14,887
2	14,89
3	14,873
4	14,835
5	14,868
6	14,825
Mean recovery	**14.863**
% RSD	**0.181983**

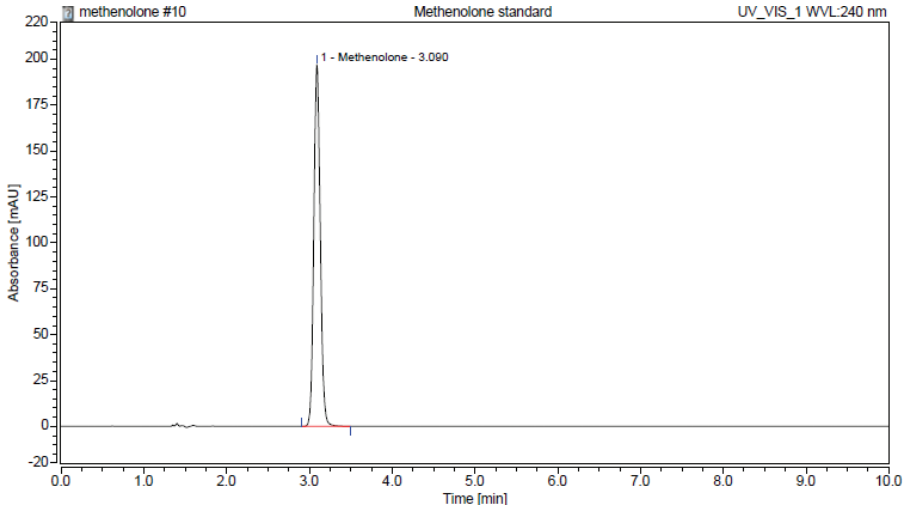

Figure 3. Chromatogram of Methenolone standard solution.

dards of Methenolone and ethenolone acetate, respectively. The retention times of analyzed compound and its ester are found to be 3.090 min for Methenolone and 7.717 min for the Methenolone acetate.

Methenolone is synthetic steroid, derivative of dihydrotestosterone (DHT) and agonist of the androgen receptor. The presence of Methenolone plays an important role in the manifestation of the type and strength of the pharmacological effect. On the other hand, the purity of the substance is crucial for its applicability in drug practice, based on the fact that the drug molecule is obtained through total chemical synthesis, which sometimes leads to appearance in its composition of side and additional substances can have severe side effects on the body.

The Methenolone itself has poor pharmacokinetic and it is marketed and applied as the acetate ester for oral administration and as the enanthate ester for intramuscular injection. The Methenolone enanthate ester is having a higher bioavailability and quite long acting. The Methenolone acetate ester has few side effects, reduces the stress on the liver and the bioavailability, and it is one of the safer steroids.

In this study, we examined a tablet form of a dietary supplement supposedly containing Methenolone acetate, as described on the package.

After proper treatment of the analyzed food supplement, identified as Sample 1, the obtained solution was injected into the chromatographic system. The corresponding result is presented on Figure 5. The chromatogram demonstrates several peaks.

One of them corresponding to the retention time of the Methenolone (3.053 min) in high amount. In addition, the chromatogram shows a peak of Methenolone acetate at 7.810 min with area 30 times less than the one for Methenolone.

The obtained result lead us to the conclusion that the identified in the label Methenolone acetate is found as impurity rather than as a main constituent.

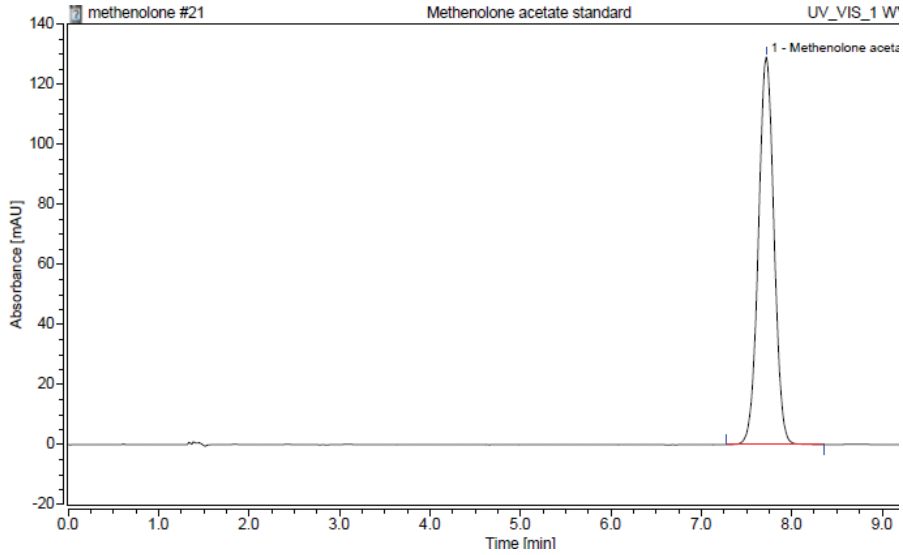

Figure 4. Chromatogram of Methenolone acetate standard solution.

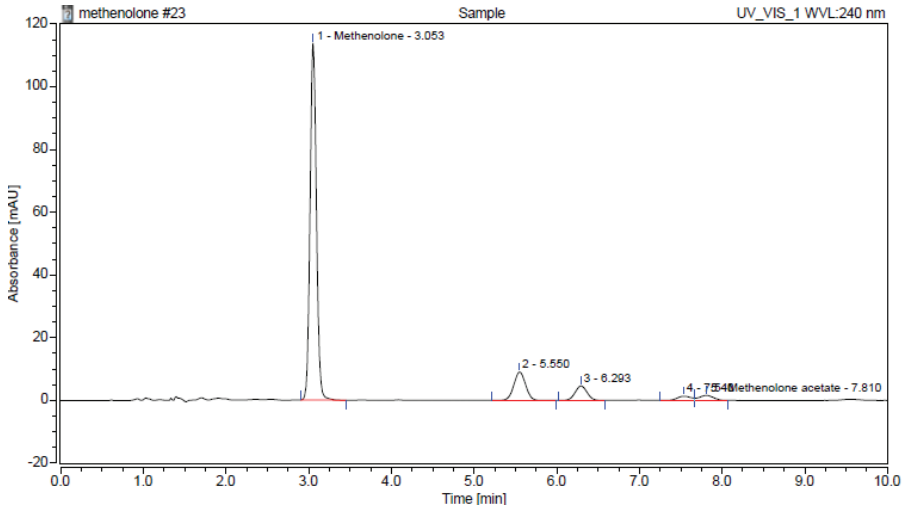

Figure 5. Chromatogram of test solution.

Conclusion

A novel RP-HPLC method for analytical characterization of the anabolic steroid Methenolone acetate in food supplement was developed. The optimal chromatographic conditions were achieved using an analytical column Kintex 5µm EVO C18 (100 × 4.6 mm); mobile phase acetonitrile:water in ratio 60:40, v/v; flow rate, 1.0 mL/min; UV-detection, 240 nm and column temperature, at 25 °C. The method has proved to be specific, linear, accurate and precise. The method identified a presence of both Methenolone and Methenolone acetate in the evaluated sample.

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References

Apffel A, Perry ML (1991) Quantitation and linearity for particle-beam liquid chromatography-mass spectrometry. Journal of Chromatography A 554(1–2): 103–118. https://doi.org/10.1016/S0021-9673(01)88441-7

Birdsong C (1986) Why athletes use drugs. Am. Pharm. 26: e43.

De Cock KJS, Delbeke FT, Van Eenoo P, Desmet N, Roels K, De Backer P (2001) Detection and determination of anabolic steroids in nutritional supplements. Journal of Pharmaceutical and Biomedical Analysis 25(5–6): 843–52. https://doi.org/10.1016/S0731-7085(01)00396-X

Elks J (2014) The dictionary of drugs: chemical data: chemical data, structures and bibliographies. Springer, 784 pp.

Schweizerischer Apotheker-Verein, Swiss Pharmaceutical Society (2000) Index Nominum 2000: International Drug Directory 17: 659–660. Jameson JL, De Groot LJ (2015) Endocrinology: Adult and Pediatric E-Book. Elsevier Health Sciences 2388. [ISBN 978-0-323-32195-2]

Kibble MW, Ross MB (1987) Adverse effects of anabolic steroids in athletes. Clinical Pharmacology 6(9): 686–692.

Llewellyn W (2011) Anabolics. Molecular Nutrition Llc. 625, 633. [ISBN 978-0-9828280-1-4]

Mellion MB (1984) Anabolic steroids in athletes. American Family Physician 30: 113–19.

Moore WV (1988) Anabolic steroid use in adolescence (editorial). Journal of the American Medical Association 260: 3404–06. https://doi.org/10.1001/jama.1988.03410230102038

Morton IK, Hall JM (2012) Concise dictionary of pharmacological agents: properties and synonyms. Springer Science & Business Media 178 pp.

Noggle Jr FT, Clark CR, DeRuiter J (1990) Liquid chromatographic and mass spectral analysis of the anabolic 17-hydroxy steroid esters. Journal of chromatographic science 28(5): 263–268. https://doi.org/10.1093/chromsci/28.5.263

Odoardi S, Castrignanò E, Martello S, Chiarotti M, Strano-Rossi S (2015) Determination of anabolic agents in dietary supplements by liquid chromatography-high-resolution mass spectrometry. Food Additives & Contaminants: Part A 32(5): 635–47. https://doi.org/10.1080/19440049.2015.1014868

Penn S (1988) Muscling in as ever more people try anabolic steroids; traffickers take over. Wall Street Journal 1.

Sandra P, Courselle P, Steenbeke G, Schelfaut M (1989) Dual channel detection-diode array and mass spectroscopy (El or Cl)–in HPLC. Journal of High Resolution Chromatography 12(8): 544–546. https://doi.org/10.1002/jhrc.1240120810

Walters MJ, Ayers RJ, Brown DJ (1990) Analysis of illegally distributed anabolic steroid products by liquid chromatography with identity confirmation by mass spectrometry or infrared spectrometry. Journal of AOAC International 73(6): 904–26. https://doi.org/10.1093/jaoac/73.6.904