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RESEARCH ARTICLE

Development and Validation of an UHPLC Method for Ostarine Determination in Dietary Supplements

Amalia Miklos[1], Amelia Tero-Vescan[1*], Lénárd Farczádi[2,3], Daniela-Lucia Muntean[1]

1. Faculty of Pharmacy, University of Medicine, Pharmacy, Sciences and Technology of Tîrgu Mureș, Tîrgu Mureș, Romania
2. Center for Advanced Medical and Pharmaceutical Research (CCAMF), University of Medicine, Pharmacy, Sciences and Technology of Tîrgu Mureș, Tîrgu Mureș, Romania
3. Faculty of Pharmacy, University of Medicine and Pharmacy „Iuliu Hațieganu" Cluj-Napoca, Cluj-Napoca, Romania

Objective: The purpose of this study was to develop a low-cost, yet sensitive and precise UHPLC method for the quantitative determination of ostarine from dietary supplements (DS) for athletes. The analytical performance of the method was verified on a DS legally acquired from a specialized website for athletes. The uniformity of mass and content of the ostarine DS was also verified. **Methods**: For the quantitative determination of ostarine a UHPLC method was developed and validated. The separation was performed using a reversed-phase C18 column, using a mixture of 75% methanol: 25% formic acid 0.1% in isocratic elution, at a flow rate of 0.5 ml/min. The uniformity of mass and content of DS was performed following the methodology described in the European Pharmacopoeia 7th Edition. **Results**: The validated method was specific and linear on the concentration range of 1-25 µg/ml and was precise and accurate at all concentration levels, according to the official guidelines for validating analytical methods. An average mass of 510 mg content was obtained for the ostarine capsules, with an RSD of 2.41%. Regarding the uniformity of the content, an average of 4.65 mg ostarine/capsule was obtained with an RSD of 1.05%. **Conclusions:** The developed UHPLC method was suitable, rapid, sensitive and allowed quantitative determination of active substance content in a DS with ostarine (92.91% ostarine/capsule from 5 mg ostarine/capsule declared by the manufacturer).

Keywords: ostarine, dietary supplements, UHPLC method, SARMs

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Introduction

The legal regime of dietary supplements (DS) is extremely permissive, leaving for the manufacturers the discretion of applying quality standards. In the US, the FDA issued a „Dietary Supplement Health and Education Act" stating that manufacturing companies are responsible for the quality and content of active substances and DS labeling while their control is the duty of the FDA if post-marketing reports of adulteration, misbranding or misuse are signaled. SARMs (Selective Androgen Receptor Modulators) are a class of highly active pharmacological substances that are in different phases of clinical study but have not yet been introduced into therapy. Their uses could target pathologies characterized by a marked protein catabolism (cachexia in neoplasic diseases, sarcopenia, muscular dystrophy etc.), osteoporosis [1], promoting male and female libido, treatment of benign prostatic hyperplasia [2].

Selective modulation of androgen receptors in the bone and muscle but without affecting genital organs (testicular atrophy, oligospermia), hair follicle (alopecia) or sebaceous glands (acne) is a cause of abusively use of these substances, especially by amateur athletes in the desire to improve their physical appearance and increase muscle mass [3]. World Anti-Doping Agency (WADA) included these substances on the doping list in 2008 [4] and since then, several analytical methods have been developed to detect doping with SARMs [5], therefore professional athletes are less exposed to the abuse of such substances than those who practice recreational sports.

Ostarine (see Figure 1), also known as GTx-024, S-22 or enobosarm, is a SARM compound that has already been included in Phase 2 clinical trials, to establish the pharmacological profile, in cancer patients with cachexia, in elderly men with low lean body mass [6], in postmenopausal women osteoporosis or breast cancer.

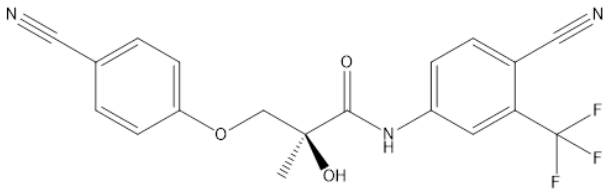

Fig. 1. Ostarine chemical structure

Since ostarine does not have a marketing authorization, it can be purchased as DS by athletes and also by those who want to improve their physical appearance. Given the increased number of pharmaceutical forms with ostarine on the market and the extensive use, sometimes in higher doses than those recommended by the manufacturer, there is a question of improving the methods of analysis of these DS. While most of the methods described in literature are LC-MS methods, the aim of our study was to develop a low cost, but at the same time rapid, sensitive and precise UHPLC method with UV detection to quantify ostarine in DS (capsules), legally purchased from a website specialized for sportsmen.

* Correspondence to: Amelia Tero-Vescan
E-mail: amelia.tero.vescan@umftgm.ro

Methods

Chemicals, reagents, solvents

Ostarine certified reference standard (CRS) was purchased from AbMole BioScience (100% purity). HPLC grade methanol and formic acid of analytical grade were purchased from Merck KGaA (Darmstadt, Germany). Magnesium stearate was purchased from Sigma Aldrich and ultrapurified water was obtained from a Millipore Direct Q system.

Preparation of standard solutions

The ostarine 200 μg/ml stock solution was prepared by weighing 1 mg ostarine on a Partner Corporation analytical balance, which was dissolved in 5 ml of methanol. Standard working solutions-at 6 concentration levels, over the concentration range of 1-25 μg/ml, were prepared by diluting the stock solution with 0.1% aqueous formic acid solution.

Preparation of sample solutions

Reconstituted samples (containing ostarine and the excipients declared by the manufacturer such as rice flour and magnesium stearate) at 5 concentration levels (1, 10, 15, 20, 25 μg/ml) were freshly prepared on the day of analysis. The solutions were prepared by weighing ostarine (0.25; 2.5; 3.75; 5; 6.25 mg), rice flour (509.75; 507.5; 506.25; 505; 503.75 mg) and magnesium stearate (1.25 mg), the only three declared components of the capsules of finished product. The extraction was made with methanol by stirring the sample for 40 minutes on a VWR magnetic stirrer at 800 rpm, then sonicated for 20 minutes and made up with methanol at 50 ml. 1ml of each solution was diluted to 5 ml with 0.1% formic acid, then filtered through nylon filters (0.45 μm).

Placebo solution was prepared by weighing the appropriate amount of rice flour (505 mg) and magnesium stearate (1.25 mg) to a 50 ml flask and the solutions followed then the extraction steps as the reconstituted samples.

Three samples for the assay of ostarine capsules were prepared by pooling the content of capsules, mixing it and weighing approximately 51 mg of powder to a 5 ml flasks and performing the extraction method described for reconstituted samples. 1ml of the solutions was diluted to 5 ml with 0.1% formic acid, then filtered through nylon filters (0.45 μm).

In order to evaluate the uniformity of ostarine content, ten samples were prepared by emptying the powder from a single capsule to 50 ml flasks and performing the extraction method described for the reconstituted samples.

Chromatographic conditions

An UHPLC method was developed and validated on a Flexar-10 UHPLC system (Perkin-Elmer) consisting of a binary pump, solvent degasser, autosampler with controlled temperature, column thermostat and PDA UV-VIS detector. Separation was performed on a reversed-phase Gemini NX-C18 3.0x100 mm, 3 μm column. The mobile phase used for the separation consisted of methanol (75%) and 0.1% formic acid (25%) in isocratic elution, with a flow rate of 0.5 ml/min. The injection volume was 5 μL and the detection wavelength was set at 270 nm. The time of analysis was 2.5 min for each sample.

The calibration curves were composed of 6 concentration levels (1, 5, 10, 15, 20 and 25 μg/ml). The 20 μg/ml standard solution is the equivalent concentration to a capsule of finished product containing the amount of ostarine declared on the label by the manufacturer (100% level). Due to the variation which may occurs in the DS, manufacturers usually trying to use less active substance to reduce manufacturing costs, the LLOQ was chosen to be at a level of only 5% of the declared content.

The analytical method was validated with regards to carry-over, selectivity, linearity, within-run and between-run accuracy and precision and analyte extraction. A total of five calibration sequences (containing a calibration curve and the apropriate types of samples) were tested during validation.

The assay, uniformity of mass and the uniformity of content of single-dose preparations were tested following the methodology described in the European Pharmacopoeia 7th Edition [7].

Results

The selectivity of the method was tested by comparing the chromatograms of placebo and LLOQ solutions, and no peaks were detected at the retention time of the analyte (1.7 minutes) (Figure 2).

The carry-over was also evaluated by injecting a blank sample (mobile phase) immediatly after a standard solution with a high concentration (25 μg/mL) and no peaks were detected in the blank solution at the retention time of the analyte.

Identification based of the similarity of the UV spectrum was performed by comparing UV spectras of placebo, sample and standard solutions, presented in Figure 3. On the overlaid spectra of placebo solution, 20 μg/ml standard solution and the sample solution, two different specific wavelenghts were observed for ostarine at 245 and 270 nm. The method was validated at 270 nm due to the higher specificity expected at that wavelength.

Linearity studies

Each of the five calibration curves injected during the validation of the method were linear with a correlation coefficient $R > 0.99$ (Figure 4).

Precision and accuracy

The accuracy and precision within- and between-run determined on 5 individual concentration levels (1, 10, 15, 20, 25 μg/mL) according to validation guidelines [8] are showed in Table I and Table II, the LLOQ being set at 1 μg/mL.

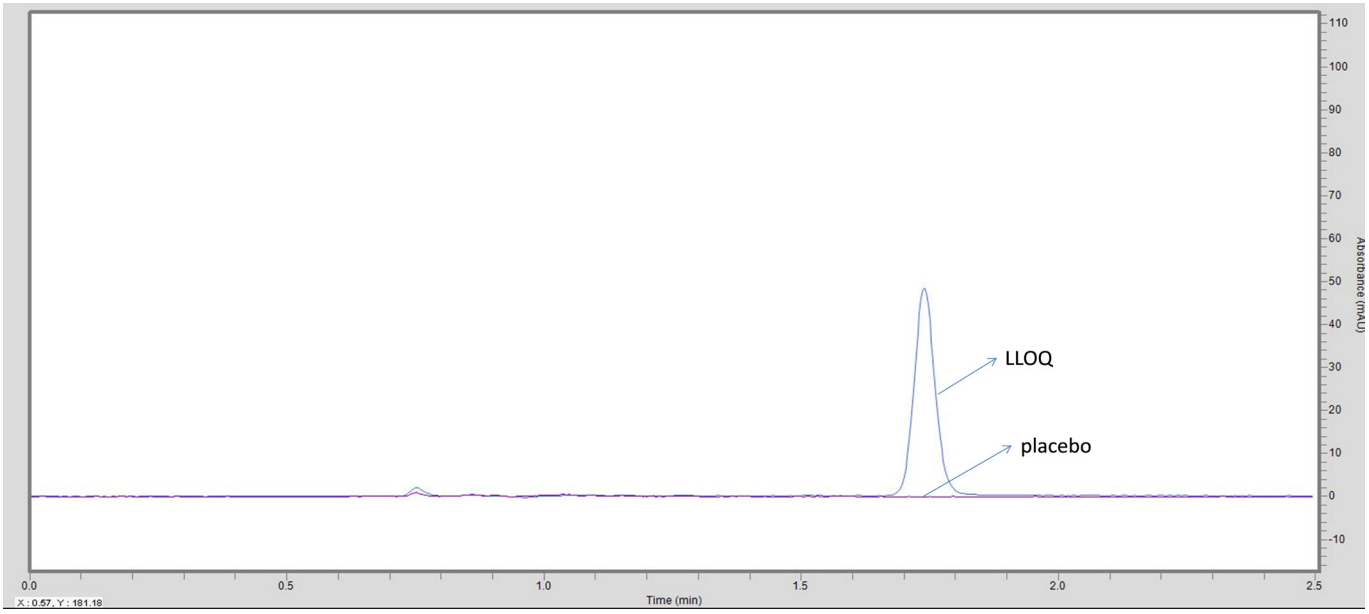

Fig. 2. Overlaid chromatograms of placebo solution and LLOQ

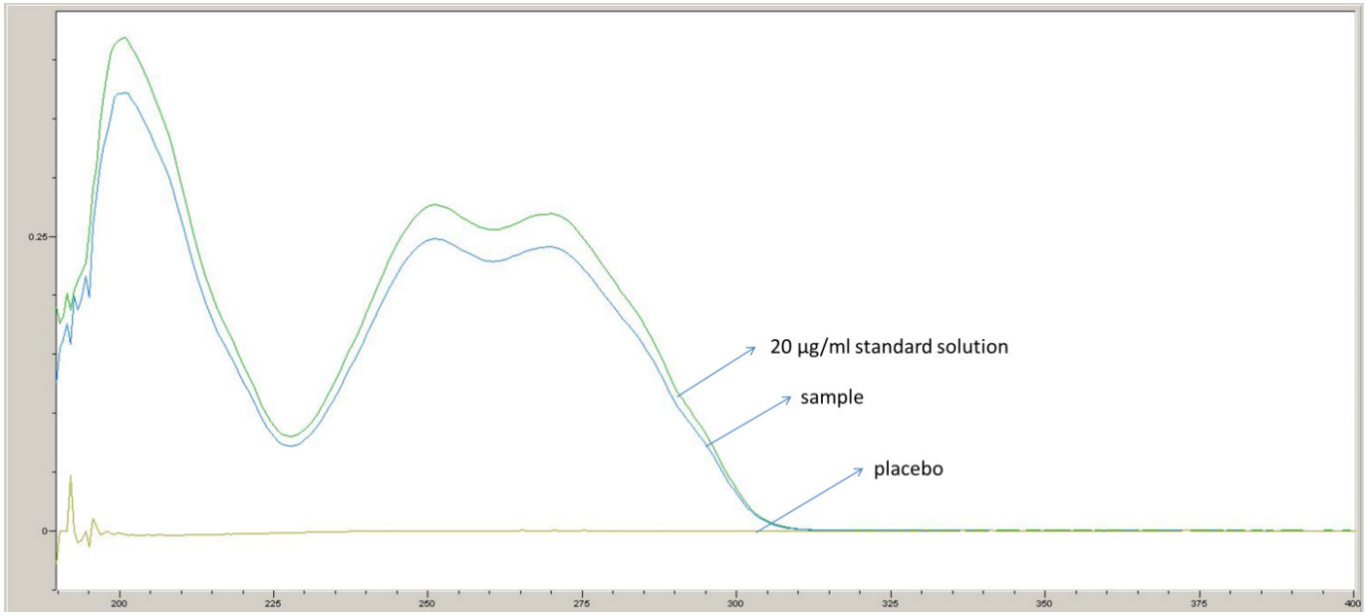

Fig. 3. Overlaid spectra of placebo solution, 20 µg/ml standard solution and sample

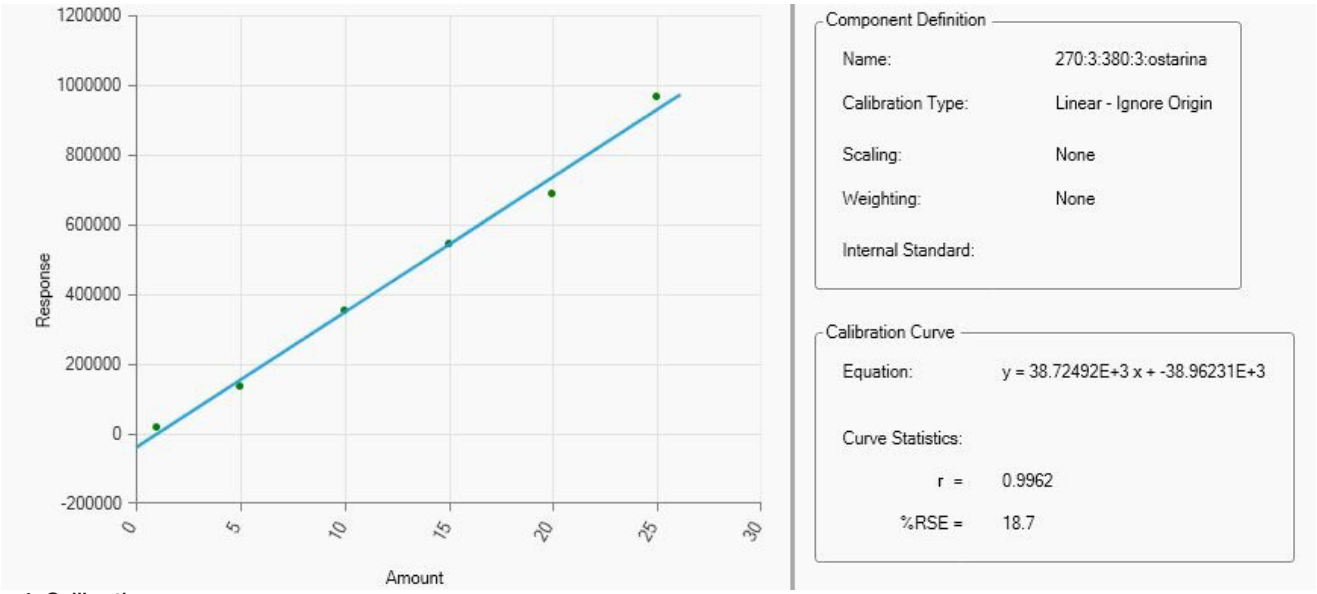

Fig. 4. Calibration curve

Table I. Within-run accuracy and precision

Nominal conc. µg/ml	Theoretical conc. µg/ml	Calculated conc. µg/ml	Accuracy (%)	Mean calculated conc. µg/ml (±SD)	Mean Accuracy % (±SD)	Precision (RSD, %)
1.39	1.36	1.52	111.81	1.52 (± 0.0191)	108.97 (± 2.0927)	1.92
	1.40	1.48	106.04			
	1.40	1.52	108.64			
	1.40	1.53	109.74			
	1.40	1.52	108.60			
10.03	10.08	9.86	97.87	9.95 (± 0.1937)	99.14 (± 1.2267)	1.24
	9.80	9.63	98.37			
	10.00	10.09	100.92			
	10.08	10.06	99.82			
	10.20	10.06	98.72			
14.93	15.00	15.25	101.72	15.18 (± 0.0773)	101.72 (±0.8382)	0.82
	14.88	15.08	101.39			
	14.96	15.14	101.25			
	14.80	15.26	103.16			
	15.00	15.16	101.08			
19.93	20.20	18.70	92.60	18.54 (± 0.3576)	93.04 (±1.4439)	1.55
	20.32	18.77	92.42			
	19.60	18.45	94.18			
	19.68	17.94	91.21			
	19.84	18.80	94.80			
25.02	24.84	26.20	105.50	26.10 (± 0.1393)	104.34 (±1.1178)	1.07
	24.80	25.98	104.79			
	25.20	26.13	103.70			
	25.00	26.25	105.00			
	25.24	25.92	102.72			

Table II. Between-run accuracy and precision

Nominal Conc. µg/ml	**Theoretical conc. µg/ml**	**Calculated conc. µg/ml**	**Accuracy (%)**	**Mean calculated conc. µg/ml (±SD)**	**Mean Accuracy % (±SD)**	**Precision (RSD, %)**
1.38	1.40	1.53	109.60	1.56 (± 0.0493)	112.41 (± 1.9128)	1.70
	1.36	1.52	111.81			
	1.44	1.63	113.40			
	1.40	1.57	112.50			
	1.32	1.51	114.73			
10.09	10.00	10.00	100.04	9.91 (± 0.0700)	98.23 (± 1.5000)	1.53
	10.08	9.86	97.87			
	10.20	9.88	96.92			
	10.16	9.83	96.77			
	10.00	9.95	99.55			
14.95	15.00	15.06	100.44	14.98 (± 0.1845)	100.21 (± 0.9971)	1.00
	15.00	15.25	101.72			
	14.88	14.81	99.59			
	14.96	14.82	99.09			
	14.92	14.94	100.18			
20.14	20.00	18.33	91.70	18.47 (± 0.1643)	91.71 (± 0.9322)	1.02
	20.20	18.70	92.60			
	20.04	18.50	92.35			
	20.28	18.29	90.20			
	20.20	18.52	91.71			
24.99	25.00	25.54	102.17	25.85 (± 0.4947)	103.42 (± 1.9315)	1.87
	25.00	26.20	104.82			
	25.00	26.48	105.93			
	24.96	25.25	101.20			
	25.00	25.74	102.99			

Regarding the extraction of the analyte, the repeatability within- and between-run was tested at one level of concentration (20 µg/ml), testing five replicates. An extraction yield of 93.98% within-run with an RSD of 1.72% and an extraction yield of 95.35% between-run with an RSD 0.88%, were obtained respectively, with a mean extraction yield of 94.67%.

Following the mass uniformity testing of single-dose preparations, an average mass 510 mg/capsule was obtained with an RSD of 2.41%.

In terms of uniformity content of single-dose preparations, an average of 4.65 mg/capsule was obtained (92.91% of the content declared by the manufacturer), with an RSD of 1.05%.

Following the assay of capsule content, an average of 4.71 mg/average capsule content was obtained (94.14% of the content declared by the producer). Figure 5 shows the overlaid chromatograms of a 20 µg/ml standard solution and a sample solution prepared from capsule contents.

Discussions

Ostarine is a substance that has been used in clinical trials but has not received Marketing Authorization Approval as the safety and efficacy are still to be demonstrated and is, therefore, marketed as a DS for athletes. If we consider the definition of DS, namely "they are oral products that contain substances such as vitamins, minerals, amino acids or plant products", ostarine is a highly active compound that cannot be included in this class. In contrast to andarine which, by hepatic transformation, is converted into several more or less active metabolites following hydroxylation, deacetylation or reduction, ostarine is active as such and is partially eliminated by urine (glucurono- and sulfoconjugated) and partly by faeces, therefore the developed HPLC-UV method could also be used for the determination of ostarine from aqueous solution, such as urine, after hydrolysis of conjugates, for the detection of suspected doping cases or in case of intoxication with unknown substances [9]. The use as a doping agent of ostarine is not limited to the human species [10] but also to racing horses or to domestic animals as growth promoters, to improve the quality of the meat (lean mass).

The pharmacokinetic interactions of ostarine with other enzyme-inducing/inhibitory substances has only been studied in drugs that are relevant for oncology, given its effects in cancer-cachexia. Studies show that ostarine did not influence the pharmacokinetics of celecoxib or rosuvastatin, but rifampicin increased by 23% C_{max} and by 43% AUC_{∞} of ostarine and probenecid increased by 50% C_{max} and by 112% AUC_{∞} of ostarine [11].

Since DS for sportsmen often contain other substances, many combinations of plant origin, there are other possibilities of pharmacological or pharmacokinetic interactions that may occur. Moreover, due to the lack of side and adverse effects described in anabolic steroids, ostarine can also be illicitly introduced into herbal or amino acid DS for athletes. Literature describes cases of DS adulterated with compounds of the SARMs class [12].

A study on the quality of DS with SARMs on 44 market products, published in 2017, shows that 9% of DS analyzed did not contain the active substance, 25% of DS contained substances not mentioned on the label and in 59% cases the amount of active substance found was different from the one mentioned on the label [13].

The tested DS with ostarine comply with the current regulations regarding the uniformity of mass and content for single-dose preparations, having an individual percentage mass deviation under 7.5 % and an individual percentage ostarine content deviation under 15%.

Regarding the content of active substance, the DS capsules have a content of ostarine very close to that declared by the manufacturer (92.91%).

Conclusions

A rapid and suitable UHPLC method was developed and validated to determine the content of ostarine from DS legally acquired from a website specialized in selling products for weightlifters, after methanol extraction of the analyte by magnetic and ultrasonic stirring.

The tested DS with ostarine are compliant with current regulations regarding assay, uniformity of mass and content testing for single-dose preparations.

These control tests are preliminary to the development of an animal doping model in order to study the pharmacotoxicological profile of ostarine.

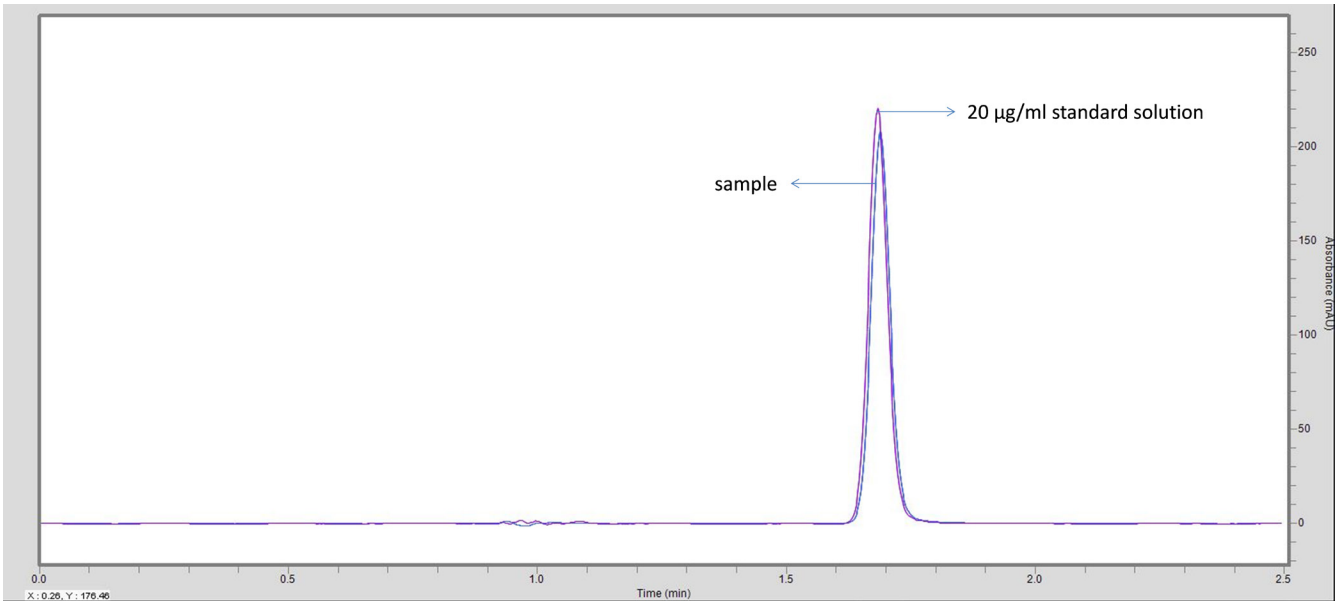

Fig. 5. Overlaid chromatograms of a 20 µg/ml standard solution and a sample solution prepared from capsule contents

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Authors' contribution

Amalia Miklos (Data curation; Methodology)
Amelia Tero-Vescan (Data curation; Writing – original draft; Writing – review & editing)
Lénárd Farczádi (Methodology; Software; Supervision; Validation; Writing – review &editing)
Daniela-Lucia Muntean (Supervision; Writing – review & editing)

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