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REVIEW

Analytical Quality by Design with the Lifecycle Approach: A Modern Epitome for Analytical Method Development

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Quality by Design is the methodical method to development concept that starts with the predefined objects. The method put emphasis on the process of development of a product, the control process, which is built on risk management and comprehensive knowledge of science. The concept of QbD applied to analytical method development is known now as AQbD (Analytical Quality by Design). Comprehension of the Analytical Target Profile (ATP) and the risk assessment for the variables that can have an impact on the productivity of the developed analytical method can be the main principles of the AQbD. Inside the method operable design region (MODR), the AQbD permits the movements of the analytical methods. This paper has been produced to discuss various views of analytical scientists, the comparison with conventional methods, and the phases of the analytical techniques.

Keywords: analytical quality by design, lifecycle approach, method operable design region

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Introduction

International Conference on Harmonization (ICH) Q8 (R1) guideline defines Quality by Design (QbD) as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management" [1]. The approach of QbD has been implemented in the manufacturing of pharmaceutical through numerous enterprises as Process Analytical Technology (PAT) and FDA guidelines. Recently FDA has approved new drug applications based on AQbD and termed it as benefits and importance of QbD in the analytical technique development cycle for accepting drug excipient interaction. Additionally, for the determination of critical quality attributes during the process, experiment, control, and continuous process verification to monitor the product quality trends; Hence this resonance attracting the pharmaceutical industry to evoke the AQbD [2]

The elementary notion of the QbD process of testing cannot put quality into an object but rather it must be constructed into the product. An essential aspect of QbD is that it understands how the choice of constraints can have an impact on the development process. The understanding attained during the process development can assist in the formulation of the products limits and the design space best suited for a particular drug.

Similar ideologies of QbD are applied in the development of analytical methods becoming Analytical Quality by Design (AQbD). In similarity to the concept of QbD, the results obtained from the AQbD process are assumed, suitable for the product and consistent in providing the envisioned performance during the lifecycle of the product. Despite a lot of discussion concerning AQbD the quality assurance personnel view AQbD as the better solution to avoid out of specifications (OOS) and out of trend (OOT) and minimize risk in method failure [3, 4].

The comprehensive knowledge that is gained while using this concept is beneficial in the establishment of the method operable design region, (MODR). This is a multidimensional region that is centered on the process properties which influences the performance of the product [1].

Safety and efficiency should be considered paramount by ensuring that pharmaceutical products are required to be healthy and easily reproduced. The process of creating the pharmaceutical products entails the production of information on the materials such as an intentional addition of an ingredient to a drug component. With the advancement in technology comes the change in both the quality and quantity of the material information that facilitates knowledge development [5].

The process of AQbD is a major stage of the control strategy in the quality system of the pharmaceutical industry. This concept constitutes the constraints and features linked with the drugs and the operating environment and their related processes. Although current Good Manufacturing Process (cGMP) regulation has been in place for a long time, many of the pharmaceutical companies are still experiencing problems in relation to issues of quality control. Quality Control is emphasized in risk management

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during the development of the product. Therefore, due to the dependency of these pharmaceutical companies for development processes to be robust, there is a great need for the AQbD concept [6]. This is an indication of the quality of this concept, and the robustness of the product in its lifecycle.

The lifecycle assessment (LCA) approach is a rooted methodical method. The technique is used in the application of environmental effects, which has been practiced for the products. The purpose of using this approach in the analytical process is the elating the development process to the requirements of the products to develop the method reliability (figure 1) [7].

Following the lack of explanatory reviews, this article aim focuses on different views of experts on the application of AQbD in the pharmaceutical quality system. Additionally, the article links the views with the lifecycle method.

Conventional Approach versus Analytical Quality by Design

The traditional process in the pharmaceutical industry was Quality by Testing (QbT). However, this becoming outdated; in the QbD philosophy there is assurance of the quality of the product, and hence it is more favored [8]. Currently, the pharmaceutical industry is using the QbD method to reduce the cost and time and assurance of the quality of the products [9]. The impacts of this process in the pharmaceutical industry have been expanded after the initialization of the FDA guidelines. The different methods in use include FDA cGMP, the process analytical technology. The target of the quality process is to ensure that the product is safe for use by the patients. The AQbD checks the Out-of-Trend (OOT), Out-of-Control (OOC) and Out-of-Specification (OOS) results [10]. This proves that the traditional process does not provide assurance of the product's safety. Hence the pharmaceutical industry can make huge improvements in the components of the product [11].

Application of Analytical Quality by Design (AQbD) advantages, expectations, and obstacles

According to pharmaceutical quality systems, analytical techniques are the primary part of the control strategy. Therefore, the implementation of QbD in manufacturing as a control mechanism will ensure predetermined performance and the quality of the product [3]. This comprises attributes and parameters related to drug product and drug substances components including instrument operating conditions, facility, finished product specification, and the related analytical methods [12].

The adoption of AQbD is expected to enhance the concept of right analytics at the right time which has an essential role in drug product development cycle [13].

The advantages of AQbD concept in product development [14] can be pointed out: the advancement of a method that is robust; the concept is applicable in the lifespan of a product; the regulations are flexible; the movement of a product inside the design area is not seen as a variation in developments; the concept allows for constant progress during the development process; the concept evades revalidation of the product.

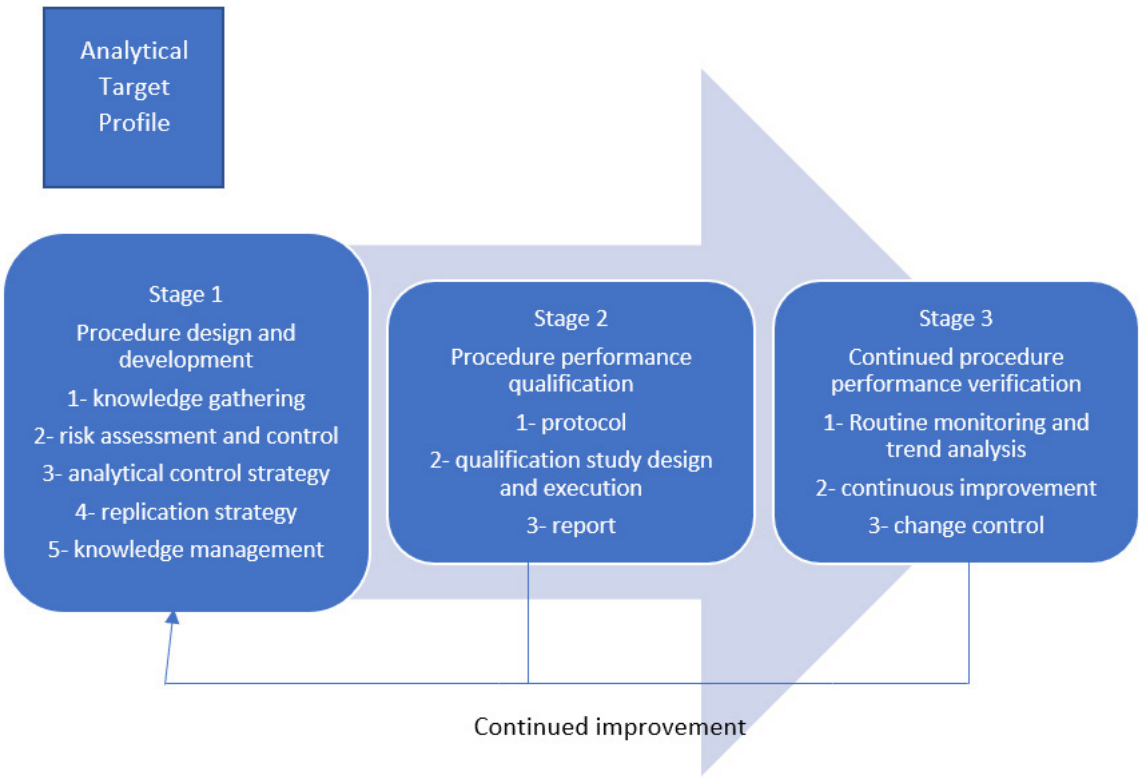

Fig. 1. The analytical procedure lifecycle [7].

Obstacles that hinder the application of the analytical quality by design are mainly that there is no full comprehension of this concept in the pharmaceutical industry and the absence of a clear description of the AQbD elements.

The USP Stimuli articles establish that the analytical method lifecycle comprises of three phases:

– Phase 1. The method design: the depiction of the demands and the nature of the process;
– Phase 2. The validation method: guarantee that the process meets the design;
– Phase 3. The life cycle management/Continued procedure performance verification confirms that the process remains in the controlled state [15].

1. Method design

Method design from point of view of AQbD incorporates the Method Operable Design Region (MODR), that means defining Analytical Target Profile (ATP), the experimental design screening and establishing Critical Quality Attributes (CQA).

1.1. ATP (Analytical Target Profile)

The analytical target profile is the necessary tool for product development, as it is outlined in the ICH Q8 guidelines. ATP describes the essential requirements to be measured which influence product advancement. The ATP is a collection of all the presentation constraints that are required for the planned analytical application [16]. An ATP is advanced for every attribute which is present in the control strategy. Analytical Target Profile defines the goal of the analytical technique development process and relating the outcomes of the method to achieve QTPP. Based on EFPIA and PhRMA ATP can be defined as a statement that defines the purpose of the method which is utilized to drive method design, selection, and development activities [3].

Analytical Target Profile is a crucial parameter in AQbD facilitating the greater constant improvement of analytical techniques and their choice when the ATP statement is approved by the regulatory authorities. Internal change control management in the pharmaceutical industry is responsible for perfect implementation of ATP to offer regulatory flexibility [12, 17].

The general ATP of the analytical procedures comprises of [18]: selecting the target of analytic process (Active Product Ingredient API and impurities); selecting the techniques to be used in process (HPLC, GC, HPTLC, Ion Chromatography, chiral HPLC, …); choosing the required method (assay or impurity profile or residual solvents).

The selected method should be precise and accurate; these are essentially the characteristics and provide vital information for the purpose of computing an unknown amount of the material for use in the process method. In the absence of accuracy and precision, a method will not be correct. In order to achieve a method which is accurate and precise, some important characteristics need to be evaluated [19]. These characteristics could be an acceptable specification, a space that is stated linearly, adequate peak determination, or else. These characteristics ensure that widespread data set for setting the constraints of the method.

1.2. Critical Quality Attributes (CQA)

The ICH Q8 guidelines describe CQA as the chemical, microbiological, physical and biological properties of a drug. These properties should be within the constraints to guarantee that the end product is of desirable quality [20]. In the instances of process related to CQA the drug products quality characteristics for example dissolution, assay, chromatographic purity, residual solvents, microbial limits, dosage unit's uniformity, water content, suspensions, viscosity in creams, the medicament in soft gelatin capsules, and medicament are regarded as vital quality properties. While in case of AQbD considering an HPLC technique development as typical example theoretical plate count, tailing of the peak, the resolution between impurities and main analyte, capacity factor, peak purity are considered as CQA [14].

As features of the CQA, the accuracy and correctness of the development process are to be calculated holistically, for the identification of the Target Measurement Uncertainty (TMU). This TMU is connected to the result of the development process [21,22]. The TMU is not a measurement that can be attained on its own; rather it is the maximum measurement that can be accepted by the Measurement Uncertainty (MU) [23].

1.3. Risk management

Risk management is defined as the systematic analytical method for the examination, regulation and, acknowledgment and validation of the threats to the quality of the finished product [24]. It is carried out throughout the lifespan of the product, which in this case is a drug product.

1.3.1. Risk assessment

Risk assessment is an essential phase of the risk management process. This phase improves the quality of the process. It can be defined as a single or combined view of the occurrence of any impairment or the degree of the impairment to the process. Risk assessment assists in increasing the quality of the development process. In addition, it is a factor for the impact of the initial variables on the analytic development process. Through the process of risk assessment, the perilous attributes that can disturb the quality of the end product are documented.

The important reasons for risk assessment are to identify the degree of dangers at the start of the analytic development of drug; to decrease the variety of CQA chosen; to acknowledge the suitable requirements, the constraints, and production regulations [15].

The known methods for risk assessment are fault tree analysis; failure mode effect analysis; risk ranking and fil-

tering; Failure modes and effects analysis (FMEA); Structured "What-if" Technique (SWIFT), the fishbone diagram [25].

There are some questions [26] in the risk assessment process as:

– What mistake can take place?
– What is the possibility of this happening?
– What are the difficulties?

In the risk assessment process, the most essential notion in the compliance phase is the concept of the decision rules. These guidelines can be defined as the acceptance or rejection of the product; this is in harmony with the measured values of the product. The constraints and uncertainty are considered in setting the acceptance limits of the possibility of making a mistake during the analytic development process. While using the established constraints, two areas are considered the acceptance and rejection areas. The product is then subjected to the tests if the product lies in the rejection area, it is considered as compliant, or otherwise, the product is declared defective [27].

1.3.2. Risk control phase

It is essential to decrease the dangers to a satisfactory level; risk control is divided into two categories. These categories are risk reduction and risk approval.

In risk reduction, there are two steps: remedial activities in order to solve the inconsistency and remedial activities to ensure the inconsistency are not repeated [28].

In risk acceptance, the steps are the assumptions of dimensions to be considered; the conclusions on the nature of the measurements; defining the following actions to be applied; managing the review of the process; risk statement, the administration can decide on the steps to be taken. The Sturdiness of the product should be examined in all the lifecycle of the product. This feature is vital in the process of selecting the best plan for authorization. The analytical processes developed at the initial stage are determined by the assortment of an inclusive procedure and the corresponding information of the product (Figure 2).

1.4. Design of experiments (DoE) in AQbD concept

The robustness of the product should be examined in all the lifecycle of the product. This feature is vital in the process of selecting the best plan for authorization. The analytical processes developed at the initial stage are determined by the assortment of an inclusive procedure and the corresponding information of the product [29].

The design space attributes and AQbD are applied to the analytical development process to intensify the suppleness of the process and reduce the dangers that can affect this process. Design of Experiments (DoE), is a credible method used to create the link between the raw products and the end products of the process. Normally the DoE is applied in the (QbD) process by defining the limits of the measurements and attributes in the development process. Experts can apply the DoE to find the operating ranges of the process [30]. Reducing the number of experiments, for example, is essential as it prevents time and money wastage in the development process. Saving time as well money is only attainable through the assessment of the potential risks while ranking them by considering the extent of their severity essential in owing to the number of prior arguments [31].

When applied correctly the DoE can provide a big improvement in precision and robustness subsequently reduce the errors realized in the development process [32]. The DoE method for validation aims at validating the method for a wide variety of concentration; this ensures that any changes in concentration within limits of design space will not need extra validation, because the changes are in the characterized area [21]. Lately, more attention has been put on the DoE applied to the analytical development process.

The DoE has three main applications: the process advancement for new processes or existing ones that need upgrading; analytical process verification; measuring the impact of the analytic methods on the products [33,34].

1.5. Method control strategy

The process of creating a control strategy is essential in ensuring that the process is performed as planned on the foundation of the ATP objectives. A process control strategy is a prearranged set of constraints that is aimed at reducing the inconsistency of the process. This method is reliant on the statistics of the whole process. The data which is created during the development and verification process is the foundation of the control strategy. The attributes that are found to have risks have to be managed. The attributes that are high risk are given additional consideration. The control strategy is defined for the attributes which have low risks and can be accomplished. This control strategy is defined and entails the suitable process appropriateness check and verification consistently. Doing this ensures that the process supplies a product with the desired attributes.

1.5.1. Process analytical technology (PAT)

PAT is a scheme for assessing, planning and regulating the quantities of the analytical process; it is done constantly in the lifecycle of a product. PAT ensures that the features and quality of the product will be as planned [35]. In the ICH Q8 guidelines PAT is described as a method that assures product remains in the established design space. In a method which is more vigorous, PAT will ensure that active regulation of the attributes and the appropriate alterations of the limits in the setting of initial materials which would otherwise affect the quality of drug are detected [36].

1.5.2. Knowledge management

This is the notion of gathering, treating and checking the data from all the previous phases. This ensures that the control strategy is accurate.

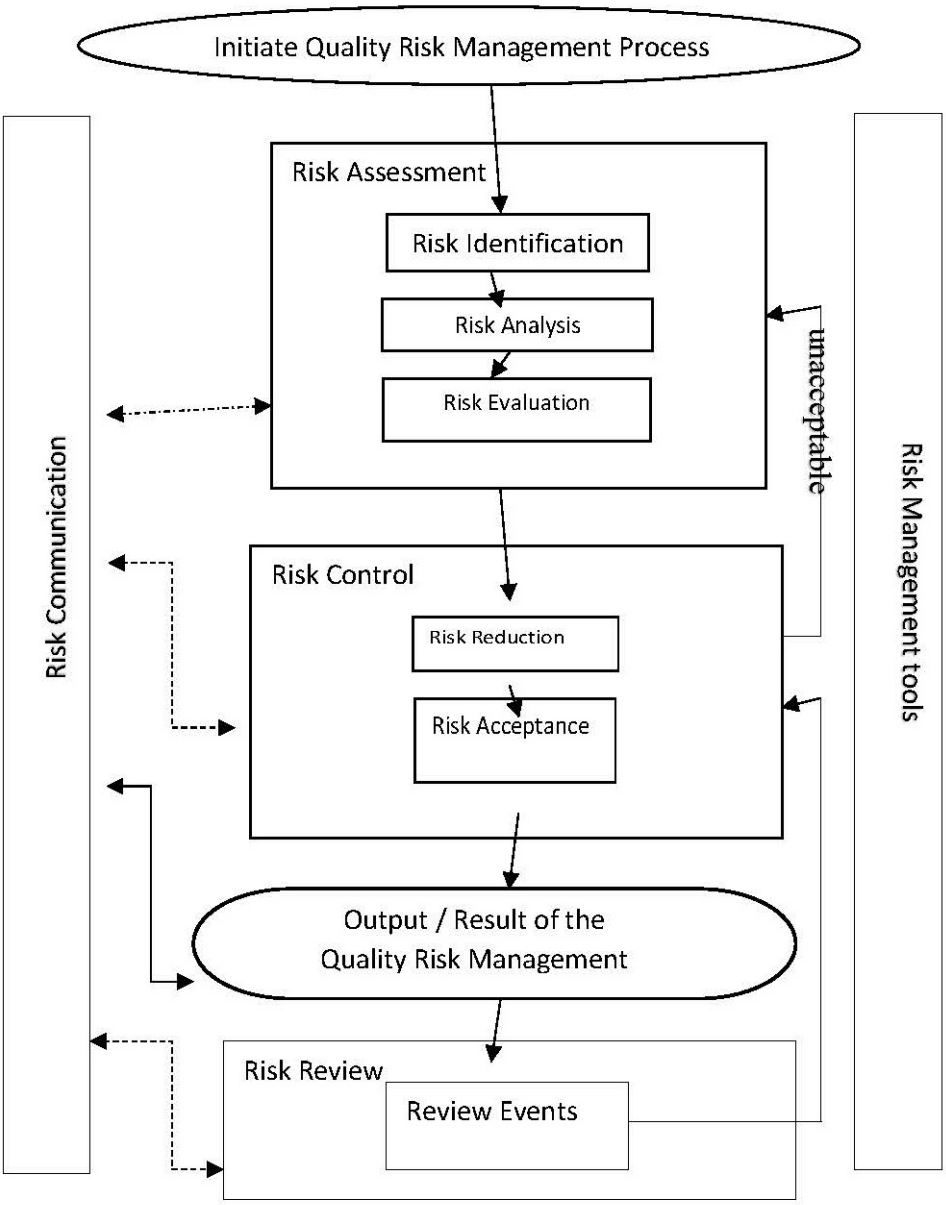

Fig. 2. An overview of risk quality management [24]. © copyright 2005 International Conference on Harmonisation (ICH) guidelines Q9.

1.5.3. Analytical control strategy (ACS)

The ATP's primary focus is the identification of the potential risks arising from making incorrect decisions. Contrary to the problem of making improper decisions, decision rules that govern the process of decision-making may not be essential all the time. First, the company should link the acceptable level of risk to the safety of patients. Therefore, the Target Measurement Uncertainty (TMU) aims at maximizing the acceptable uncertainty to meet the ATP hence ensuring an accomplishment of the fitness-for-purpose necessary for the process of analysis. ACS, on the other hand, plays an essential role in providing consistency of TMU during the whole process of analytical procedure (Figure 3).

2. Method validation

Method validation entails clarifying that the chosen method will provide information that meets the guidelines of the ATP, in the predicted conditions. Subsequently, the attribute presentation requirements must be put in place before the predicted presentation of the analytical development process. The analytical techniques used in the process of presentation of the prerequisite study should be recognized from the information that is accessible. Method validation can also be understood as the process of verifying and recording of any evidence, method, and apparatus which should be installed correctly. This would, in turn, ensure that the process leads to the results which had been predicted [39].

3. Continued procedure performance verification / Lifecycle Management

The last phase of the AQbD process, this phase involves linking the critical quality attributes, the analytical target profile, the MODR, and risk assessment of the process with preceding information considerations. The lifecycle

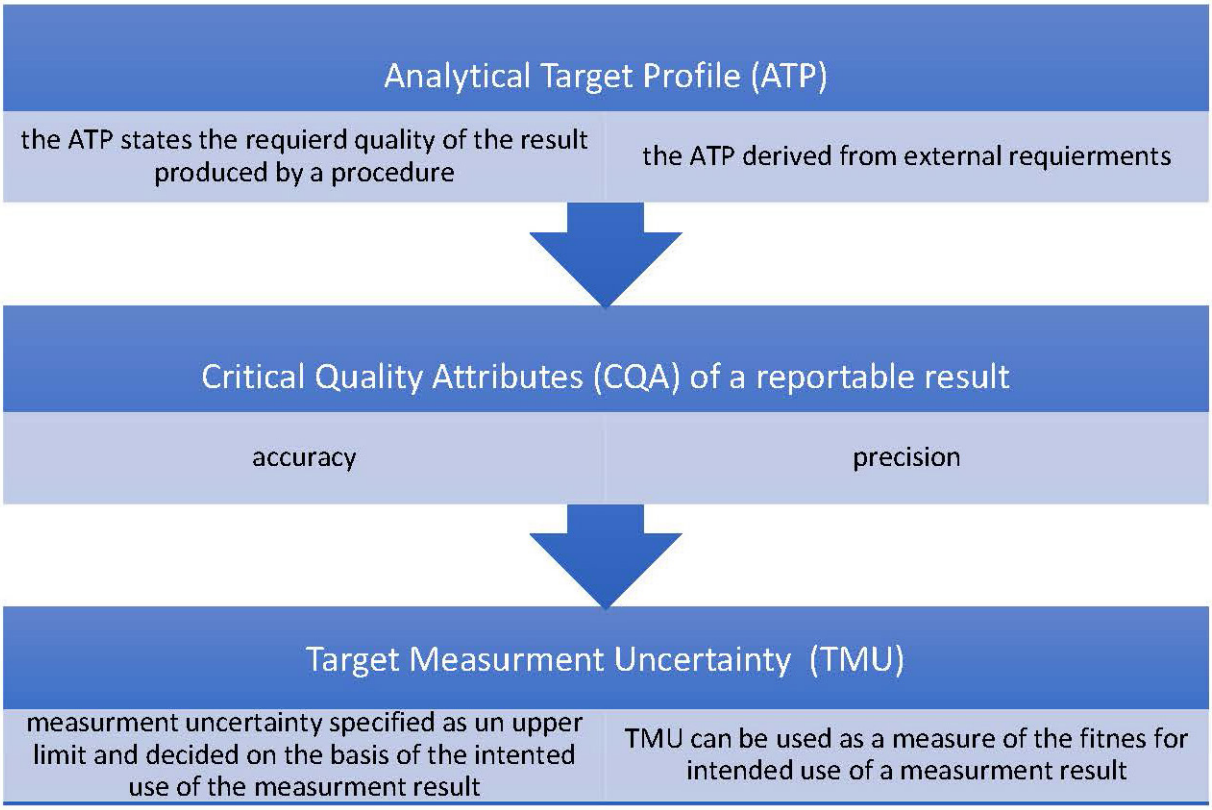

Fig. 3. An illustration of how ATP, CQA, and the TMU relate to one another [9]. © copyright 2016 U.S. Pharmacopeial convention (USP).

management of the process offers the background for describing the method of development of the analytical process, ensuring that it meets planned standards throughout the life of the product [8]. The lifecycle method is determined by the methods which satisfy the constraints, suitable risk assessment is done to reduce the chances of the product failing to meet the standards.

The following stages can advance the analytical development of the product lifecycle [40] advancement of the measuring; advancement of the decision rule; advancement of the ATP; choosing the analytical process that meets the set guidelines.

The adaptation of the previous concepts and the existing concept are used in the verification of the analytical process to support the consistency of the product that is to be examined. And for the development of the reliability of the analytical method throughout the increasing of the understanding and decreasing of the variability. This is done to ascertain that the analytical process correlates with the planned objectives specified in the analytical process. The lifecycle concept is an addition of the existing advantages of the AQbD concept [24].

4. Regulatory prospect

In line with pharmaceutical guidelines, the analytical process plays an important part in the control strategy. The analytical process ensures that the planned performance and quality of the finished product is achieved through the use of the analytical QbD in the drugs manufacturing process [41]. The execution of AQbD is depended on the notion of correct examination on the appropriate time; this adopts a vital role in the drug production process. Hence, analytical QbD execution in the production process as a control strategy ensures that the product meets the set standards [3]. Currently, the problem relating to the faultiness in the analytical method is becoming more prominent especially concerning the departments responsible for the transfers and quality control, considering the robustness of the whole process. The FDA's letter to the pharmaceutical companies which gives more interest to the reliable analytical techniques has in the recent past given rise in the Quality control. Therefore, the success of the company depends on the Quality by Design (QbD) employed in the analysis of the methods [16].

Conclusions

Quality by Design (QbD) is a concept extensively used now in the pharmaceutical industry than the traditional process. This method reduces product unpredictability and the connected dangers that can be incurred. The execution of Analytical Quality by Design (AQbD) in the product development is done through understanding the critical quality attributes, risk assessment and determination of the design space. The execution of QbD to the analytical development process has its advantages. The process provides a systematic approach; with the approach one is likely to explain the rudimentary and misleading concerns. In addition, the Design of experiments (DoE) strategy helps the assessor with more useful and precise data about the analytical process development. Through having a good

understanding of the ATP and the MODR, one can create an analytical procedure that is suitable for the demands of the ATP.

Consequently, it is important to use the application software of a computer to get more accurate data of the investigation. The AQbD approach to the lifecycle of the analytical method performance focuses on the envisioned purpose of the analytical procedures. This allows for the basic interpretation of the analytical process. Nonetheless, it is the ATP which incorporates the TMU in the analytical development process. The decision rule permits acceptable probabilities to be established, these probabilities are made with the aim that all the dangers can be handled.

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Conflict of interests

The authors declare no conflicts of interest.

Abbreviations

QbD = Quality by Design
AQbD = Analytical Quality by Design
QbT = Quality by Testing
MODR=Method Operable Design Region
OOS = Out-of-Specification
OOT=Out-of-Trend
ICH = International Conference of Harmonization
PAT = Process Analytical Technology
FDA = Food and Drug Administration
cGMP = current Good Manufacturing Process
LCA = Life Cycle Assessment
TMU = Target Measure Uncertainty
QTPP = Quality Target Product Profile
ATP = Analytical Target Profile
CQA= Critical Quality Attributes
RA = Risk Assessment
DoE = Design of Experiment

Authors' contribution

Maher Abdulrazaaq Alhakeem (Conceptualization; Methodology; Resources; Validation; Visualization; Writing – original draft; Writing – review & editing)
Mihaela Violeta Ghica (Conceptualization; Methodology; Writing – original draft; Writing – review & editing)
Cristina Dinu Pîrvu (Conceptualization; Methodology; Writing – original draft; Writing – review & editing)
Valentina Anuța (Conceptualization; Methodology; Writing – original draft; Writing– review & editing)
Lăcrămioara Popa (Conceptualization; Methodology; Resources; Supervision; Validation; Visualization; Writing – original draft; Writing – review & editing)

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RESEARCH ARTICLE

New UHPLC Method for Cannabidiol Determination in Hard Capsules

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Objectives: The aim of the study was to propose a new UHPLC method for the determination of cannabidiol (CBD) from supplements and drugs available on the Romanian market. **Materials and methods**: The HPLC assay of CBD was achieved by using a Phenomenex Gemini NX-C18 column. The mobile phase consisted of 70% acetonitrile and 30% water. Elution was performed in isocratic mode and the detection was done at 208 nm. The method was tested on hard capsules containing 150 mg of CBD. **Results and discussions**: The retention time of CBD was 2.8 minutes. Regression analysis showed good linearity over the 1-100 ug/ml concentration range. The lowest limit of quantification was established at 1 µg/ml. The method was developed by using reconstituted capsules. The substance proved low stability in solution at room temperature and stability at temperatures between 2-8ºC. The recovery of reconstituted samples was 96.77%. The commercial capsules had a very low content of 15-20% from declared content. **Conclusions**: The proposed method can be used for CBD determination in different pharmaceutical formulations – hard and soft capsules with coconut oil as excipient.

Keywords: cannabidiol, UHPLC, assay, hard capsules

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Introduction:

Cannabidiol (CBD), with the IUPAC naming: 2-[(1R,6R)-3-methyl-6-prop-1-en-2-ylcyclohex-2-en-1-yl]-5-pentylbenzene-1,3-diol is an alkaloid found in the species of Cannabis sativa and Cannabis ruderalis; usually it occurs in higher concentrations in Cannabis sativa [1, 2, 3].

CBD is a white crystalline powder with a low melting point of 63º C, practically insoluble in water 11.6 mg/L but soluble in ethanol, methanol, acetonitrile, dimethylsulfoxide (DMSO) [3].

From the pharmacological point of view CBD has a low affinity for CB1 and CB2 receptors. Many studies demonstrated that the substance is an antagonist on the CB1 and CB2 receptors. The alkaloid proved also an antagonist effect of the GPR 55 receptor, inverse agonist on GPR 3, 6 and 12 receptor and a partially agonist on 5 HT_{1A} receptor, having as a consequence antidepressant, anxiolytic and neuroprotective effects [4-6]. The oral route bioavailability is between 20-33% due to its lipophilic character (log P 6.33) and due to the first hepatic passage; as a result of these characteristics CBD is classified in the second class in Biopharmaceutic Classification System (BCS) because it has a low solubility but a high permeability [7]. Other effects are anti-dystonic [8], antiemetic [9,10] and anti-inflammatory [11-13].

As a result of the low solubility in water we can found on the pharmaceutical market formulas that contain CBD dissolved in different types of oils due to the much higher solubility. THC can be found besides CBD, a substance needing a prescription in some pharmaceutical formulations which is prohibited in many countries or it can be prescribed under various restrictions due to the fact that cannabis extract is included in the oily phase. In Romania CBD could be find as supplements, soft capsules that contain cannabis oil, and formulations, hard capsules with 150 mg CBD. The number of HPLC published methods for CBD determination is low, therefore the aim of this study was to propose a new UHPLC method for CBD determination in hard capsules.

Material and methods

Chemicals and reagents

The reference substances and reagents used were: CBD – 99.5 % purity obtained from Trigal Pharma, ACN - Acetonitrile supplied by SLW Chemicals.

Instrumentation and chromatographic equipment

Chromatographic equipment consisted of UHPLC Flexar 10 system (Perkin Elmer). The analysis was performed on a Gemini NX-C18 column, 3.0 x 100 mm, 3 µm. Other equipments: a water purification system - Direct Q3 System (Millipore); magnetic stirrer with heater– VWR Hotplate Advanced Series; refrigerated ultramicrocentrifuge 5430 R (Eppendorf).

Stock and quality control samples

The stock solution of 2 mg/mL was obtained by dissolving 20 mg of CBD in 10 mL of acetonitrile. A number of six concentrations were further obtained: 1 µg/ml, 5 µg/ml, 10 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml. The quality control samples were stored in the freezer at 5ºC.

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Extraction procedure

Ten capsules were weighted empty and with the mixture of CBD and coconut oil in order to determine the average mass of the content. A mass of 427 mg of was transfered in a 10 ml volumetric flask and extracted with ACN. The resulted composition was stirred at 1200 rpm for 60 minutes, without heating; the next step was to centrifugate at 12000 rpm for 5 minutes and in the end from the solution obtained a volume of 0.1 ml has been taken diluted 50 times with ACN.

A quantity of a reconstitued samples equivalent to 15 mg of CBD and 25 mg of coconut oil was extracted by applying the same procedure as for the hard capsules.

Calibration curve and linearity

Calibration curve consisted of a blank sample, a placebo sample (sample with coconut oil and ACN) and 6 non-zero samples, ranging from 1-100 µg/mL. The calibration curve plotted the peak areas of the 6 non-zero samples.

Results

HPLC method development

During the method development, several columns have been tested: Zorbax SB-Solvent Saver plus, Supelcosil LC-18, Luna C18 (2) and Gemini NX-C18. Due to the relatively high tendency of decomposition of CBD under temperature effect, the column temperature was set at an optimum temperature of 13 ºC and the samples were kept at 10 ºC.

The chromatography analysis was finally conducted on a Gemini NX-C18, 3.0 x 100 mm column, 3 µm, at 13 °C. The analysis time was set for 3.5 minutes. The elution was isocratic and a mixture of 70% ACN and 30% water as a mobile phase was used, whereas the flow rate was set for 1 mL/min and the injection volume at 10 µl. The chromatograms were obtained at 208 nm, however spectra were recorded between 200-400 nm.

At the begining of the chromatographic method development the purpose was to find a suitable mobile phase which leads to adequate peak's shape and short analysis time. Initially CBD was eluted with a mixture of 40% water and 60% acetonitrile gradient composition, resulting in broad peak with a retention time of 4 minutes. The chromatographic behaviour of the analyte allowed the reducing the percentage of water to 30% which determined the CBD retention time of 2.8 minutes and a total run-time of 3.5 min, providing symmetric and narrow peak. Based on the UV spectra, the optimal wavelength was set at 208 nm.

Analytical performances of the method

Specificity

The retention time of the CBD in the optimised chromatographic conditions was 2.8 min. No interferences were detected at the retention time of the CBD on the chromatogram of a blank sample (Figure 1).

Selectivity

Good selectivity was observed as no peak from the coconut oil interfered with CBD. The peaks showed small variability, RSD% being less than 5%.

Calibration curve and carry-over

The calibration curve was obtained as area versus concentration by using 6 calibration points. The concentrations ranged between 1-100 µg/ml and each calibration point was determined by replicate analysis (n=5). The value of the correlation coefficient was higher than 0.99. No carry-over was observed by injecting high-concentration standard before blank sample analysis.

Accuracy and precision

Accuracy and precision were calculated at three levels of concentration: low L (10 µg/ml), intermmediate I (25 µg/ml) and high H (100 µg/ml). The intra-day and inter-day variability were less than 2% at all the 3 concentrations tak-

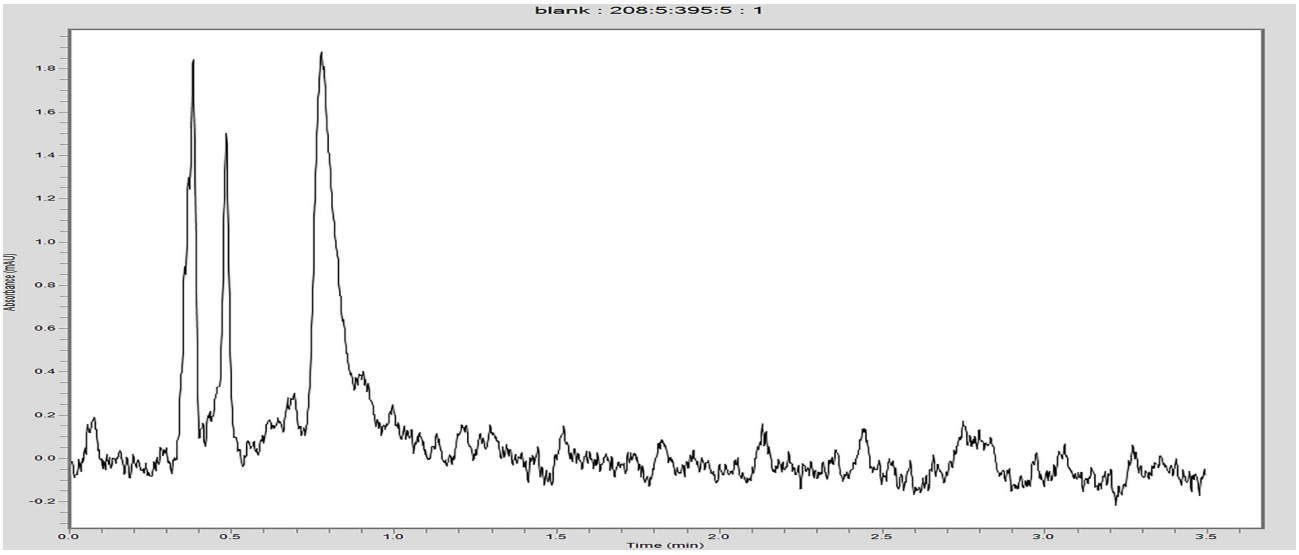

Fig. 1. Chromatogram of a blank sample

en into consideration, showing that the method was precise and accurate. The CV for intra-day assays ranged from 0.11% to 1.84% while the inter-day values of CV were between 0.31-0.51%. The mean accuracy for the intra-day assay ranged between 97.83%-102.99% while the inter-day accuracy was between 98.34%-102.81% (Table I).

Stability

It has been observed that if the CBD capsules are storaged at room temperature the concentration of CBD is 5 to 6 time smaller compared to when it is kept at temperatures between 2-8 ºC, also the CBD solution kept at room temperature gets colored into yellow. No specific storage conditions were labelled on the studied commercial samples. The indication for storage at low temperature is not a common label for this substance and commercial products. Our findings confirm the Sigma Aldrich storage indication at 2-8ºC.

Limits of quantification

These two parameters were analyzed on acetonitrile solutions, diluted from the stock solution. The lowest limit of quatification was 1 µg/ml (Figure 2) and the highest limit of quantification was 100 µg/ml (Figure 3).

Table I. Accuracy and precision for within batch and between-batch assays (n=5)

Level	Nominal concentration, µg/mL	Intra-day		Inter day	
		RSD %	Mean accuracy	RSD %	Mean accuracy
L level	10	1.84	99.19	0.50	102.5
I level	25	0.29	97.83	0.31	98.34
H level	100	0.11	102.99	0.51	102.81

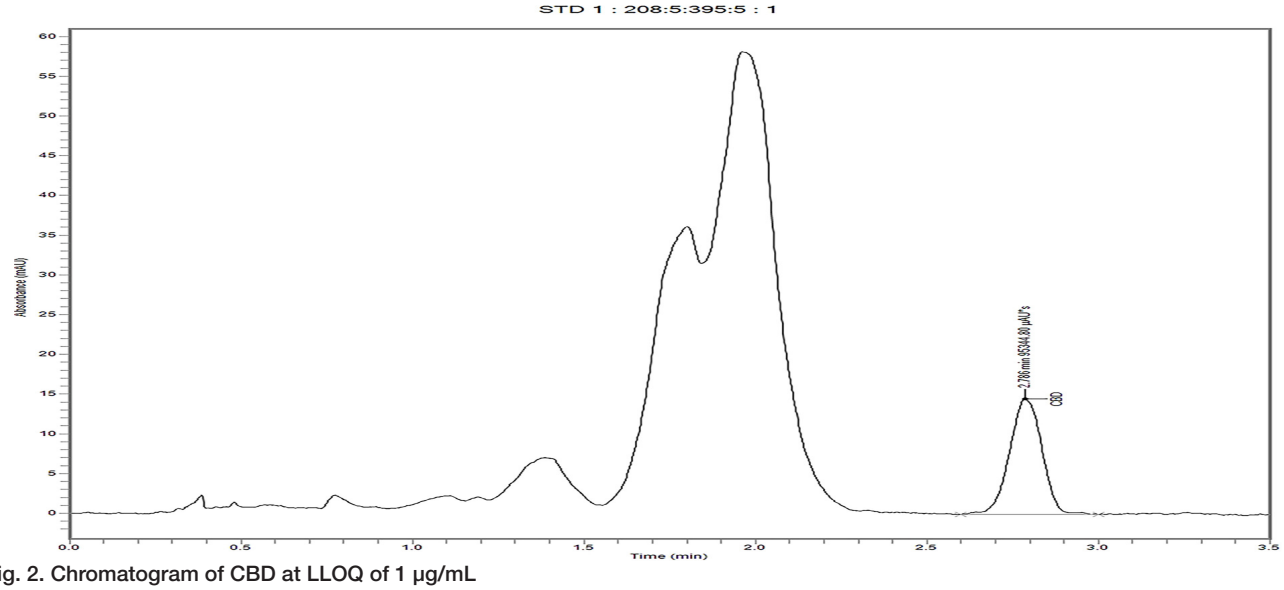

Fig. 2. Chromatogram of CBD at LLOQ of 1 µg/mL

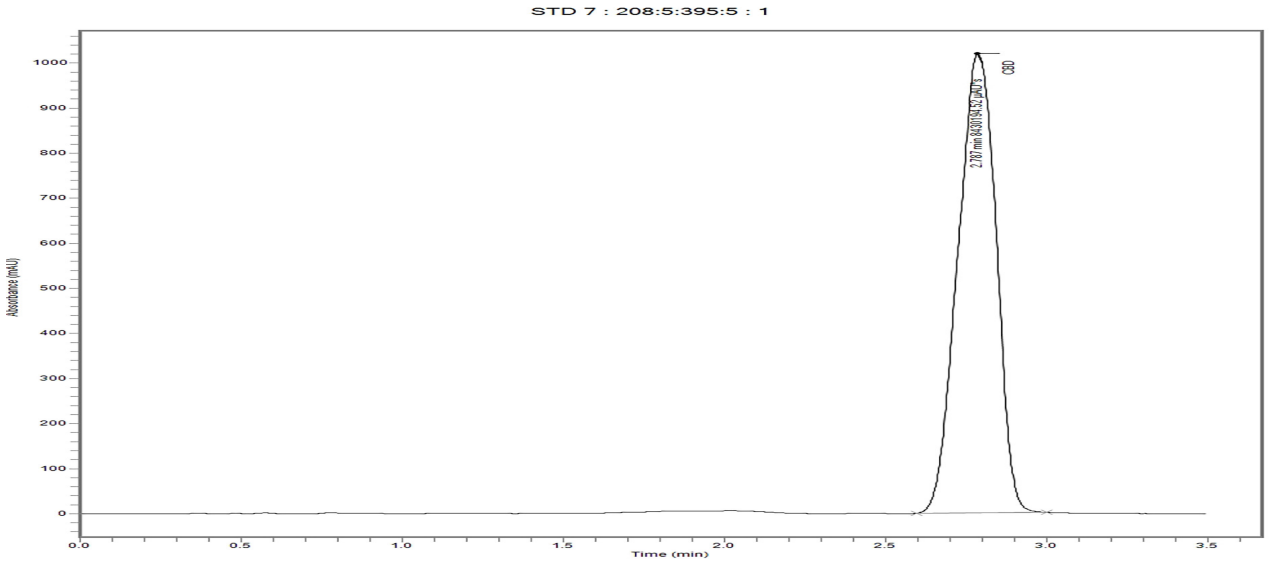

Fig. 3. Chromatogram of CBD at ULOQ of 100 µg/ml

Method application for CBD determination in the pharmaceutical product

The HPLC UV method was applied for CBD determination in capsules. The CBD content of the capsules kept at room temperature was about 15-20% of the declared concentration, while on the reconstituted capsules which were kept at temperatures between 2-8ºC for 7 days, the CBD content was 96.77%.

Discussions

In this study, a new HPLC-UV method was developed for the screening of CBD in hard capsules. A simple mobile phase composition allowed a rapid analysis of CBD (retention time of 2.8 min) on a middle-bore HPLC column, providing symmetric and efficient peak.

The CBD content of capsules was 96.77% on the reconstituted samples while on the ones found on the market it was ranged within 15-20%. Beneath the storage mentions an important factor which could conduct to low CBD concentratios may have been the fact that the capsules were close to the expiry date. However a decrease of concentration of 80% before expiration date indicates that in this case the storage below 8 °C is necessary.

There are few literature data regarding CBD determination in formulations. *Ravula A* et al. proposed a similar method in terms of efficiency and analytical performances [14], but in comparison with the method published by *Zgair A* et al. with a retention time about 8.3 minutes, the current method is closed to a high-throughput one [15].

Conclusions

The method obtained is simple, accurate, precise and specific and it can be used for CBD determination in formulations, hard capsules with cannabidiol and coconut oil.

Acknowledgements

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Authors' contribution

Alexandru Robert Vlad (Data curation; Formal analysis; Resources; Software; Validation; Writing – original draft; Writing – review & editing)
Lenard Farczadi (Investigation; Resources; Software; Supervision; Validation; Writing – review & editing)
Silvia Imre (Conceptualization; Data curation; Formal analysis; Methodology; Project administration; Writing – review & editing)
Adriana Daniela Ciurba (Conceptualization; Funding acquisition; Methodology; Project administration; Writing – review & editing)
Todoran Nicoleta (Conceptualization; Formal analysis; Funding acquisition; Writing – review & editing)
Redai Emoke (Visualization; Writing – review & editing)
Antonoaea Paula, Assist prof. (Conceptualization; Validation; Writing – review & editing)
Muntean Daniela Lucia (Funding acquisition; Project administration; Software; Writing – review & editing)

Conflict of interest

None to declare

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RESEARCH ARTICLE

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

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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.

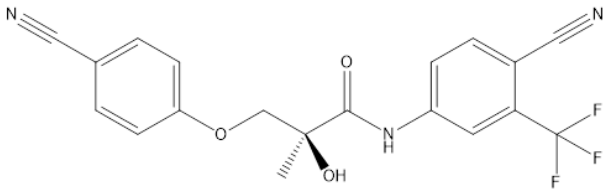

Fig. 1. Ostarine chemical structure

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.

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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.

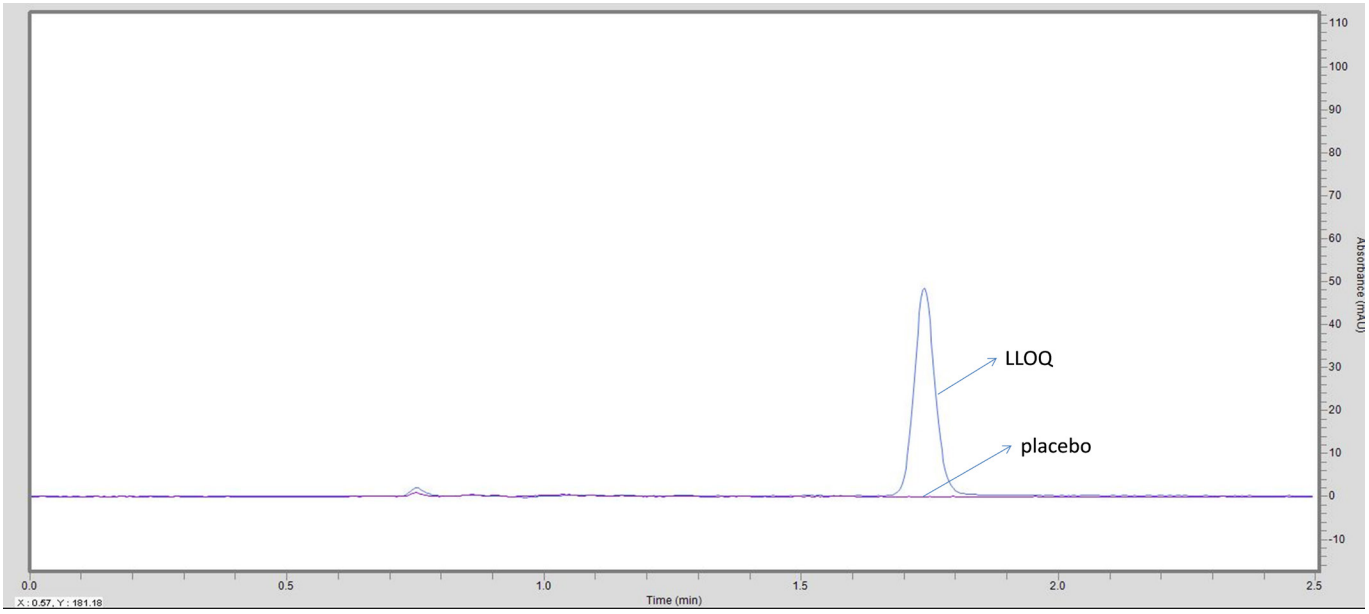

Fig. 2. Overlaid chromatograms of placebo solution and LLOQ

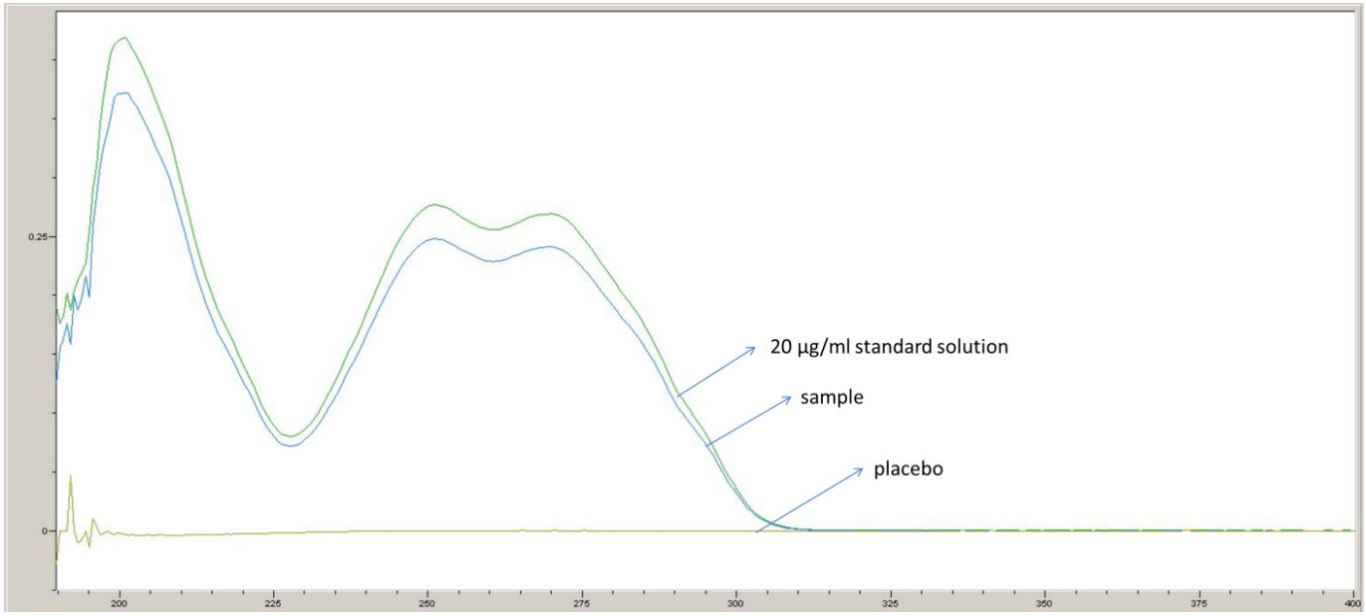

Fig. 3. Overlaid spectra of placebo solution, 20 µg/ml standard solution and sample

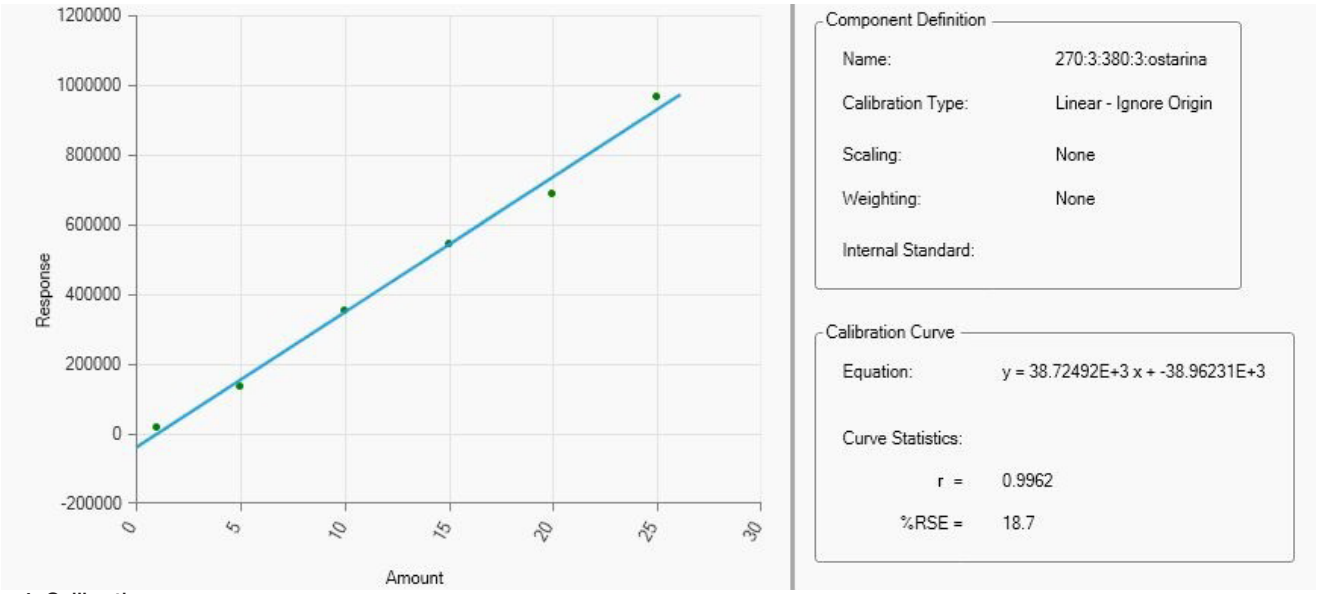

Fig. 4. Calibration curve

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.

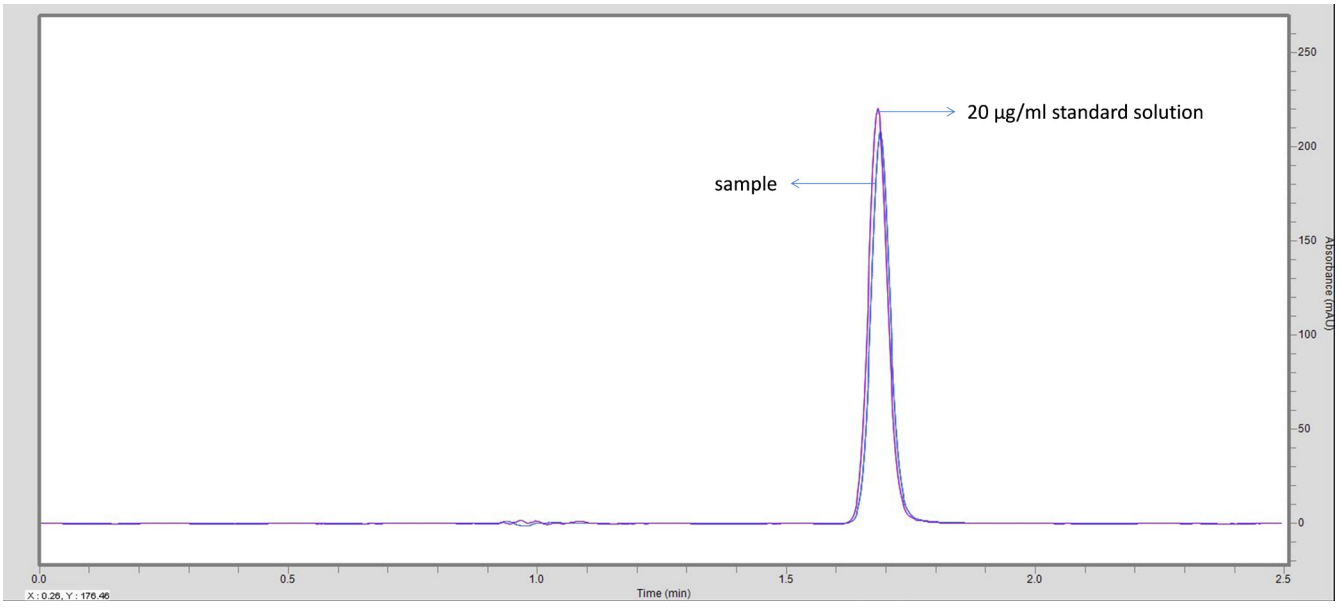

Fig. 5. Overlaid chromatograms of a 20 µg/ml standard solution and a sample solution prepared from capsule contents

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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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RESEARCH ARTICLE

Development and Validation of an UV-Spectrophotometric Method for the Assay of Strontium Ranelate and HPLC Stability Testing from Bulk and Pharmaceutical Dosage Form

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Objective: The present work offers a fast, reliable and easy UV spectrophotometric method for the assay of strontium ranelate from bulk samples and pharmaceutical dosage form.
Methods: The proposed method uses 0.1% V/V trichloroacetic acid as dissolution medium for spectrophotometric analysis, by signal detection at 321 nm. The method was validated according to the currently in-force international guidelines for linearity, accuracy, precision, robustness, limit of detection and quantification.
Results: The method was found to be linear in the range of 5-100 µg mL-1 (R2 > 0.999). Method accuracy was found in-between 98.87-100.41%, showing good linear correlation as well (R2 = 0.9997). The concentrations for limit of detection and limit of quantitation were found 1.13 µg mL-1 and 3.77 µg mL-1, resp. The proposed method showed good intra- and interday precision, with low RSD values of 0.53-1.24% and 1.11%, resp.
Conclusions: Stability studies performed by both HPLC and UV spectrophotometric methods revealed that the active substance is highly susceptible to acidic hydrolysis, oxidation and exposure to high temperature.

Keywords: strontium ranelate, UV spectrophotometry, validation, stress stability, HPLC

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Introduction

Strontium ranelate (SrR, Protelos, Osseor), chemically is the di-strontium salt of 2-(2-carboxy-4-cyano-5-[N,N-di(carboxymethyl)amino]thiophene-3-yl) acetic acid (ranelic acid) (Fig. 1), is used in the treatment of post-menopausal osteoporosis, having a positive risk/benefit ratio, and represents a viable alternative when medication with other anti-osteoporotic agents is futile [1]. The active substance is freely soluble in aqueous media at pH < 2, presenting a decreasing solubility by reaching the neutral domain. It is practically insoluble in organic solvents [2].

Literature data revealed that only a few UV spectrophotometric methods have been reported for strontium ranelate so far [3-6]. Further analytical methods imply RP-HPLC determination [7-10] and capillary zone electrophoresis [11] for the determination of the active substance.

According to the guidelines Q1A (R2) and Q1B published by the International Conference on Harmonization, the forced degradation studies of the active substances are helpful for the identification of the possible degradation products and also may be applied for the evaluation of the intrinsic stability of the molecule. The stress stability

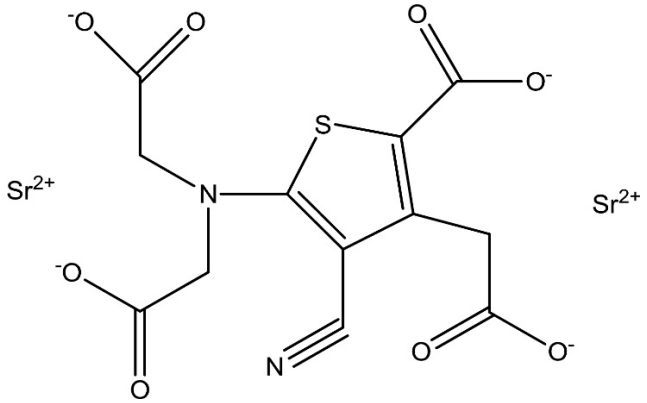

Fig. 1. Chemical structure of strontium ranelate

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testing of an active substance according to the mentioned directives should include the effect of temperature, humidity, oxidative conditions, photolysis and hydrolysis across a wide pH range. Also, it is specified that photolysis should be performed under a light source which provides an illumination greater than 1.2 million lux, and an energy in the near UV of not less than 200 Wh/m^2 [12,13].

Taking into consideration that the already available UV spectrophotometric methods for the assay of strontium ranelate use a multicomponent solvent system, having a prolonged sample preparation time, our main objective was to develop and validate a high throughput, cost-effective and accessible method for the assay of strontium ranelate from both bulk samples and pharmaceutical dosage form. Moreover, we aimed to test the stability of the active substance in accordance with the currently in-force international guidelines with two different analytical methods using an already available HPLC method and the currently presented UV spectrophotometric method.

Material and methods

Reagents

Strontium ranelate (SrR) standard was purchased from Sigma Aldrich (St. Louis, USA) and bulk active pharmaceutical ingredient (strontium ranelate octahydrate) was obtained from Dishman Pharmaceuticals and Chemicals (Ahmedabad, India). Trichloroacetic acid (TCAA) and trifluoroacetic acid (TFA) was used from Merck (Darmstadt, Germany). Water, purified was obtained by means of a Milli-Q water purification system (Millipore, Merck, Germany). Osseor® 2-g granules for oral suspension (Lés Laboratoires, Serviér, France) was purchased from local pharmacies. Selectivity studies were performed using mannitol (Pearlitol 300DC, Ph. Eur., Roquette Pharma, France), maltodextrin (Lycatab DSH, Ph. Eur., Roquette Pharma, France) and aspartame (Ph. Eur., Sigma Aldrich, USA), excipients of the original product.

Preparation of standard solution

Standard solution was prepared by dissolving 4 mg SrR in TCAA 0.1% V/V solution in a 100 mL volumetric flask, and completed to the mark with the same solution, obtaining a final concentration of 40 μg mL^{-1}.

Apparatus and spectrophotometric method

UV spectrophotometric determination was performed using a Shimadzu 1800 UV-VIS (Shimadzu Co., Kyoto, Japan) spectrophotometer, special optical glass (OS type, Hellma Analytics, Müllheim, Germany) cuvettes with an optical path of 10 mm. For the evaluation of the optimal determination wavelength a scanning run (200-400 nm) was carried out and TCAA 0.1% V/V was used as a blank solution. For robustness studies a Labomed UVD-3200 (Labomed Inc., Los Angeles, USA) spectrophotometer was used for comparison. Furthermore, method robustness was tested for the type of the cuvettes used for routine analysis, as the specification of OS type cuvettes indicates that it is useable in the range of 320-2500 nm. Absorbance of SrR stock solution was evaluated for both OS type and QS (Suprasil® quartz glass, Hellma Analytics, Müllheim, Germany) type cuvettes, for which the recommended working interval is greater, lying between 200-2500 nm.

Method validation

Linearity. – Method linearity was assessed in the range of 5-100 μg mL^{-1} in seven points (5, 10, 20, 40, 60, 80, 100 μg mL^{-1}), repeated five times for each concentration. Solutions were prepared by dilution with TCAA 0.1% V/V from a stock solution of 100 μg mL^{-1}.

Selectivity – The selectivity of the method was investigated considering the quantitative and qualitative composition of Osseor® 2g granules for oral suspension. Placebo formulation was prepared using 4.0 g mannitol, 0.4 g maltodextrin, and 0.02 g aspartame per dose. Selectivity was evaluated by comparing the absorbance spectrum of individually prepared samples of the excipients, placebo mixture, and placebo spiked with SrR. All samples were prepared under the same conditions using TCAA 0.1% V/V as dissolution medium.

Accuracy (recovery) – The accuracy of the method was tested using placebo mixture samples spiked with SrR, at five concentration levels (50%, 75%, 100%, 125%, and 150%) of the working concentration, repeated three times for each concentration.

Robustness – Method robustness was verified for individual changes in detection wavelength (321 nm ± 2 nm), temperature (4°C vs. 25°C), instrumentation (Shimadzu 1800 vs. Labomed UVD-3200), pH (2.0 ± 0.2) and cuvette type (OS vs. QS).

Precision – The precision of the method was evaluated for both intraday- (repeatability) and intermediate precision. Six individual samples were prepared on the same day and on two different days by two analysts. Samples were prepared from Osseor® 2g granules corresponding to 3.12 mg SrR anhydrous.

Limit of detection (LOD) and limit of quantification (LOQ) – LOD and LOQ were calculated from the calibration plot as $3.3\sigma/S$ and $10\sigma/S$, resp., where σ is the standard error of the intercept and S represents the slope of the calibration plot.

Statistical analysis

Statistical analyses were carried out using Minitab 17.0 (Coventry, UK) and Statistica 8.0 (Tulsa, USA) software for the validation of the UV spectrophotometric method.

Method linearity and the normal distribution of the residuals was tested using the Shapiro-Wilk's test (confidence interval of 95%). Statistical significance was considered if both of the following criteria are met: the W value for SrR was greater than the critical tabulated value and $p > 0.05$. ANOVA F-test and its test for lack of fit (confidence limit

of 95%) was used for the assessment of the significance of the calibration curve.

Student's t-test was used for statistically evaluating the intraday and intermediate precision results (confidence level of 95%).

Stress stability testing

Stability testing was performed for acidic – (with 0.1 M HCl) and alkali hydrolysis (with 0.1 M NaOH), oxidative stress (3% H_2O_2), thermal degradation (60ºC and 120ºC for 2 h) and photolysis (under a 125W UV lamp for 2 h). For the acidic –, and alkali hydrolysis, oxidative stress conditions three samples, with two replicates were prepared individually for time points of 1, 2 and 7 days. Thermal degradation and photolysis studies were also performed from two replicate samples under the specified conditions.

Sample preparation for HPLC determination – for stability testing 4 mg of SrR and 6.24 mg of Osseor® were weighed in 50 mL volumetric flasks. For acidic and alkali hydrolysis and oxidative stress conditions 2 mL of 0.1 M HCl, 0.1 M NaOH and 3% H_2O_2 were added, resp. The samples were held in closed dark chambers until sampling. Before analysis, the samples were completed with TFA 0.1% V/V, stirred on an ultrasound bath for 2 minutes and filtered through a 0.45-µm Whatman® nylon filter (General Electric Healthcare, UK) in brown HPLC vials. The first 2 mL of the filtered solution were discarded. For thermal degradation studies and photolysis the volumetric flasks were completed with TFA 0.1% V/V after weighing, stirred on an ultrasound bath for 2 minutes and filtered through a 0.45-µm Whatman® nylon filter in brown HPLC vials, prior to analysis.

Sample analysis was performed according to the method described by Kovács et al. [9].

Sample preparation for UV spectrophotometric determination - for stability testing 4 mg of SrR and 6.24 mg of Osseor® were weighed in 50 mL volumetric flasks. For acidic and alkali hydrolysis and oxidative stress conditions 2 mL of 0.1 M HCl, 0.1 M NaOH and 3% H_2O_2 were added, resp. The samples were held in closed dark chambers until sampling. Before analysis, the samples were completed with TCAA 0.1% V/V. For thermal degradation studies and photolysis the volumetric flasks were completed with TCAA 0.1% V/V after weighing.

Results and discussion

Absorption spectrum and selectivity. – The absorption spectrum of the stock solution revealed that strontium ranelate has an absorbance maximum at λ = 321 nm. Selectivity studies elucidated that there is no interference between strontium ranelate and the selected excipients at λ = 321 nm. Furthermore, no change in absorbance maximum was observed between the two types of cuvettes tested (Fig. 2).

Linearity. – The method was found to be linear in the range of 5-100 µg mL^{-1} (R^2 = 0.9999). The normal distribution of residuals was evaluated by the Shapiro-Wilk's test, indicating that the residuals follow a normal distribution, as the W_{SrR} is greater than the critical tabulated value, W_c, and $p > 0.05$.

Accuracy (recovery). – The recovery of placebo spiked samples were found between 98.87-100.41% for the tested range of 50-150% of the working concentration, fulfilling the requirements of international standard to be in-between 95-105%. The mean recovery was found to be 99.24%. The linearity of the tested samples showed a good correlation with R^2 = 0.9997.

Robustness. – The method was found to be robust for all tested changes, the obtained concentrations lying between 98.23-102.16%.

Method precision. – The precision of the method returned low RSD% values for both the intraday (0.53-1.24%) and intermediate precision (1.11%).

Limit of quantification and limit of detection. – Based on the regression analysis LOD and LOQ values were calculated, resulting in 1.13 µg mL^{-1} and 3.77 µg mL^{-1}, resp.

The presented method offers a greater linearity interval in comparison to the already available UV-spectrophotometric methods for the assay of strontium ranelate, where depending on the dissolution medium and method peculiarities linearity ranges of 2-20 µg mL^{-1} [3], 4-28 µg mL^{-1} [4], 5-55 µg mL^{-1} [5] and 5-50 µg mL^{-1} [6] are reported. Although the current method only approximates the lower limits of the disclosed methods (5 µg mL^{-1} vs. 2-4 µg mL^{-1}), the upper limit is substantially superior when compared to literature data (100 µg mL^{-1} vs. 50-55 µg mL^{-1}). Method accuracy shows similar recovery intervals to the referred methods. Our method is more bounded regarding the LOD and LOQ values (1.13 µg mL^{-1} and 3.77 µg mL^{-1}, resp.) when compared e.g. to the values presented

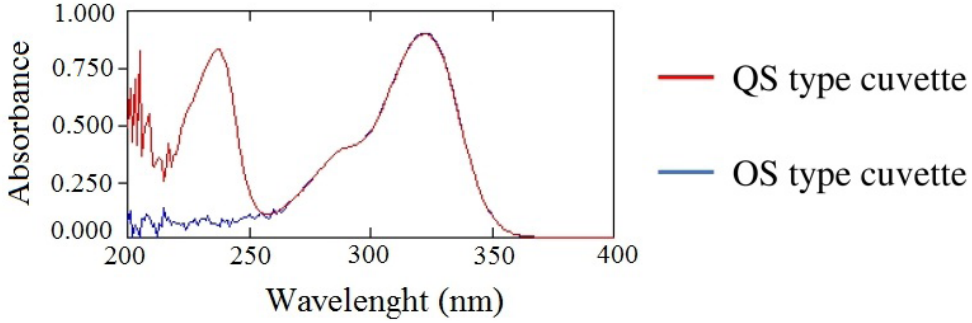

Fig. 2. Absorbance spectrum of strontium ranelate using QS (special quartz) and OS (special optic) type cuvettes

by Swami et al. [4] of 0.013 μg mL^{-1} and 0.043 μg mL^{-1} for LOD and LOQ, resp. Finally, as the previous studies lack the robustness testing of the method, the newly developed technique assessed the impact of general variables (detection wavelength, instrumentation, cuvette type) on method performance and tested them during the validation procedure (Table I).

Stress stability testing. – The active substance proved to be highly susceptible to acidic hydrolysis, oxidative stress and thermal degradation, especially at high temperatures. Alkali conditions, UV light or lower thermal impact has only a negligible effect on the stability of SrR. The results are in concordance with the finding presented by Swami et al. [7], as the active substance subjected to acidic hydrolysis and oxidative stress (1 M HCl and 3% H_2O_2 for ½ hour) presents high degradation (77.15% and 80.89%, resp.), whilst under thermal impact (60°C for ½ hour), alkali hydrolysis (1 M NaOH for ½ hour) and UV irradiation (24 hours) only slight decomposition of SrR was observed (94.77%, 97.59% and 98.19%, resp.).

The stability testing results are similar to the ones obtained in our previous HPLC studies [9]. The degradation profile presents the same level, to a certain extent, regarding hydrolysis, oxidative and photolytic studies for both HPLC and UV-spectrophotometric determinations (Table II and III).

Conclusions

The presented UV spectrophotometric method proved to be adequate for the routine analysis of strontium ranelate

Table I. Analytical merits of the developed UV-spectrophotometric method

Parameter	Results	Statistical results
Linearity (μg mL^{-1})	5-100 (y = 0.0242x + 0.0116)	R^2 = 0.99991 (n=7) W_{SrR} = 0.911 (p = 0.40)[a] Cpk = 2.43[d]
Accuracy (%)	98.87-100.41	R^2 = 0.9997 (n=5)
Intraday precision (RSD, %), n=6	0.53-1.24	$t_{\text{analyst 1, day 1 vs. analyst 2, day 1}}$ = 1.546 (p = 0.15)[b] $t_{\text{analyst 1, day 2 vs. analyst 2, day 2}}$ = 0.656 (p=0.53)[b]
Inter-day precision (RSD, %), n=24	1.11	$t_{\text{analyst 1, day 1 vs. analyst 2, day 2}}$ = 0.580 (p = 0.57)[b] $t_{\text{analyst 1, day 1 vs. analyst 1, day 2}}$ = 0.298 (p = 0.77)[b] $t_{\text{analyst 1 vs. analyst 2}}$ = 1.614 (p = 0.12)[c]
Instrument precision (RSD, %)	0.33	-
LOD (μg mL^{-1})	1.13	-
LOQ (μg mL^{-1})	3.77	-

[a] W_c = 0.850, critical value of Shapiro-Wilk's test; [b] Critical value of t = 2.228, df = 10; [c] Critical value of t = 2.074, df = 22; [d] C_{pk} > 1.33 (limit of acceptance for process capability)

Table II. Stress stability test results of strontium ranelate bulk samples and Osseor® by RP-HPLC

Strontium ranelate bulk sample, % of degradation by HPLC							
	Stock solution	NaOH 0.1M	HCl 0.1M	H_2O_2 3%	Thermal degradation		UV light exposure
					60°C / 2h	105°C / 24h	
Day 1	3.55	Nil	8.11	8.20			
Day 2	9.32	Nil	10.54	27.96	17.19	100.00	2.70
Day 7	31.97	0.96	37.75	43.02			
Osseor® samples, % of degradation by HPLC							
	Stock solution	NaOH 0.1M	HCl 0.1M	H_2O_2 3%	Thermal degradation		UV light exposure
					60°C / 2h	105°C / 24h	
Day 1	3.65	Nil	6.80	8.97			
Day 2	6.76	Nil	9.86	32.13	4.38	100.00	0.26
Day 7	23.61	0.59	35.65	57.84			

Table III. Stress stability test results of strontium ranelate bulk samples and Osseor® by UV-spectrophotometry

Strontium ranelate bulk sample, % of degradation by UV spectrophotometry							
	Stock solution	NaOH 0.1M	HCl 0.1M	H_2O_2 3%	Thermal degradation		UV light exposure
					60°C / 2h	105°C / 24h	
Day 1	2.26	Nil	8.24	3.74			
Day 2	7.69	Nil	12.91	9.59	9.90	87.87	3.20
Day 7	25.73	0.95	26.60	61.25			
Osseor® samples, % of degradation by UV spectrophotometry							
	Stock solution	NaOH 0.1M	HCl 0.1M	H_2O_2 3%	Thermal degradation		UV light exposure
					60°C / 2h	105°C / 24h	
Day 1	2.53	Nil	2.48	3.27			
Day 2	7.70	Nil	11.11	6.91	14.24	97.98	2.86
Day 7	17.40	0.72	26.55	50.34			

from both bulk samples and pharmaceutical dosage forms. The method offers a high throughput, low cost sample measurement, using conventional apparatus and single component solvent system (TCAA 0.1%). The validated method according to the currently in-force international guidelines presents an appropriate linearity in the range of 5-100 μg mL^{-1} and a mean recovery of 99.24%. Moreover, the method proved to be applicable regardless of the type of cuvettes, thus might ease the analytical transfer between control laboratories. Furthermore, taking into consideration the precision of the method validated from Osseor® 2 g granules for oral suspension, this determination might represent an alternative to the currently available analytical methods. Both the HPLC and UV spectrophotometric methods proved to be adequate for the determination of the degradation profile of strontium ranelate, the method having its limitations in the quantification of the formed impurities. The stability studies of the active pharmaceutical ingredient from both bulk samples and pharmaceutical dosage form revealed that it is highly susceptible to acidic hydrolysis, oxidative stress and heat being slightly influenced in alkali media and UV light exposure.

Authors' contribution

Béla Kovács (Conceptualization; Validation; Writing – original draft)
Réka Molnár (Formal analysis; Validation)
Előd Ernő Nagy (Supervision; Writing – review & editing)
Éva Katalin Kelemen (Funding acquisition; Writing – review & editing)
Blanka Székely-Szentmiklósi (Writing – review & editing)
István Székely-Szentmiklósi (Project administration; Writing – original draft)
Boglárka Kovács-Deák (Formal analysis; Writing – original draft)
Árpád Gyéresi (Writing – review & editing)

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Conflict of interest

None to declare.

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RESEARCH ARTICLE

The Statistical Analysis of Pharmacokinetic Parameters in the Context of Bioequivalence Testing of Two Anthelmintic Formulas Based on Ivermectine and Triclabendazole in Sheep

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Conducting bioequivalence studies is an essential step during the market authorization process of generic pharmaceutical formulations, for both human or veterinary use. The aim of the present study was to evaluate the pharmacokinetics of triclabendazole sulphoxide, the main metabolite of triclabendazole, and ivermectin in order to evaluate the bioavailability and bioequivalence of a novel sheep anthelmintic formulation of oral suspension for sheep treatment containing triclabendazole 50 mg/mL and ivermectin 1 mg/mL compared to the reference product. In order to determine relative bioavailability of the test product with respect to the reference product the study was conducted on 36 clinically healthy sheep, following an unicentric, randomized, cross-over, two-sequence, two-treatment and 14-day wash-out study design. For the determination of triclabendazole sulphoxide and ivermectin sheep plasma concentrations, two rapid, selective high performance liquid chromatography coupled with mass spectrometry (LC-MS/MS) methods were developed and validated. The measured plasma concentrations of triclabendazole sulphoxide and ivermectin were used for the pharmacokinetic analysis and the determination of bioequivalence between the test product with regards to the reference product. The noncompartmental analysis of the pharmacokinetic data for both triclabendazole sulphoxide and ivermectin showed similarities between first-order kinetics of the test and reference product. The relevant pharmacokinetic parameters (Cmax, AUClast, AUCtot) were determined and the bioequivalence between the test and reference product could be concluded.

Keywords: ivermectin, triclabendazole, pharmacokinetics, bioequivalence

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Introduction

Triclabendazole is an anthelmintic drug which is used for the treatment of liver fluke, Fasciola hepatica and Fasciola gigantica, in sheep and other livestock. Triclabendazole cannot only be used for treating liver fluke in livestock but may also be used in cases of human fascioliasis [1].

Following oral administration and absorption triclabendazole is rapidly metabolized in the liver. Triclabendazole and its two metabolites, triclabendazole sulphoxide and triclabendazole sulphone, have high activity against the migratory juvenile stages of liver fluke. Both metabolites show important plasma protein binding which explains their relatively slow elimination from the animal's organism. Triclabendazole sulphoxide and triclabendazole sulphone are also the main two unconjugated metabolites present in bile. The metabolic mechanism of triclabendazole is complex, but helps maintaining concentrations levels of triclabendazole and its metabolites in the bloodstream over longer periods of time which in turn contributes to its high efficacy against the fluke [2].

Triclabendazole sulphoxide is the main active metabolite of triclabendazole and the major metabolite detected in the host [2]. Thus, the bioequivalence of a product containing triclabendazole can be evaluated by analyzing the pharmacokinetics of the metabolite in plasma and comparing it between the generic (test) and reference product.

Ivermectin is a mixture of two chemically modified avermectins (B1a and B1b) and it is a very widely used drug against a wide array of nematode species. It is lipophilic and thus easily soluble in organic solvents but not water soluble [3]. Due to this it tends to accumulate in fat tissue and persist for longer periods of time in the body not only due to this accumulation but also due to its low plasma clearance. For bioequivalence purposes the pharmacokinetics of avermectin B1a in plasma were analyzed and compared between the test and reference product, as avermectin B1a makes up more than 90% of ivermectin.

In order to further extend the spectrum of use, the antiparasitic drugs are often used in combined therapy [4]. In some cases, the combined therapy may even yield higher efficacy than the separately applied treatments [5]. Due to the multiple compounds, however, the bioequivalence studies performed on combined products are more difficult and complex, from both analytical point of view as well as due to the increased number of pharmacokinetic analyzes which need to be carried out.

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Bioequivalence studies are an essential step in the process of obtaining marketing authorization for generic medicinal products in the USA and Europe. As at the time of the study no generic products containing a combination of triclabendazole and ivermectin were marketed, the launch of a generic product is very important. Generic medicinal products not only offer consumers a choice, but can also satisfy demand in areas where the originator product is scarcely available or not available. On the other hand due to competition the authorization of generic product leads to a decrease in prices and makes products more accessible to a larger population, especially in underdeveloped countries

The aim of the present study was to evaluate the pharmacokinetics of triclabendazole sulphoxide, the main metabolite of triclabendazole, and ivermectin in order to evaluate the bioavailability and bioequivalence of a novel sheep anthelmintic formulation, an oral suspension containing triclabendazole 50 mg/ml and ivermectin 1 mg/ml, compared to the reference product.

Methods

Subjects

The study was conducted on a total of 36 healthy male and female sheep in accordance with the following guidelines: Good Clinical Practice guidelines [6], EMA Guidelines for the Conduct of Bioequivalence Studies for Veterinary Medicinal Products [7,8,9] as well as other applicable regulations or laws enforceable in Romania.

The study protocol was reviewed and approved by the Ethics Committee of the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca.

Sheep were deemed to be healthy judged by means of a medical and standard laboratory examination (normal hematology, clinical chemistry and urinalysis).

Study design

The study consisted of two study periods. All animals received in each of both periods of the study a single dose of product, 0.6 ml/kg bodyweight (either test or reference formulation). The first period of the study was followed, after a 14 days washout period, by the second period of the trial. The sheep were fast from food from the morning of the day of the drug administration until 24 hours after administration. Water was allowed ad libitum until 2 hours before and 2 hours after administration. Products administered were the test product Trimectin 50 mg/ml + 1 mg/ml Oral Suspension for Sheep (ivermectin 1 mg, triclabendazole 50 mg) (Pharma VIM Kft., Hungary) and the reference product Fasimec Duo 50 mg/ml + 1 mg/ml Oral Suspension for Sheep (ivermectin 1 mg, triclabendazole 50 mg) (Elanco Animal Health, UK).

Venous blood samples (5 mL) were drawn from the jugular vein, from the neck area, of each subject in tubes containing anticoagulant (K3EDTA) before the administration (time 0.0) and at 0.5; 1.0; 1.5; 2.0; 3.0; 4.0; 6.0; 8.0; 10.0; 12.0; 14.0; 16.0; 18.0; 20.0; 22.0; 24.0; 28.0; 36.0; 48.0; 72.0; 96.0; 120.0; 144.0; 168.0; 216.0; 264.0; 336.0; 408.0; 480.0 hours post dose administration. Samples were centrifuged using a Centurion Scientific K241R Centrifuge, at 5000 rpm for 10 minutes within 60 minutes after collection. The plasma was separated and transferred to two test tubes (sample and backup sample). The test tubes were securely closed, labeled and immediately frozen for storage at -20 °C until analysis.

Analysis of plasma samples

Sheep plasma concentrations of triclabendazole sulphoxide and ivermectin (avermectin B1a) were determined using validated high-performance liquid chromatography coupled with mass spectrometry (LC-MS/MS) methods which were developed in-house. A typical chromatogram for triclabendazole sulphoxide and its internal standard are presented in Figure 1.

Pharmacokinetic and statistical analysis

Data from all evaluable subjects were included in bioequivalence assessment. All 36 subjects finalized the study and

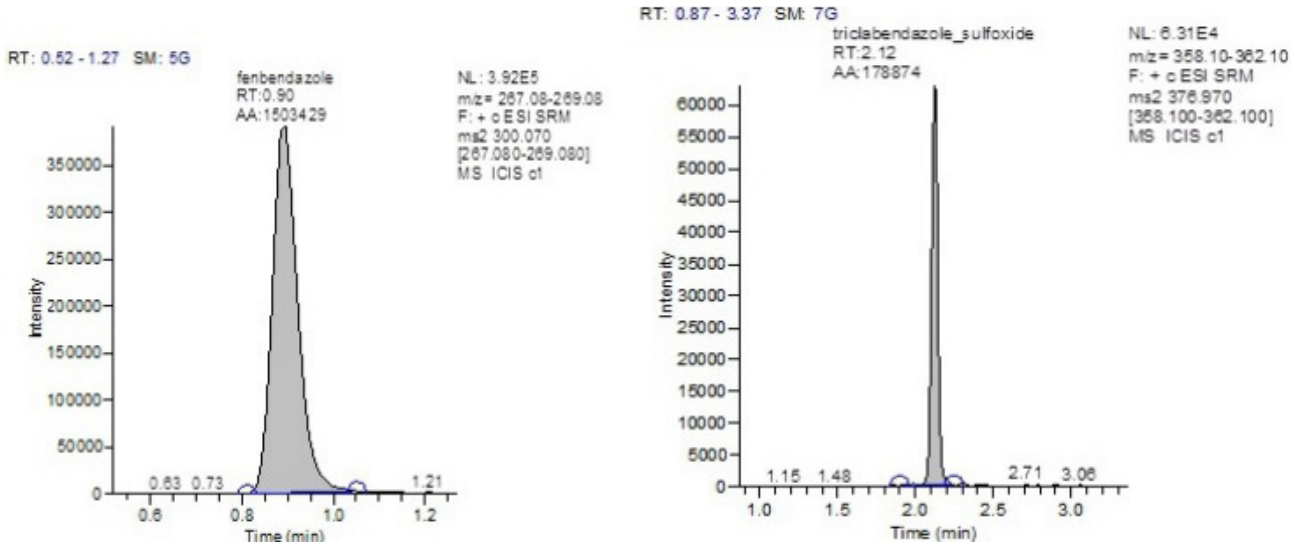

Fig. 1. A typical chromatogram for triclabendazole sulphoxide and its internal standard (fenbendazole)

were included in the statistical analysis of pharmacokinetic data. Noncompartmental pharmacokinetic analysis method was carried out for both triclabendazole sulphoxide and ivermectin in order to determine the pharmacokinetic parameters. For bioequivalence assessment for all the primary pharmacokinetic parameters considered (Cmax, AUClast) a confidence interval of 90% for the ratio of the population means T/R (Test/Reference) was calculated. All these pharmacokinetic parameters were planned for analysis using ANOVA, after data logarithmic transformation. A reference 90% confidence interval of 0.8 – 1.25 was chosen.

In addition, summary statistics were performed, including arithmetic mean, harmonic mean, geometric mean, SEM, standard deviation, median, range. For Tmax comparison, non-parametric tests were carried out (Kruskal-Wallis and Friedman test) on untransformed data. For MRT (mean residence time) and Thalf the same statistical tests were applied as for primary parameters (Latin-square ANOVA with determination of 90% CI of the ratio of means T/R after data log-transformation and descriptive statistics). The pharmacokinetic analysis was performed using Kinetica 5 (ThermoLabsystems, USA) [10].

In order to evaluate a possible statistical or clinical significance of the pharmacokinetic interaction, an analysis of variance (ANOVA) was performed on the main calculated pharmacokinetic parameters, using general linear model procedures, in which the sources of variation were the subject and the treatment. All calculations and evaluations were analyzed taking into consideration the current applicable guidelines [11,12].

Results

All sheep plasma samples obtained during the study were analyzed using the fully validated LC-MS method developed in-house. Improbable results obtained during initial assay of samples were reanalyzed in accordance with the current guidelines and incurred samples were also analyzed on the last day of analysis as part of the validation process.

During incurred samples analysis the percent difference between concentrations obtained for the initial analysis and the concentrations obtained after reanalysis was not greater than 20% of their mean for 74% of samples for triclabendazol sulphoxide and 84% of samples for ivermectin, respectively.

The mean plasma concentration curves for triclabendazole sulphoxide and ivermectin were determined, for both test and reference product, and are shown in Figures 2, 3, 4 and 5 respectively.

The mean values for triclabendazole sulphoxide Cmax were 56.0 (+/- 17.1) µg/ml for test and 54.4 (+/- 20.1) µg/ml for the reference product. For ivermectin Cmax mean values were 41.2 (+/-8.7) ng/ml for test and 42.2 (+/-10.5) ng/ml for reference product, respectively.

Statistical analysis was carried out for the obtained data. After applying ANOVA the 90% confidence intervals for the ratio of means of triclabendazole sulphoxide and ivermectin "Test/Reference" for the highest concentrations (Cmax) were 0.98-1.12 for triclabendazol sulphoxide and 0.92-1.05 for ivermectin. The 90% confidence intervals for the ratio of means of triclabendazole sulphoxide and ivermectin "Test/Reference" for the area under the curve up to the last measurable concentration (AUClast) were 0.88-1.07 for triclabendazole sulphoxide and 0.86-1.06 for ivermectin. The mean pharmacokinetic parameters for triclabendazole sulphoxide and ivermectin as well as the statistics for them are shown in Table I and Table II.

Discussions

Incurred sample reanalysis testing passed criteria stipulated in current bioanalytical method validation guidelines [13,14] as the percent difference between concentrations obtained for the initial analysis and the concentrations obtained after reanalysis was within the +/- 20% of their mean for more than 67% of the reanalyzed samples, for each triclabendazol sulphoxide and ivermectin. Thus, the LC-MS method used for analysis being validated and in-

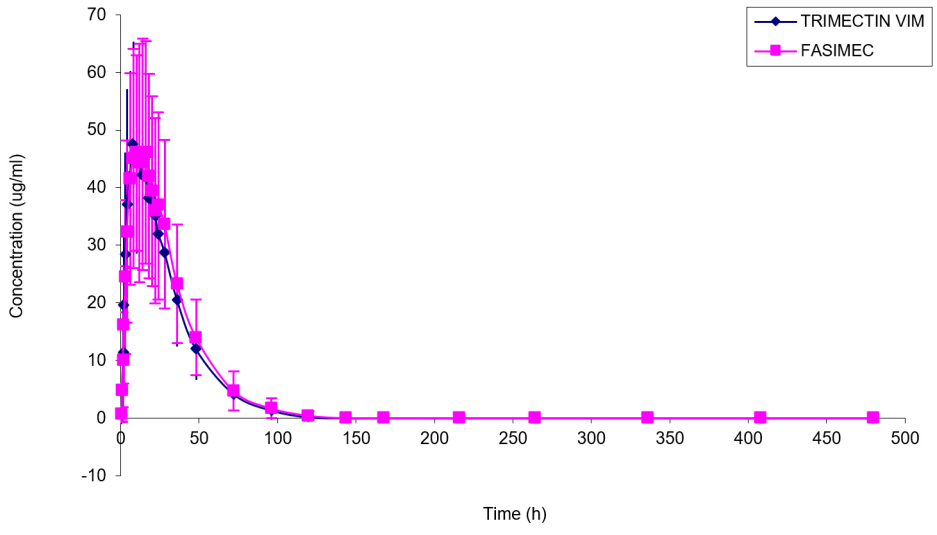

Fig. 2. Mean plasma concentration curves for triclabendzole sulphoxide in bioequivalence testing of two anthelmintic formulas for sheep

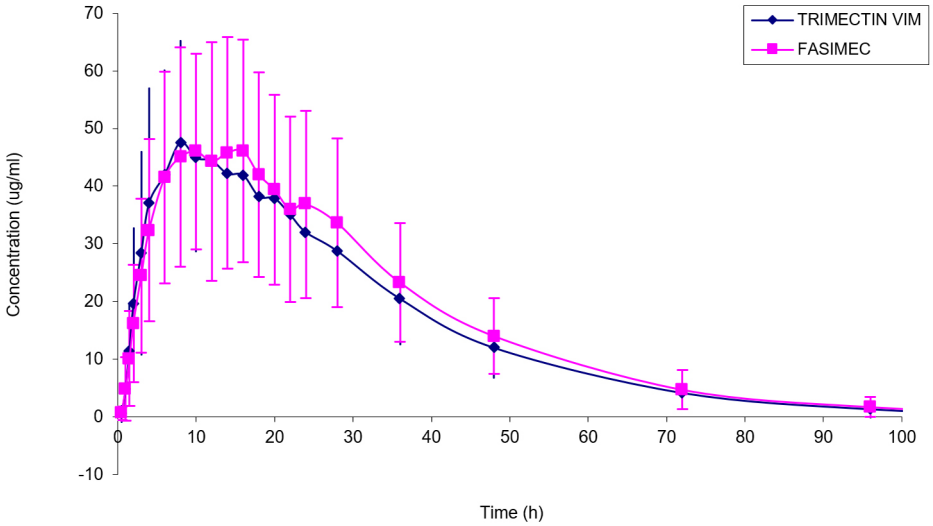

Fig. 3. Mean plasma concentration curves for triclabendzole sulphoxide in bioequivalence testing of two anthelmintic formulas for sheep, zoomed in for the interval 0-100 h

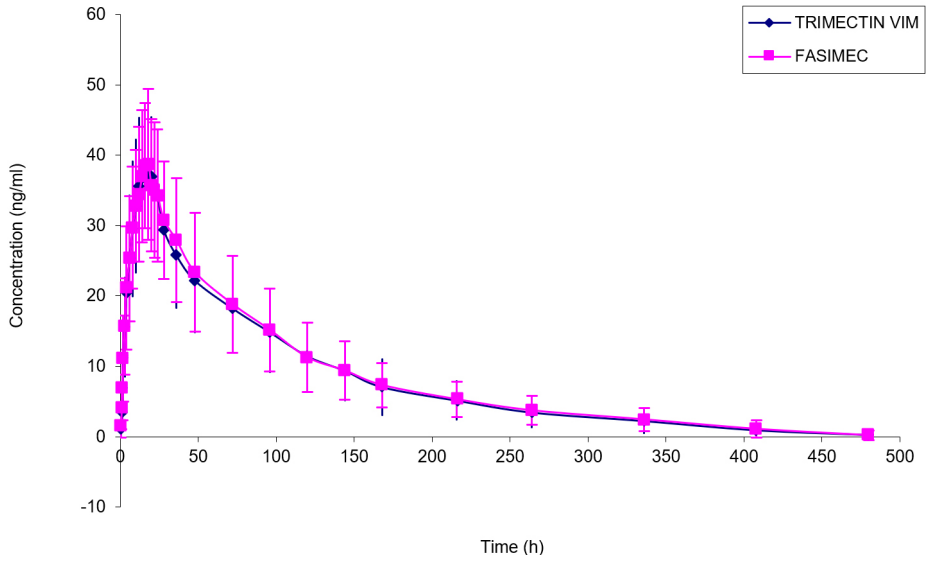

Fig. 4. Mean plasma concentration curves for ivermectin in bioequivalence testing of two anthelmintic formulas for sheep

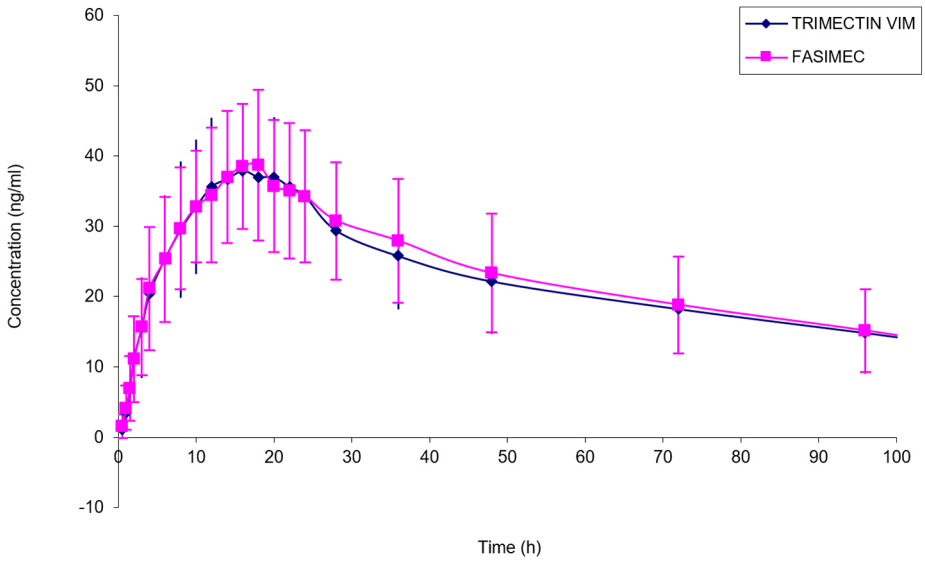

Fig. 5. Mean plasma concentration curves for ivermectin in bioequivalence testing of two anthelmintic formulas for sheep, zoomed in for the interval 0-100 h

Table I. Statistics of primary, secondary and additional pharmacokinetic parameters of triclabendazole sulphoxide in test and reference groups

Parameter	TEST Trimectin		REFERENCE Fasimec Duo		Geometric mean ratio T/R	p* value ANOVA	90% confidence interval (%)
	Mean	SD	Mean	SD			
Cmax (µg/ml)	55.997	17.124	54.402	20.100	1.05119	0.2184 NS	0.98 - 1.12 (ANOVA, NS)
Tmax (h)	11.583	5.6334	11.833	4.6935	0.978873	-	χ^2=3.841 (Fried, K-W, NS)
AUClast (ng/ml*h)	1655.6	443.85	1803.3	750.6	0.969058	0.5863 NS	0.88 - 1.07 (ANOVA, NS)
AUCtot (ng/ml*h)	1702.4	445.88	1847.7	755.64	0.970008	0.5870 NS	-
Thalf (h)	15.517	3.7018	15.594	3.5047	-	0.8828 NS	-
MRT (h)	28.334	5.5840	29.388	4.5714	-	0.1017 NS	-

* significance for p<0.05; MRT – mean residence time; NS – not significant

Table II. Statistics of primary, secondary and additional pharmacokinetic parameters of ivermectin in test and reference groups

Parameter	TEST Trimectin		REFERENCE Fasimec Duo		Geometric mean ratio T/R	p* value ANOVA	90% confidence interval (%)
	Mean	SD	Mean	SD			
Cmax (µg/ml)	41.220	8.6637	42.182	10.462	0.981343	0.6191 NS	0.92 - 1.05 (ANOVA, NS)
Tmax (h)	17.500	4.0107	18.333	5.6061	0.954545	-	χ^2=3.841 (Fried, K-W, NS)
AUClast (ng/ml*h)	3804.3	1295.3	3926.9	1310.5	0.957828	0.4913 NS	0.86 - 1.06 (ANOVA, NS)
AUCtot (ng/ml*h)	4001.0	1338.7	4172.6	1389.2	0.949778	0.4068 NS	-
Thalf (h)	84.295	27.945	91.045	27.042	-	0.1977 NS	-
MRT (h)	118.360	30.72	124.970	29.278	-	0.1901 NS	-

* significant difference for p<0.05; MRT – mean residence time; NS – not significant

curred sample analysis testing passing acceptance criteria, the plasma concentrations determined for the study samples can be considered accurate.

Based on the obtained plasma concentrations statistical analysis of data was carried out. The primary pharmacokinetic parameters (Cmax, Tmax, AUClast) were calculated for triclabendazol sulphoxide and ivermectin and used for the bioequivalence evaluation of the test product with regards to the reference product.

The parametric 90% confidence interval for the ratio T/R period of the mean pharmacokinetic and the significance of the difference of Tmax values based on Friedman and Kruskal-Wallis test are also shown.

The 90% confidence intervals for the ratio of means of triclabendazole sulphoxide and ivermectin "Test/Reference" respectively, were within the conventional bioequivalence range of 80-125 % for all primary parameters. The difference between means is not statistically significant for the Tmax of the test and reference products (Friedman and Kruskal-Wallis test) for neither triclabendazole sulphoxide nor ivermectin.

Thus, we were able to consider that all criteria described in current guidelines for bioequivalence testing were met for the test product [11,12].

Conclusion

The bioequivalence could be concluded between the studied test and reference products due to the calculated 90% confidence interval around the ratio of means (Test/Reference) of log transformed data falling within the reference acceptance range for bioequivalence of 0.8 – 1.25 for all primary pharmacokinetic parameters of both triclabendazole sulphoxide and ivermectin. The Friedman and Kruskal-Wallis tests showing no significant difference between Tmax of reference and test products for both triclabendazole sulphoxide and ivermectin.

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Authors' contribution

Lenard Farczadi (Formal analysis; Methodology; Validation; Writing – original draft)
Laurian Vlase (Methodology; Supervision; Writing – review & editing)
Orsolya Melles (Formal analysis; Project administration)
Ramona Tolomeiu (Investigation)
Octavia Tamas-Krumpe (Investigation)
Andreea Buta (Investigation)
Laurentiu Ognean (Conceptualization; Investigation; Supervision)

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RESEARCH ARTICLE

Dental Students' Tobacco Smoking Habits, Second-hand Smoke Exposure, and Training in Cessation Counselling at the University of Medicine Pharmacy Sciences and Technology of Târgu Mureș

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Objectives: To describe tobacco smoking habits, attitudes, second-hand smoke exposure, and training in cessation counselling at the University of Medicine Pharmacy, Sciences and Technology of Târgu-Mureș (UMPSTTM), as baseline data for the first Romanian university to implement a Smoke Free University Project. **Methods**: A cross-sectional survey was administered in 2014 among dental students at UMPSTTM to explore their smoking habits, attitudes toward smoking and tobacco control policies, exposure to second-hand smoke, interest in quitting, and their knowledge about cessation counselling. We used core questions of the Global Health Professions Student Survey (GHPSS) and added specific items related to the Smoke Free University Project. Data were analysed by SPSS v22 software. We compared our results with those of the GHPSS Survey. **Results**: 581 dental students, 73.1% of the target population (n=795), completed the questionnaire. 38.7% were current smokers. Approximately 1 in 5 (22.6%) current smokers admitted smoking inside university buildings, although 80.7% were aware of the smoking ban. 44.2% of current smokers plan to quit smoking. Nearly half of the students (48.9%) were exposed to second-hand smoke in their current homes, 78.1% in public places and 33.3% inside the university buildings. Only 21.0% of all participants received any formal training on how to help future patients quit. **Conclusions**: Tobacco use prevalence was higher among future dentists than in the majority of respondents to the GHPSS. Changes in dental school education are needed to promote personal smoking cessation, as well as to educate dentists on how to support their future patients quitting.

Keywords: smoking, dental students, smoking cessation, training

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Introduction

According to Geoffrey Rose: "It is better to be healthy than ill or dead. That is the beginning and the end of the only real argument for preventive medicine. It is sufficient" [1].

Tobacco use is a man-made pandemic with a huge impact on public health. Smoking is not just a bad habit it is a severe addiction affecting body and mind, leading to serious conditions, disability, and death. Smoking is an important risk factor for many oral diseases, such as mucosal and periodontal diseases or cancer [2]. There is growing evidence, that oral status reflects the general physical and mental health, and periodontitis may be indicative of other chronic diseases like atherosclerosis, stroke, myocardial infarction and diabetes [3]. The progress of numerous oral diseases is reversible, and survival rates for early diagnosed oral cancers are high, if treated in the initial stages [4].

Dentists have an important role in preventing initiation of tobacco and promoting cessation. According to the FDI World Dental Federation "oral health professionals are in a unique position to contribute to tobacco control". They have the opportunity, responsibility and obligation to change their patients' behaviour, to protect them of falling victim to the world epidemic. [5] To increase the capacity of dentists and other health professionals to curb tobacco use, UMPSTTM was the first health professional university in Romania to launch a comprehensive smoke-free initiative. The initiative includes assessment of smoking habits, attitudes toward smoking, exposure to second-hand smoke, level of knowledge related to smoking cessation methods, with the aim of promoting non-smoking among students and ensuring a smoke free environment in the medical campus.

Methods

Design

A cross-sectional survey of Dental Faculty students from all study years (1 to 6) was made in March 2014 as part of the Smoke Free University project. Trained data collectors distributed the questionnaires and answer sheets designed for immediate electronic scanning. From a target population of 795 students, 581 (73.1%) completed the questionnaires. This study was approved by the Institutional Ethics Committee of UMPSTTM.

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Measurement

The 72-item questionnaire included: 42 core questions from Global Health Professions Student Survey GHPSS and 30 questions specific to our study. Questions were structured into 7 sections as follows:

Section 1: Demographics (age, gender, year of study, faculty, language of study);

Section 2: Tobacco use (experimenting cigarette smoking and/or other tobacco products, age of smoking the first time, days of smoking and/or using other tobacco products during the past month, age of starting smoking regularly, smoking and/or using other tobacco products on university campus and/or in buildings during the past year, smoking status of parents and their cardiovascular or pulmonary diseases;

Section 3: Addiction and cessation (time elapsing to light up the first cigarette after waking up in the morning, actual willingness to quit and/or change for other tobacco products, attempts to quit during the last year, getting help or advice on cessation, quitting advice to patients depending on the smoker status of the health professional);

Section 4: Environmental tobacco smoke (ETS) especially in confined spaces of the university concerning smoking ban and its enforcement);

Section 5: Attitudes toward smoking (opinion on smoking ban policy, health professional's role model and its importance in smoking cessation counselling for patients);

Section 6: Knowledge and training (level of knowledge of tobacco use and cessation techniques);

Section 7: Smoke Free University program (asking students about their intention to advice patients to quit smoking and whether they feel capable of doing that based on their current knowledge, their interest attending the tobaccology course, willingness of being involved in the Project and their opinion on the impact of the Project on smoking habits of students and teachers, and finally the students' opinion about placing specially designed smoking areas outside the university buildings, perceived smoking behaviour at UMPSTTM);

Data were analysed using IBM-SPSS v.22 software. Bivariate analyses were conducted using chi square test ($p<0.05$). Results were compared with GHPSS data of third year dental students around the world, and Global Adult Tobacco Survey GATS data collected in Romania 2011.

Results

Demographic characteristics

Respondents were 63% females aged between 19-24 years old.

Smoking behaviour

Most of the respondents (81.6%) reported having experimented with cigarettes (even only one or two puffs). Most of them (76.0%) admitted smoking initiation before 18 years old, while 24.0% experienced cigarettes for the first time later at the university.

According to the current smoker definition (anyone who has smoked in the last 30 days prior to the survey), there were 61.3% non-smokers, 38.7% smokers, 9.4% daily smokers (9.01% of females and 10.23% of males). Prevalence of smoking was nearly the same in each year. Overall prevalence of females was 38.5% that of males 39.1% with no significant gender difference (p=0.48).

The age of smoking initiation was under 19 years in 66.2% and 33.8% started over 19 years.

Table I. shows the current use of other tobacco products (at least once in the past month) Prevalence decreased significantly from 18.4% of 1st year students to 6.7% of 6th year students. (p=0.04).

As a marker of nicotine dependence, 31 students (13.7%) admitted to smoking the first cigarette within 10 minutes after waking up in the morning, 105 (46.6%) smoked the first cigarette between 10 and 30 minutes. Among daily smokers, 30 students (54,5%) lit up the first cigarette within the first 30 minutes after waking up in the morning.

Among all current smokers, 100 students (44.4%) were interested in quitting smoking as soon as possible. Past attempts to quit in the previous year were reported by 102 students (45.3%). 56.3% thinks that they will definitely quit smoking in the following five years ("probably quits" 33.9%). Only a minority (1.8%) anticipates continued smoking beyond five years ("probably continues" 8.1%). Among 225 current smokers only 103 (45.7%) received help or advice when trying to quit smoking.

Most students were aware of smoke free policy in hospitals (80.7%) and university buildings (82.6%). 60.2% report that this policy is enforced efficiently, while 34.3% do not; almost 1 in 10 did not realize there was a smoking ban in all educational facilities. 188 students (2.5% of all students and 83.5% of current smokers) admitted having

Table I. Prevalence of current tobacco use by year of study and gender

Year of study	Current cigarette smokers				Current use other tobacco products			
	Total% (N)	Female% (N)	Male% (N)	P value	Total% (N)	Female% (N)	Male% (N)	P value
1	35(36)	33.3 (22)	37.8(14)	0.64	18.4(19)	9(6)	35.1(13)	0.001
2	38.1 (37)	36.8 (25)	41.4 (12)	0.66	25.7 (24)	19.1 (13)	37.9 (11)	0.049
3	39.9 (55)	43.2 (38)	34 (17)	0.28	10.9 (15)	8 (7)	16 (8)	0.14
4	41.6 (50)	40 (28)	44 (22)	0.66	17.5 (21)	8.5 (6)	30 (15)	0.002
5	37.5 (24)	32.4 (12)	44.4 (12)	0.32	10.9 (7)	10.8 (4)	11.1 (3)	0.96
6	39 (23)	43.2 (16)	31.9 (7)	0.38	6.77 (4)	2.7 (1)	13.6 (3)	0.1
All years	38.7 (225)	38.5 (141)	39.1 (84)	0.48	15.5 (90)	10.1 (37)	24.6 (53)	< 0.001

smoked on campus including 51 (8.8% of all students and 22.6% of current smokers) who reported smoking inside the university buildings.

38 students (6.6% of current students and 42.2% of current users) declared that they had used other tobacco products (e.g. chewing tobacco, snuff, bidis, cigars, hookah or pipes) on the campus and 15 students (2.6% of all students and 16.6% of current users) admitted use inside the university buildings.

Exposure to second-hand smoke SHS

During 7 days prior to the survey, 48.9% indicated they were exposed SHS in their homes, and 16.0% admitted daily exposure. Outside their homes, 78.1% indicated any SHS exposure and 17.6% admitted daily exposure.

In the university's central building 33.3% of non-smokers were exposed to SHS I the 7 days preceding the survey. About 1 in 3 students (29.4%) reported exposure 1-4 of the previous 7 days, while 6.6% reported exposure every day in the preceding week. In the inpatient university clinics, 31.3% of non-smokers realized SHS in confined spaces. In students' dormitories, 72.4% of the non-smokes reported second-hand smoke exposure.

Attitudes toward smoking policies

Attitudes towards legislation of tobacco control and the awareness of their future role model for patients differ significantly across non-smoker and smoker students. Table II. summarizes these attitudes related to the actual smoking status.

Students often tolerate their smoking peers even when they smoke inside the university buildings where smoking is banned. One-third (33.3%) considered that it is not their "business" if they realize someone smoking inside the university buildings, although 37.4% criticized this behaviour. 17.4% reported that they would refer smokers to designated smoking areas, but only 10.3% affirmed they would inform smoking persons that they behave illegally.

Training of dental students about tobacco cessation

21% of participants stated that they had received formal training in smoking cessation, although 91.9% of the students wanted to be trained in this regard. Most students indicated that they learned about the dangers of smoking (71.9%), the importance of recording tobacco use as part of the patient's general medical history (73.1%), and they were informed about nicotine replacement therapy options (90.7%). The training is lacking information about reasons why people smoke (33.4%), practical smoking cessation approaches (21.2%), significance of providing educational materials to support cessation to patients who want to quit smoking (23.3%), and options of pharmacotherapy to support tobacco cessation programs (27.4%).

Based on their current knowledge, only 27.9% are convinced that they are able to deliver smoking cessation advice to smoking patients, while 55.3% feel that they were probably able to support the patients Most students (63.4%) declared that they would be interested attending special tobacco course about the risks of smoking, benefits of cessation, cessation techniques, and basics of nicotine replacement therapy.

Discussion

Overall prevalence of current smoking in Romania (a middle income country) was 26.7%, being more than two times higher among men (34.9%) than women (14.5%). An inquiry upon the prevalence of smoking among medical doctors (n=1,136) in Romania revealed the overall prevalence of 43.2% (50.1% of males and 38.6% of females) [7]. It is far from 2025 WHO suggestion of 15.0-19.9%. According to Professor Richmond: "Leadership from the medical profession is essential if the world is to reduce preventable diseases caused by smoking" [8].

Our results provide evidence based data about smoking habits and attitudes of dental students at UMPSTTM. Their smoking prevalence of 38.7% was considerably higher compared to Romanian adult overall population (22.6% in age group 15-24 years), and especially high among female students (38.5%) contrasted to 16.7% of Romania's adult female population [6]. However, our study showed no significant gender difference of cigarette smoking.

The Global Youth Tobacco Survey (2009) indicates that 13.5% of Romanian school students aged 13–15 years have smoked at least one cigarette in the past 30 days. The

Table II. Dental students' attitudes toward tobacco control and quit smoking support, stratified by their smoking status

N=581	Percentage "of yes" answers to the questions			
Variables	Overall %	Non- smokers %	Smokers %	p value
Should tobacco sales to adolescents be banned?	92.9	96.0	87.9	<0.0001
Should advertising be completely banned?	77.5	83.1	68.4	<0.0001
Do you agree with smoking ban in restaurants?	82.6	92.1	67.4	<0.0001
Do you agree with smoking ban in discos/bars/pubs?	58.2	73.0	34.7	<0.0001
Do you think that smoking in all public spaces should be banned?	73.9	85.4	55.8	<0.0001
Should health professionals get cessation training?	91.9	95.7	86.6	<0.0001
Are health professionals role models?	71.8	73.8	68.8	0.11
Should health professionals give quitting advice routinely?	90.4	94.4	84.0	<0.0001
Should health professionals advise stopping other tobacco products?	86.4	88.3	81.8	0.007
Do health professionals have a role in giving advice?	91.7	92.1	91.1	0.38
Do chances of quitting improve if health professional gives advice?	78.4	81.7	73.2	0.01
Are health professionals who smoke less likely to advise patients to stop smoking?	44.5	47.5	39.9	0.08

European School Survey Project on Alcohol and Other Drugs (2011) Report shows that among 15- to 16-year-old Romanian students 29% smoked in the last 30 days. Another study from 2016 reported 24.1% prevalence of smoking in the last 30 days among 15 years old adolescents living in Târgu Mureș [9], this prevalence increases among 19-24 years old dental students according to our study to 38.7%. These numbers mean that between 13 and 24 years adolescents are at an increased risk of becoming smokers.

Comparing our results with GHPSS dental data of European countries (Table III.) female rates are similarly high and Romania was on the 4th place of the negative list of 16 countries. Although, overall prevalence rate of smoking was high, daily smoking was less frequent among dental students compared to the 15-24 age group of Romania's general population. Previous surveys in Romania about smoking habits of dental students found similarly high smoking rates [10, 11]. This might have been due to the lack of tobacco-control programs of professional education. Lacking of relevant programs even now explains why there was no difference by academic years of smoking rates in our survey.

Current use of other tobacco products was higher among male students as was in all European countries from the GHPSS study [12].

Many students started smoking before entering university, which is consistent with GATS findings with highest proportion of initiation at age 17-19 [6].

"Health workers should look at themselves before trying to convince others to quit smoking" [9]. Results of our survey showed not only a widespread use of tobacco among dental students, but also lack of willingness to quit smoking in more than half of current smokers. It is a considerably high proportion if compared with 33.6% of current smokers interested in quitting in the general population of Romania [6]. This can also be related to the fact that less than half of current smokers among dental students received any help or advice on smoking cessation. Only a small percentage of participants think they will be smokers after 5 years, however they are identical with the nicotine dependent subsample.

Table III. Prevalence of cigarette smoking in selected European countries according to Dental Global Health Professions Student Survey compared with UMPSTTM data of 3rd year dental students.[12]

Country	year	Total%	Male%	Female%
Republic of Moldova	2008	65.2	69.1	-
Macedonia	2009	52.5	52.4	52.5
Bulgaria	2009	52.2	49.3	54.7
Kyrgyzstan	2008	44.0	60.9	27.8
Russian Federation	2006	43.7	53.3	37.9
Greece	2009	39.1	31.7	43.5
Romania (UMPSTTM)	2014	38.7	38.5	39.1
Bosnia Herzegovina	2006	36.1	34.1	37.1
Czech Republic	2006	33.3	29.4	34.4
Albania	2005	30.1	38	27
Lithuania	2006	29.6	61.7	22.9
Slovakia	2006	29.3	23.1	32.1
Serbia	2006	28.5	24.7	31
Armenia	2006	28.4	60.2	7.8
Latvia	2009	19.6	32.4	32.1
Slovenia	2007	17.9	-	17.6

Second-hand smoke exposure

In Romania, despite of implementing smoking ban in indoor areas of public places including hospitals and educational facilities exposure did not change to ETS [13].

Our results of SHS exposure resemble those of GHPSS study in high rate European countries (32 of 48) [12]. More than 2/3 (78.1%) of UMPSTTM dental students reported SHS exposure outside their homes during the week prior to data collection. (comparing Republic of Moldova 79.2%, Kyrgyzstan 78.2% Slovakia 71.4%, Slovenia 74.4%) [12].

One third of non-smoker dental students (33.3%) reported exposure to SHS in university buildings however 47.5% of the general population realized tobacco smoke in universities of Romania [6]. We found higher prevalence of reported indoor SHS exposure in our dental students' homes contrasted to the Romanian adult population (48.9 % versus 35.4% of GATS) [6]. Indoor exposure is mounting (72.0 %) if related to the dental students' dormitories-Despite the fact that dental students were mostly aware of non-smoking policy rules, they disrespected them while admitting that they did not realize any control. Attitudes of dental students toward smoking regulation policies and health professional's role in tobacco cessation counselling depended heavily on their smoking status. Smokers were less likely to support smoking control policy, like smoking ban in all confined public spaces, restaurants, discos/bars/pubs, and they did also not support complete ban on advertising tobacco products and tobacco sales to underage people. Smoking status also proved to decrease the willingness to be trained in smoking cessation counselling and to provide patients advice to quit. Both smokers and non-smokers agreed on health professional's role model. Comparing the share of relevant opinion of our dental students (91.7%) with those of other European countries, 8 countries out of 10 placed behind UMPSTTM with highest value in Latvia (90.5% and the lowest in Slovakia 56,8%). Findings were the same regarding the opinion of providing health professionals with specific training on cessation techniques (UMPSTTM 91.9%, Latvia 90.0% and Macedonia 78.0%). Results of our study and those of GHPSS indicate that dental schools failed to meet this demand. Except Moldova (63.1%) among other European countries, less than half of dental students have ever received any formal training in smoking cessation counselling. The lowest value shows Slovakia (14.0%) and UMPSTTM was in the lower third (21.0%) of the range [12].

"Dental practice in the 21st century will increasingly move from a restorative orientation to one of broader promotion of health and well-being. It is unconscionable to not include aggressive tobacco intervention in that new

paradigm." [14] Discouraging tobacco use among dental students, offering them a smoke free environment and including education about tobacco in their medical curricula may help to translate ideas into action. We hope that the Smoke Free University project is a good start and will help in developing efficient tobacco control program at all Romanian universities.

Limitations and strengths of the study

Our data were based on anonymous voluntary answered questionnaires, this fact constitutes the strength and in same time the limitation of the study. Results of this study provided reliable baseline data of dental students' smoking behaviour, attitudes, cessation plans, and their knowledge about smoking cessation counselling.

Conclusions

Tobacco use prevalence is higher among future dentists compared to Romania's general population and to most of the countries taking part in the GHPSS study. Our students' attitude is ambiguous, as they disregard already implemented antismoking regulations, their willingness to quit is alarmingly low, but most of them are aware of the role model of health professionals and consider that they should receive academic education about smoking cessation interventions. Behavioural changes of dental students toward tobacco smoking are urgently needed by introducing trainings about smoking cessation counselling in the academic curricula.

Authors' contribution

Márta Germán-Salló, PhD (Conceptualization; Investigation; Methodology; Project administration)
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Dalma Bálint Szentendrey, PhD (Conceptualization; Data curation; Investigation; Validation; Visualization)
Enikő Nemes-Nagy, PhD (Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Resources; Supervision; Validation)
Mihály Imre László, Dr. (Data curation; Formal analysis; Investigation; Methodology; Software; Validation; Visualization)
Zita Fazakas, PhD (Conceptualization; Investigation; Methodology; Supervision; Validation)
Edith Simona Ianosi, PhD (Conceptualization; Data curation; Investigation; Methodology; Validation)
Pál István Kikeli, PhD (Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Supervision)
Zoltán Ábrám, PhD (Conceptualization; Funding acquisition; Investigation; Methodology; Project administration; Resources; Writing – review & editing)
Péter Balázs (Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Writing – review & editing)

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Conflict of interests

None to declare.

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