# Development of an Analytical Methodology for the Qualitative and Quantitative Characterization of Capsules with Andarine, in Order to Use them to Investigate the Pharmacotoxicological Profile of the Substance

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*Andarine is one of the most commonly used doping substance between selective androgen receptor modulators (SARMs). The purpose of our study is to characterize qualitatively and quantitatively andarine capsules purchased legally via the Internet through a rapid and precise UHPLC method and the uniformity of mass, content and dissolution assays were determined following the methodology of the European Pharmacopoeia 8th Ed. Regarding the uniformity of mass, the capsules are within the permissible limits, but regarding the active substance content, an average of 4.99 mg of andarine/capsule was obtained (with an RSD of 5.41%) from the 25 mg/capsule declared by the manufacturer.*

*Keywords: andarine, SARMs, UHPLC, method*

Andarine, also known as S-4, acetamidoxolutamide or GTx-007 is one of the first synthesized selective androgen receptor modulator (SARM). SARMs present an important binding affinity to androgen receptors (AR) located in the muscular and bone tissues exhibiting anabolic effects [1].

Moreover, compared to the endogenous hormone testosterone, SARMs lack virilizing effects [2-4].

Therefore, andarine detain tissue-specific affinity, being a candidate as therapeutic agent in pathologies characterized by muscle wasting and bone tissue damages [5, 6].

Even if it was discovered more than two decades ago, this substance has failed to obtain marketing authorization, although it has been shown to be effective in different stages of clinical trials in cachexia, breast cancer, hypogonadism, osteoporosis [7-12].

Currently, andarine is a prohibited substance for athletes, being included since 2008 in the S1 class of the Word Anti-Doping Agency (WADA) Prohibited list [13].

Despite of this fact, S-4 is still used by performance athletes to improve sports performance and the researchers' interest in developing methods for the identification and quantification of andarine or its metabolites in various biological matrices (plasma, urine, hair) is steadily increasing [14- 18].

At the same time, this substance is available for purchase via the Internet as dietary supplements in the form of powders, capsules or oily liquid forms. Dietary supplements are not controlled by the manufacturer in terms of the declared content and also may contain other undeclared substances that can cause risky health effects [19, 20].

Besides this, SARMs represent a topic of interest and a temptation for doping amongst both athletes and teenagers who want to improve their physical appearance.

The latest scientific articles are centered on developing methods to unmask doping in various biological matrices, but there are only a few articles regarding the control of dietary supplements that can be purchased in shops or via the Internet.

This fact is a cause of concern regarding their possible abuse, because they do not have a clearly determined pharmacotoxicological profile [21- 24].

Therefore, the purpose of our preliminary study was to determine qualitatively and quantitatively the andarine content in dietary supplements (finished products, capsules, with a declared content of 25 mg andarine/capsule), legally obtained via the Internet, through a fast, simple, precise and validated UHPLC method.

The uniformity of mass and content of the capsules were also tested and due to the difficulties encountered during the development of the analytical method in extracting the analyte, the capsules were subjected to dissolution profile testing.

These preliminary tests represent an important step in designing an animal doping model for the future investigation of the pharmacotoxicological profile of andarine.

## Experimental part
### Chemicals, reagents
Andarine reference standard (($S$)-3-(4-Acetylaminophenoxy)-2-hydroxy-2-methyl-$N$-(4-nitro-3-trifluoromethylphenyl)propionamide) was purchased from Sigma-Aldrich

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(St. Louis, USA). HPLC grade methanol, hydrochloric acid and acetonitrile were purchased from Merck (Darmstadt, Germany) and sodium hydroxide, trisodium phosphate, sodium lauryl sulfate and formic acid from Sigma-Aldrich. Ultrapure water was produced by a Merck Millipore (Burlington, USA) Direct-Q3 water purification system.

*Preparation of solutions*

A stock solution of andarine was prepared in methanol at a concentration of 200 μg/mL. Standard calibration solutions were prepared on the day of analysis (1, 5, 10, 15, 20 and 25μg/mL) by appropriate dilution of the stock standard solution (200 μg/mL) with 0.1% aqueous formic acid.

During method validation, blank (mobile phase), placebo solutions, but also reconstituted samples were prepared.

Reconstituted samples (containing andarine and rice flour, the only excipient declared by the manufacturer) at 5 concentration levels (between 1-25 μg/mL) were prepared on the day of analysis. The solutions were prepared by weighing andarine and rice flour according to table 1 and the extraction was made with methanol by stirring the sample for 40 min on the hotplate magnetic stirrer at 800 rpm, then sonicated for 20 min. Each of the solutions obtained were diluted 25 fold (0.4 mL diluted to 10 mL) with 0.1% formic acid, then filtered through nylon filters (0.45μm) before being transferred to HPLC vials and injected into the HPLC system.

The placebo solutions were prepared by weighing the appropriate amount of rice flour equivalent to the content of one capsule (545 mg) to a 50 mL flask, following the same extraction steps with methanol as the reconstituted samples and finally making a 25 fold dilution (0.4 mL diluted to 10 mL) with 0.1% formic acid.

To determine the uniformity of andarine content, samples were prepared by extracting with methanol the powder from a single capsule in a 50 mL flask, similarly to the extraction method described for the reconstituted samples and making a 25 fold dilution (0.4 mL diluted to 10 mL) with 0.1% formic acid. Samples were filtered through nylon filters (0.45 μm) before being transferred to HPLC vials to be injected into the HPLC system.

The samples for average assay testing were prepared by pooling the content of capsules, mixing it and weighing approximately 57 mg of powder to a 5 mL flasks and performing the extraction method described for reconstituted samples, then making a 25 fold dilution (0.4 mL diluted to 10 mL) with 0.1% formic acid.

*Analytical methods*

The uniformity of mass and the uniformity of content of single-dose preparation were determined following the methodology described in the European Pharmacopoeia 8th Edition [25].

For the quantitative determination of andarine, an UHPLC method was developed and validated on a Flexar-10 Perkin Elmer chromatographic system, using a reversed-phase Gemini NX-C18 3.0 x 100 mm, 3 μm column. The mobile phase consisted of formic acid 0.1% (25%) and methanol (75%) in isocratic elution. The run time was 2.5 min and the flow rate was set to 0.5 mL/min. The column and autosampler tray temperature was set at 25 and 20°C, respectively. The injection volume was 5μL for each sample and the analytical wavelength was set at 244 nm.

The described UHPLC method was validated with regards to selectivity, carry-over, linearity, within-run, between-run accuracy and precision and analyte extraction.

Linearity of calibration curves was determined by calculating the coefficient of correlation (R) for the mean curve, generated by the calibration standards, at six concentration levels, in the range of 1-25 μg/mL (1, 5, 10, 15, 20 and 25 μg/mL).

In order to determine the dissolution profile, an adapted method from the European Pharmacopoeia 8th Edition, method A, for prolonged release dosage forms was used [25].

Even though the tested capsules are conventional-release solid dosage forms, our purpose was to apply a more complex dissolution method which would help release as much as possible of the active substance, taking into account that difficulties were encountered during the development of the extraction method of andarine from capsules. In this regard, the capsules were tested twice in a two-phase dissolution test (acidic phase and buffer phase), two different buffer dissolution media being used for each of the tests, both containing an adjuvant: assay 1 - with pancreatic enzyme powder and assay 2 - with sodium laurylsulfate.

In both dissolution tests, one capsule was added to each of the six cuvettes of the Type 1 dissolution equipment with baskets (Erweka DT 80) containing 750 mL of acid dissolution medium (0.1M hydrochloric acid) thermostated at 37°C, after which stirring was performed at 100 rpm for 2 h. 1 mL samples from each well were collected at 30, 60 min , 90 and 120 min. 0.2M trisodium phosphate solution was then added and the *p*H of each well adjusted to 6.8 using a 2M sodium hydroxide solution. In this phase adjuvant agents were added in each of the two tests: a pancreatic enzyme mixture with enzyme activity of amilase 39000 units, lipase 50000 units and protease 1000 units determined by an European Pharmacopoeia method, for assay 1 and 3 g sodium laurylsulfate (for a final concentration of 10 mM or 0.3% of sodium laurylsulfate) in each of the six cuvettes for assay 2. 1 mL samples of each well were collected at 135 min, 150 min and 165 min. All samples were filtered through nylon filters (0.45μm) and transferred to HPLC vials in order to be analysed.

| Reconstituted samples | | | |
|---|---|---|---|
| Andarine (mg) | Excipient (mg) | Volume (mL) | Final concentration (μg/mL) |
| 1.25 | 545 | 50 | 1 |
| 1.25 | 54.5 | 5 | 10 |
| 1.88 | 54.5 | 5 | 15 |
| 2.50 | 54.5 | 5 | 20 |
| 3.13 | 54.5 | 5 | 25 |

**Table 1**
RECONSTITUTED SAMPLES PREPARATION

## Results and discussions

Regarding the selectivity and the carry-over, no peaks were detected at the retention time (1.7 min) of the analyte in placebo samples and no peaks were detected in blank samples when injecting them immediately after a high concentration sample (25 μg/mL standard solution). The results of the five calibration curves presented a good linearity, with a correlation coefficient R > 0.99 for all of them.

The accuracy and precision evaluated according to validation guidelines using standard samples at 5 different concentration levels (1, 10, 15, 20, 25 μg/mL), reveal a mean within-run accuracy between 94.54-111.84% and precision 0.42-2.07%, respectively. The mean between-run accuracy was 92.76-109.59% and precision between runs was 0.94-2.47%, respectively.

The accuracy and precision of within-run extraction yield were determined using reconstituted samples at 5 levels of different concentrations and resulted in yields between 64.18-78.01%, with variation coefficients between 1.11-2.40% and between-run yields (also tested at 5 different concentration levels) were between 62.72-77.91%, with coefficients of variation between 1.56-2.34%, respectively.

In table 2 the average yields of extraction and the variation between concentration levels are presented.

| Extraction yield | Average yield between all concentration levels (%) | Variation between concentration levels (RSD) | Average extraction yield (%) |
|---|---|---|---|
| Within run | 69.24 | 7.63 | 69.07 |
| Between run | 68.90 | 8.46 | |

It is important to note that andarine molecules present a significant adherence to the rice flour excipient, probably because of the starch content. The first indication for this is the need for a long extraction time which had to be extensively studied and optimized. Secondly, during method development, when trying to filter a standard sample through a cellulose filter (chemically a polysaccharide like starch), we noticed a significant drop in andarine peak areas measured using the UHPLC method, compared to the same solution being filtered through nylon filters and analyzed using the same method.
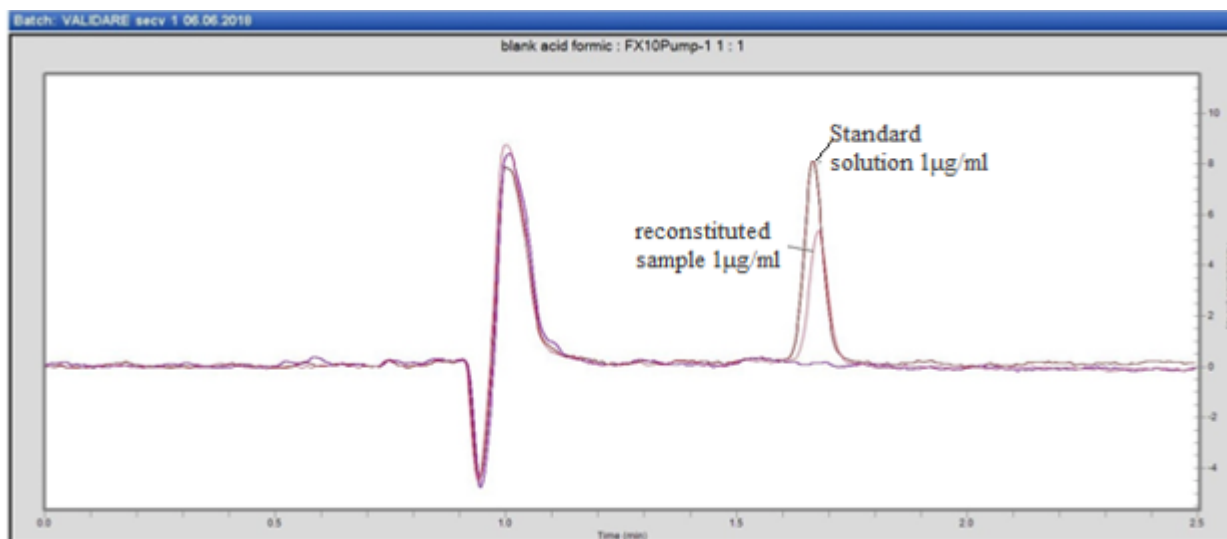


Fig. 1. Overlaid chromatograms of placebo solution, 1μg/mL standard solution and the 1 μg/mL reconstituted sample
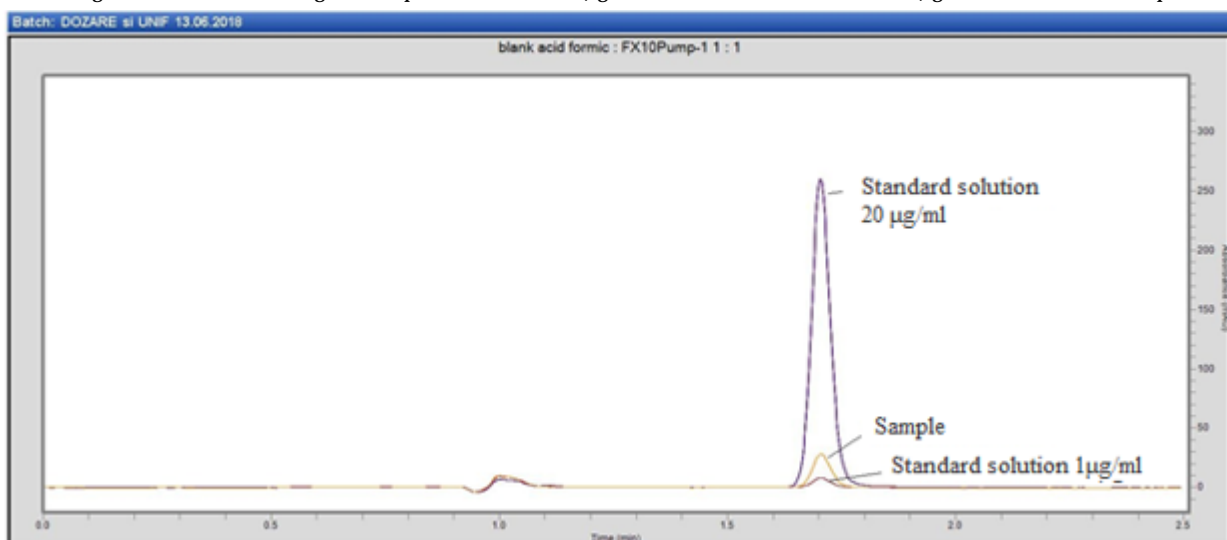


Fig. 2. Overlaid chromatograms of a 1 μg/mL standard solution, 20 μg/mL standard solution and a sample solution prepared from finished product

| Sample | Powder weighed (mg) | Calculated andarine content for 570mg of powder (mg) | % to the teoretical content | Average (mg) |
|--------|---------------------|------------------------------------------------------|------------------------------|--------------|
| 1 | 56.65 | 4.92 | 19.67 | |
| 2 | 56.58 | 4.88 | 19.51 | 4.98 |
| 3 | 56.60 | 5.14 | 20.57 | |

**Table 3**
AVERAGE ANDARINE CONTENT OF
THE POWDER
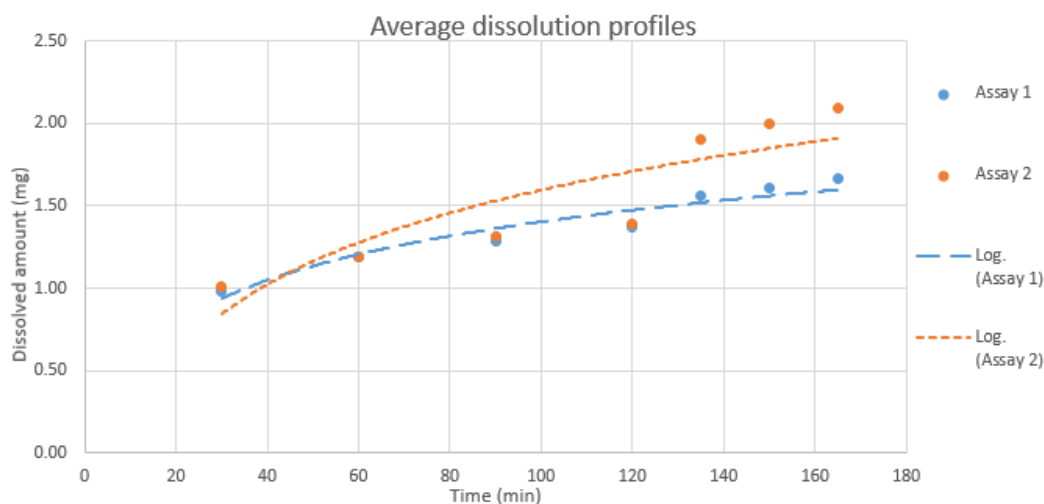


Fig. 3. Average dissolution profiles for assay 1 (with pancreatic enzyme powder) and assay 2 (with sodium laurylsulfate)
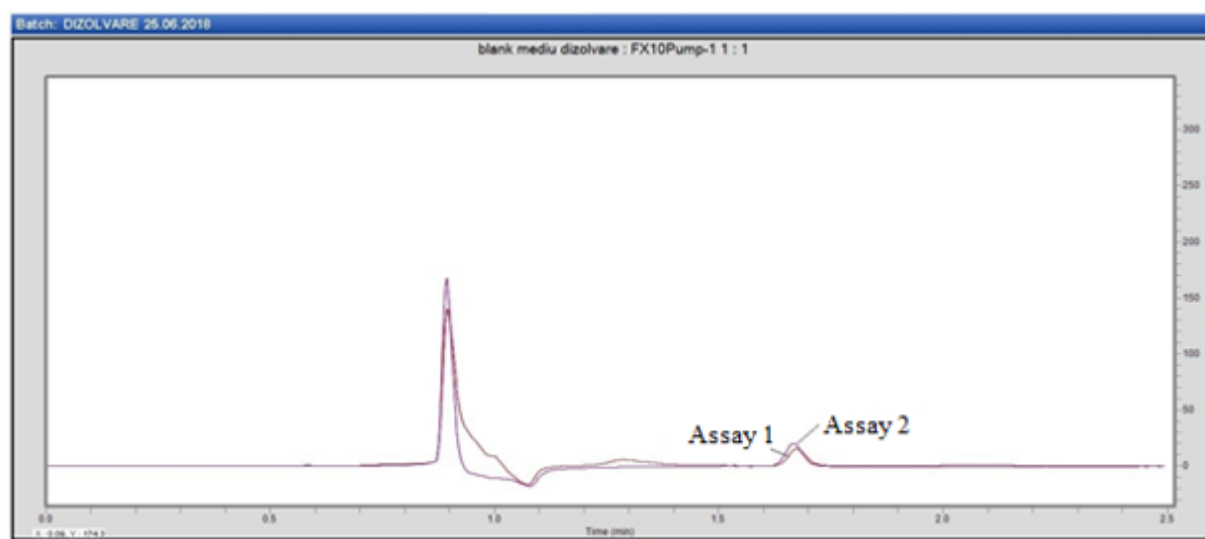


Fig. 4. Overlaid chromatograms of the samples representing assay 1 and assay 2 (sampling time 165 min)

This effect can be seen in figure 1: despite the long time of extraction and complex method, not all the added andarine content was extracted from the reconstituted sample, this difference between standard and reconstituted sample of theoretically the same concentration, confirming that there is some interaction between andarine and the excipient.

Regarding the uniformity of mass testing, an average mass of 570 mg content was determined per capsule, with an RSD of 2.05%. The percentage deviation for individual capsule content was in the range -2.85 and +2.63%.

Regarding the uniformity of the content, a 5.41% RSD was obtained after testing 10 individual capsules with an average content of 4.99 mg andarine/capsule.

Figure 2 shows obvious underdosing of the capsules, about five times less compared to the quantity declared by the manufacturer, after applying the extraction yield

The results obtained for the assay of three separate samples of powder from the capsules, presented in Table 3, further confirm the fact that the actual andarine content of the finished product is below the values declared by the manufacturer.

*Dissolution profile*

After 165 min, following the dissolution assay 1, an average of 1.66 mg of the total content of andarine and an average of 2.09 mg of andarine following dissolution assay 2 was released from capsules (fig. 3).

Thus, it appears that the presence of sodium laurylsulfate as an emulsifying agent facilitates the release of andarine compared to the pancreatic enzyme powder (added to ensure the digestion of rice flour components, specifically starch), but still not the whole quantity contained in the capsules was released for either test (fig. 4).

## Conclusions

The dietary supplements with andarine comply with the test for uniformity of mass having an individual percentage deviation under 7.5 %.

Even though the uniformity of content complied with current regulations, the tested capsules are underdosed, containing about 20% of declared label content.

Moreover, there are concerns about the *in vivo* release of the active substance, given the difficulties encountered in both the extraction of the analyte and the dissolution profiles.

The characterization of these capsules is preliminary to a study regarding the pharmacotoxicological profile of andarine in an experimental animal doping model.

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