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Development and Validation of a UV Spectrophotometric Method for the Determination of Dioscin in Tribulmon Tablets

Baigalmaa Dovchinsuren[1], Dorjbal Enkhjargal[2], Wang Qin Hu[3]

[1]Department of Pharmacy Technician, School of Pharmacy, Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia; [2]Department of Pharma Chemistry and Pharmacognosy, School of Pharmacy, Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia; [3]Department of Chemistry, Mongolian Medical College, Inner Mongolia University for Nationalities, Tong Liao, China

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Corresponding Author
Baigalmaa Dovchinsuren MS
Department of Pharmacy Technician, School of Pharmacy, Mongolian National University of Medical Sciences, Ulaanbaatar 15120, Mongolia
Tel: + 976 – 9901-2910
E-mail: baigalmaa.d@mnums.edu.mn

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Objective: Develop and validate a novel, sensitive, precise and straightforward UV spectrophotometric method to determine the amount of dioscin in tribulmon tablets. **Methods:** tribulmon tablets were prepared in the laboratory from medicinal plants *Tribulus Terrestris L*, native to Mongolia. Extract solutions were prepared from a single batch of tribulmon tablets, and the amount of dioscin was determined by UV spectrophotometric method using a calibration curve of known solutions of the dioscin. A chromogenic reaction was used to determine the amount of dioscin in the tablets. The maximum absorption was 201 nm. Validation parameters were evaluated following ICH guidelines. **Results:** The correlation coefficient of linearity for the UV spectrophotometric method was 0.9996. The standard working solution was linear from 3.2 to 18.9 μg/mL. The Limit of Detection (LOD) and Limit of Quantitation (LOQ) were 1.9 μg/mL and 5.9 μg/mL, respectively. The recovery test accuracy was ranged from 97.9 to 100%. The %RSD for repeatability and intra-day precision was 0.45%, 0.13–0.50%, respectively. The %RSD for robustness was 0.26% to 0.40%, respectively. **Conclusion:** Based on the results of the proposed UV spectrophotometric method, the method we developed to determine dioscin content in tribulmon tablets from *Tribulus Terrestris L* is sensitive, precise, stable and straightforward. The test's validation parameters comply with ICH requirements.

Keywords: Spectrophotometry, Validation, Tablets, Dioscin, *Tribulus Terrestris L*

Introduction

Tribulus Terrestris L. (TT) is an annual plant belonging to the *Zygophyllaceae* family. Fully grown, it is about 30–70 cm tall with feathery leaves and bright yellow flowers. The fruits are stellate with angled thorns, which gave the plant its common name - puncture vine [1] (Figure 1). It is typically distributed throughout the world's tropical and subtropical regions. In Mongolia, it grows in the Gobi Desert and the steppes. Since ancient times, it has been used in traditional Mongolian

medicine to treat various diseases, improve stamina, reduce blood pressure, and as a diuretic for the kidney and urinary tract [2]. Parts of the plant have many pharmacological properties, including aphrodisiac, anti-inflammatory, antimicrobial and antioxidant effects. Products and preparations made from parts of terrestrial plants are especially popular among athletes and people with health problems and diseases such as hormonal imbalances, sexual problems, heart problems, various kidney and skin diseases [2]. The extract of this herb stimulates the production of luteinizing hormone, which directly affects testosterone production. Luteinizing hormone regulates the expression of 17β-hydroxysteroid dehydrogenase, an enzyme that converts androstenedione to testosterone. TT extract is mainly recommended for men's health and vitality [3]. Also, its antioxidant effect may enhance erectile function, knowing that oxidative stress is associated with endothelial dysfunction. Nitric oxide mediates the formation of cyclic guanosine monophosphate (cGMP); this mechanism can promote erection by vasodilation and increased blood supply to the corpora cavernosa [4, 5].

Preclinical studies have focused on animal models of human diseases that affect spermatogenesis and androgen secretion (cytotoxic drugs that affect the gonads, castration, and diabetes) [6]. The results of clinical trials of TT extract in men can be divided into two main categories: the first is the assessment of the effects of erectile dysfunction (the quality of an erection and the intensity of libido), the second is the comparison of changes in testosterone secretion with the control group at the end of the study. However, existing studies have not clarified the controversy over the actual efficacy of TT, on the one hand, due to differences in results (e.g., testosterone and dihydrotestosterone, accurately quantified gonadotropin levels, etc.); on the other hand, due to subjective assessment (especially if the endpoints were based on self-assessment of standardized questionnaires on topics such as the International Index of Erectile Function (IIEF), the Global Effectiveness Questionnaire (GEQ)) [7, 8].

TT contains important secondary metabolites such as saponins, polyphenolic compounds, and alkaloids. Steroid saponins mainly include furostanol and spirostanol. It is believed that saponins of furostanol are biogenetic precursors of spiro analogs. More than 70 different compounds were identified today [9]. Besides, Mulinacci et al. have noted that many researchers have focused on the extraction, purification, and determination of the structure of saponins found in plants with more than 30 different glycosides of saponins [10]. Testing using the analytical HPLC-ELSD method resulted in a wide variation in both saponins and saponins, depending on the geographic region. Analysis of market products showed significant fluctuations in protodioscin content from 0.17 to 6.49% [11]. A comparative study of the biologically active components of TT, growing in four flower regions in southern Bulgaria, found dioscin with concentrations from 1.51 mg/g to 4.35 mg/g [11].

The quality and effectiveness of herbal medicines are directly related to the quality of herbal raw materials. The three critical steps at the very beginning of the production process are 1) cultivation/collection of natural whole plants or plant parts, 2) sorting, drying, and grinding of plant material, and 3) inappropriate or targeted solvent extracts of herbal materials to enrich or incorporate "active" or "marker" compound [12].

Validation is a concept that is fundamental to good manufacturing practice and any quality assurance program [13]. Some of the major pharmacopeias contain monographs that set out standards for herbal medicines. The main advantage of an official monograph published in a pharmacopeia is that the standards are defined and available, and the analytical procedures used are fully validated. This is very important as validation can be quite time-consuming [14]. The Bulgarian "Tribestan" was the first standardized preparation [13], described in Bulgarian patent applications and German articles. Standardization of TT products is difficult due to the observed quantitative and qualitative dynamics of TT saponins. Scientific literature standardization of preparations is sparse and TT product [15]. The ISO/IEC International Standard requires validation of non-standard methods, laboratory-developed methods, standard methods used outside the intended field of application, and extensions and modifications of standard methods to confirm that the methods are suitable for the intended use. The International Council for Harmonization (ICH) of Technical Requirements for Registration of Medicinal Products for Human Use has developed a harmonized text to validate analytical procedures. The document includes definitions for eight characteristics of validation [16].

Despite numerous reports determining biologically active compounds of TT grown in different geographical regions, we are not aware of any study investigating the dioscin content of Mongolian original TT extract. Therefore, it is vital to develop and validate non-standard quality control methods for dioscin

and other natural products. We conducted a study of the tablet production technology of *Tribulus Terrestris L*, which grows in Mongolia, and obtained the tablets. This study aimed to validate the spectrophotometric method for the determination of the biologically active substance dioscin in tribulmon tablets.

Materials and Methods

The herb TT was harvested in 2019 in the Dundgobi province, dried after harvest, and made into tablets. As part of the study, the authors made tribulmon tablets in the chemical laboratory of the Mongolian Medical College in Tong Liao, Inner Mongolian University of Nationalities. Validation of the spectrophotometric method for determining the amount of dioscin in tablets was performed in 2020 in the laboratory of drug quality of the National Reference Laboratory for Food Safety of the Main Agency for Specialized Inspection of Mongolia. The development of new tribulmon tablets and the spectrophotometric method for determining the amount of biologically active substances contained in the tablets were prospective validations. To identify potentially dangerous manufacturing errors in the tribulmon tablets, it necessary to confirm the sample size with a 95% confidence level and 99% reliability. According to Bayes success run theorem, a sample size of 299 tablets from the same series were used as samples.

Chemical and reagents

For all of the analyses, the analytic grade reagents and solvents were used, including methanol, ethanol and perchloric acid. The tribulmon tablets contained the following excipients: lactose, microcrystalline cellulose, polyvinylpolypyrrolidone, magnesium stearate, talc, and hydroxypropyl methylcellulose. Our dioscin reference substance had a purity of 96.1%. A Thermo Scientific Evolution 300 UV-VIS spectrophotometer was used.

Preparation of tribulmon tablets

We made an ethanol extract of TT to make table tribulmon tablets. We conducted a pilot study to select the appropriate conditions and parameters of extraction. The optimal conditions for obtaining liquid extract were using 70% ethyl alcohol as a solvent with a solid-to-liquid ratio of 1: 6, an extraction time of 6 hours, and a single extraction. The TT was refluxed with ethanol to obtain a liquid extract. The extraction parameters were optimized using a one-way experiment and an orthogonal test. Then, column chromatography on HPD-100 microporous adsorption resin was used to purify the crude TT extract. A 70% ethanol solution was used for desorption and collected, concentrated in a vacuum and dried with a lyophilization apparatus. The dry extract was mixed with an excipient, and tribulmon tablets were obtained using the wet granulation method and compressed (Figure 2). We conducted studies previously to select suitable excipients such as binders, disintegrants, lubricants required to make the tribulmon tablets. The quality of the obtained tablets met the requirements of the Mongolian National Pharmacopoeia (2011).

Preparation of sample solution from tribulmon tablets

Following the pharmacopeia quality control method for tablets, 20 tribulmon tablets were taken from one batch of pills and were crushed, and a powder sample was prepared. An 0.100 g sample of tribulmon tablet powder was placed into a 100 mL volumetric flask, diluted with a small amount of methanol, and sonicated for 30 minutes until the powder was completely dissolved. After sonication, the volume was made up to 100 mL with methanol. This solution was centrifuged and filtered, and 2 mL of the solution was transferred into a test tube, 4 mL of perchloric acid was added, the tube capped and placed in a water bath at 25 ^{0}C for 30 minutes, after which the reaction was cooled in ice water. The absorbance of this solution was measured with a spectrophotometer at 201 nm. Perchloric acid was used as a blank.

Preparation of standard stock dioscin solution

We made the dioscin standard solution by dissolving 19.78 mg standard grade dioscin in 5 mL of methanol in a 25 mL beaker. It was carefully transferred to a 25 mL volumetric flask and diluted with methanol to achieve a final volume of 25 mL, with a final dioscin concentration of 79.1 μg/mL.

Preparation of diluted dioscin working standard solutions

In the next step, 0.20, 0.40, 0.60, 0.80, 1.0, 1.20 mL of the standard dioscin solution was measured and placed in 10 mL tubes with a stopper and then heated until the solvent evaporated. After that, 5 mL of perchloric acid was added, the tubes placed in a water bath at 25^0 C for 30 minutes to develop

the color fully, and then cooled in an ice bath for 5 minutes. After stabilization, the absorbance of the diluted working solutions was measured spectrophotometrically. Perchloric acid was used as a blank. The plot was constructed with concentration on the y-axis and absorbance on the x-axis, yielding a straight line.

Method validation

We used the spectrophotometric method for determining the content of dioscin in tribulmon tablets following the validated method of the International Council for Harmonization Q2 (R1). Validation was performed on the parameters of Specificity, Linearity, Limit of Detection (LOD), Limit of Quantitation (LOQ), Accuracy, Precision, Robustness [17, 18].

Selectivity and specificity

We determined the selectivity and specificity of our method to measure dioscin in the presence of the excipients and other components in the tribulmon tablets.

Accuracy

We determined the accuracy of UV spectrophotometric analysis to detect dioscin in solutions made from tribulmon tablets using recovery studies at three different levels in the percentage of dioscin per tribulmon tablet: 80%, 100%, and 120%. We measured 0.03 g, 0.05 g, and 0.07 g of tribulmon tablet powder and dissolved each in 100 ml of methanol, respectively. We took 10 ml of each of those solutions and added 0.4 ml of standard solution to it. We then measured 2 ml of those solutions again and added 4 ml of perchloric acid. We placed the final solutions in a water bath at 25^0 C for 30 minutes and cooled them in ice water. We then re-analyzed these final solutions using our UV spectrophotometric analysis method. The dioscin concentrations were 5.2 μg/mL, 6.5 μg/mL, and 7.8 μg/mL.

Precision

To check our method's repeatability, we measured the absorbance of a single solution made from tribulmon tablets six times and calculated the %RSD. The test solution was prepared using tribulmon tablet powder 0.1 g using the above method, and the concentration of the solution was 6.5 μg/mL. The sample was prepared 6 times and measured 6 times. Intraday accuracy was checked testing one batch of tribulmon tablet solution over 1 day using our proposed method of UV spectrophotometric analysis at 201 nm.

Robustness

We tested the robustness of this method by measuring the wavelength variation. To do this, we checked to see if the maximum absorption decreased or increased by 1 nm, using the tribulmon tablet solution. For these tests, a sample was taken 0.15 g of a tablet was prepared following the above method, and the concentration of the solution was 9.8 μg/mL.

Study design

A total of 12 tablets of dioscin were measured three times (initial, 6 hours and 12 hours) in the experiment. Identification tests were needed to ensure the identity of the dioscin in the tribulmon sample. We achieved this by comparing the spectrum of tribulmon sample solutions to a dioscin working standard solution. Accuracy was assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g., 3 concentrations/3 replicates each of the total analytical procedure).

Statistical analysis

Friedman's test for the non-parametric two-way ANOVA was used to analyze the accuracy/recovery at three different levels. And Friedman test for the non-parametric repeated measure ANOVA was used to analyze the precision of the stability of tablets at three different times. The relationship between the absorbance and concentration variables was tested by Pearson's correlation. A critical p-value of < 0.05 was used. All analyzes were performed using SPSS (version 26.0).

Ethical statement

The study was approved by the Research Ethics Committee of Mongolian National University of Medical Sciences (No. 2017/3-05).

Results

Before testing to determine the maximum absorption (λ max), the absorption was measured with standard solutions and solutions from tablets in the UV range from 190 to 450 nm. The wavelength of the maximum absorption was 201nm.

Table 1. Correlation between dioscin concentration and absorption for the dioscin standard solution.

Concentration (mg/mL)	Absorbence (nm)	Correlation coefficient
0.034	0.205	0.99
0.063	0.388	
0.095	0.566	
0.127	0.755	
0.158	0.926	
0.198	1.121	

Table 2. Accuracy/recovery studies for the different concentrations of the three tribulmon solutions.

Level (%)	Amount of reference solution added to pre-analyzed sample (μg/mL)	Amount recovered (μg/mL)	Recovery %	p-value
80	5.2	5.3	101.9	0.532
80	5.2	5.2	100.0	
80	5.2	5.1	98.1	
Mean ± SD %RSD			100.0 ± 1.92 1.92	
100	6.5	6.5	100.0	
100	6.5	6.4	98.5	
100	6.5	6.3	96.9	
Mean ± SD %RSD			98.5 ± 1.54 1.56	
120	7.8	7.6	97.4	
120	7.8	7.7	98.7	
120	7.8	7.6	97.4	
Mean ± SD %RSD			97.9 ± 0.07 0.76	

Table 3. Results of repeatability study for the tribulmon solution.

Amount taken (μg/mL)	Dioscin absorbance (nm)
6.5	0.389
6.5	0.392
6.5	0.389
6.5	0.390
6.5	0.393
6.5	0.389
Mean ± SD	0.390 ± 0.002
%RSD	0.449

Selectivity and specificity

Placebo solutions did not affect light absorption. In the placebo test, the absorbance value was almost the same as for the solvent, indicating that the inactive ingredients added to the placebo solution did not interfere.

Linearity and range

A linear response to increasing concentration of the working standard solution was observed in the range of concentrations from 3.2 to 18.9 μg/ml following Beer's law (Table 1). The linear regression equation was $y = 0.1712x - 0.002$. Pearson correlation

Table 4. Stability of the UV spectrophotometric measurements of tribulmon in solutions with the passage of time.

Dioscin concentration in tablet (μg/mL)	Absorbance (nm) (n=3) Analysis timetable (hours after preparation)				p-value
	Initial analysis	6 hours	12 hours	Mean ± SD	
3.3	0.201	0.199	0.200	0.200 ± 0.001	0.130
6.5	0.389	0.390	0.391	0.390 ± 0.001	
9.8	0.582	0.583	0.581	0.582 ± 0.001	
13.0	0.773	0.772	0.768	0.771 ± 0.003	
16.3	0.956	0.960	0.959	0.958 ± 0.002	
19.5	1.148	1.146	1.149	1.148 ± 0.002	

showed there is a strong correlation between absorbance and concentration ($r = 0.99$, $p < 0.000$).

Accuracy

The accuracy of the analytical method was consistent with the accepted reference test results. The accuracy of the method was assessed by adding three levels of standards. To the tribulmon tablet, solution was added a standard stock solution equivalent to 80%, 100%, and 120%. The recovery of the test accuracy was 100%, 98.5%, and 97.9% (Table 2).

Precision

The absorbance of the tribulmon tablet solution was measured six times, and the relative standard deviation (%RSD) was calculated to test the repeatability of the method. The %RSD of repeatability and intra-day precision was 0.45%, 0.13% - 0.50%. The results indicate excellent repeatability and intra-day precision using our method (Tables 3, 4).

Figure 1. *Tribulus Terrestris L.* growing in Mongolia.

Figure 2. Tribulmon tablets obtained from *Tribulus Terrestris L.* in laboratory.

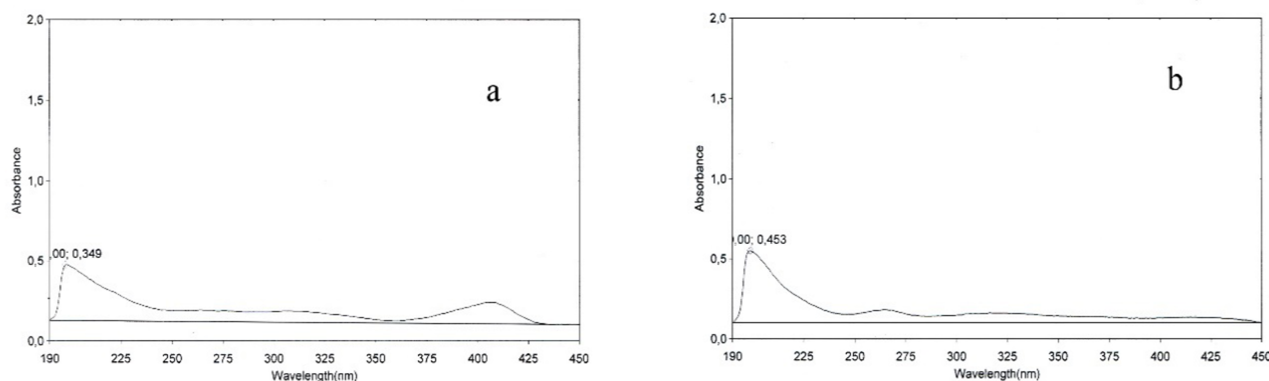

Figure 3. Absorption of standard solutions (a) and solutions of tablets (b) in the UV range from 190 to 450 nm.

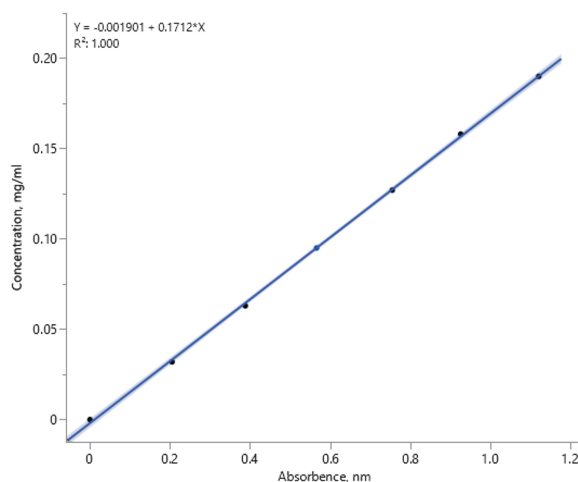

Figure 4. Calibration curve of working solution of standard dioscin in methanol.

Discussion

Spectrophotometry is a powerful method that does not require expensive solvents, equipment, time and is suitable for drug research. But saponins have no chromophore activity and are therefore difficult to measure with a spectrophotometer. Therefore, often when determining saponins with sulfuric acid, phosphoric acid, and with a high content of chlorins acid, there is a change in the color and fluorescence of anhydrous water. The reaction mechanism is dehydration and the formation of new double bonds. After double bonding, condensation, and other reactions, a diene system is formed, and ultimately the action of the acid leads to the formation of cationic carbon, resulting in colored ions [15].

Ultrasonic extraction (UAE), reflux (RE), low-pressure reflux (LPRE) and Soxhlet extraction (SE) have been developed to determine the content of steroidal saponins in TT [16]. We used the reflux method to obtain the liquid extract from TT, calculated the saponin release for each experiment, and continued to study the appropriate extraction conditions to obtain high yields. Based on the one-way and orthogonal study, suitable extraction conditions were selected. The purification and enrichment of the total saponin are the key to quality control of manufacturing drugs from plants. In recent years, macroporous adsorption resins (MARs) have received particular attention in the separation and enrichment of plant materials for laboratory and industrial purposes. This is because they have unique absorption properties such as optimal pore structure, different surface functional groups, low operating costs, low solvent consumption, and easier regeneration [17, 18]. The purity of the total saponin of the dry extract obtained from TT influenced the stability and accuracy of the spectrophotometric method for determining the

amount of dioscin in tablets. Bulgarian studies have identified a steroidal saponin known as the saponin furostanol, which has a protodioscin content of at least 45% [19]. In our study, the content of dioscin in the dry extract was 52.3%, with a tablet weight of 0.100 ± 0.02 g. Each tablet contained 25 mg of dry extract, and our study showed that the content of dioscin was 12.93-13.03 mg/g (12.93-13.03%).

Linearity methods are the ability to obtain test results that are directly proportional to the analyte concentration within a specified range. The range is the interval between high and low analyte levels that were used in the procedure. Good linear correlations were obtained between the absorbance and the concentration of the standard substance in the selected range of 3.2-18.9 µg/mL. Pearson correlation showed a strong correlation between absorbance and concentration with a correlation coefficient r = 0.9996, more than 0.997 [20]. The Limit of Detection (LOD) is the smallest amount of an analyte in a sample that can be detected. The Limit of Quantitation (LOQ) is the smallest amount of an analyte that can be quantified. The method for validating the developed analytical method is a systematic process for acceptability checking based on several parameters such as linearity, LOD, and LOQ when determining if the analytical method is well suited for experimental purposes [21].

Accuracy is the measure of how the value obtained by a certain procedure is consistent with the accepted true value. Our method's accuracy for measuring the dioscin concentration in the tribulmon tablets was assessed by comparing the test results of samples obtained at three levels of concentration. The ICH recommendation defines the limits of the variability in the parameters tested. Therefore, we tested the method by measuring the relative variability of the standard deviation and checking it is within the range of change. The recovery of the accuracy of the tests was 97.9-98.5%, which indicates that the recovery rate is stable, accurate, and reproducible (For assay mean recovery 97%-103%) [22].

Precision indicates the extent of differences in repeated measurements of the same thing and is acceptable below 20%. %RSD of 6 replicates test was 0.45% and less than 2% [23]. The content of each compound in the samples taken at certain time points did not differ significantly, and the %RSD values ranged from 0.13% to 0.50%, respectively. In precision tests, the analyte maintained good reproducibility throughout the assay procedure.

The assessment of stability should be considered at the design stage and depends on the type of procedure and deliberate modification of the method's parameters. If measurements are sensitive to change in analytical conditions, analytical conditions should be appropriately controlled or precautions should be included in the procedure [24]. Experimental results showed that the change in %RSD values were within the acceptable theoretical limit < 2%RSD [25]. Our method was not subject to slight, deliberate fluctuations indicating that it is reliable and robust under normal conditions.

Validation of dioscin determination methods in tablets tribulmon is the basis for preclinical, clinical studies, the development of normative documents on the effectiveness and safety of this drug, and regulatory registration. Our spectrometry method is used to check the quality of the drug in the laboratory. In the future, we will develop a method for determining the amount of biologically active substances in tribulmon tablets using high-performance equipment. Comparative studies of chromogenic saponin reactions will also be conducted to improve the spectrophotometric method.

Our study has a limitation. We focused mainly on the determination of the dioscin content of TT extract grown in Mongolia. Our next step will be comparing the dioscin content in TT extracts from different geographical regions using the UV spectrophotometric method.

Conclusion

We found UV spectrophotometric method for measuring dioscin in tribulmon tablets using a chromogenic reaction to be sensitive, accurate, precise, stable, and straightforward. Based on the results of the validation parameters, the methods are ICH compliant and will be used for routine analysis to determine dioscin in TT-derived tablets.

Conflict of Interests

The authors state no conflict of interest.

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