A Simple Method for Determination of Protodioscin in *Tribulus Terrestris* L. and Pharmaceuticals by High-Performance Liquid Chromatography Using Diode-Array Detection

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Abstract: The *Tribulus terrestris* L. (Zygophyllaceae) is a known medical plant used in traditional and folk medicine worldwide. The steroid saponine protodioscin, an active component found in this plant, serves as a marker for quality control of plant raw materials. In this study, we developed a simple and selective method for determination of protodioscin using convenient High-Performance Liquid Chromatographic (HPLC) laboratory equipment. The detection was performed by Diode-Array Detector (DAD). The proposed method was fully validated and according to the validation results, it was accurate and precise. It was proven to be linear over a protodioscin concentration range of 10,9 to 544.9 µg/mL. The low values of limit of detection (LOD) and limit of quantitation (LOQ) demonstrated adequate sensitivity (16.0 µg and 48.6 µg protodioscin per g plant material, respectively). The proposed method was successfully applied for quality control of a raw plant material intended for use in pharmaceutical industry, as well as for determination of protodioscin in a commercially available pharmaceutical formulation. The positive identification of protodioscin in analyzed samples was done by comparison of retention times of chromatographic peaks and their UV spectra. The content of protodioscin in analyzed samples was: 0.65 – 0.73 % in a raw plant material, and 0.38 % in tablets, respectively.

Keywords: Protodioscin, *Tribulus terrestris* L, Steroid saponins, HPLC.

1. INTRODUCTION

It has been indicated that the public health problem connected with erectile dysfunction affects 50 % of man, aged 40-70, according to the National Institutes of Health of USA [1]. Despite availability of effective conventional medicaments, plant-derived and herbal remedies continue to provide a popular alternative for people seeking to improve quality of life [2]. The plant *Tribulus terrestris* L. (TT) has long been used in the traditional Chinese and Indian medicines for treatment of various ailments and popularly is claimed that improve sexual functions in man [3, 4]. This medical plant has been for long considered as an energizer and vitalizer [3, 4]. Its fruits and seeds are of great importance in oriental medicine because they are used as an aphrodisiac, diuretic and anthelmintic, as well as in treating coughs, kidney failure [5, 6], and urinary aliments [7]. This medical plant is also used in various ayurvedic formulations in combination with other medical plants in treatment of osteoarthritis [8]. Despite high administrated doses results showed a good safety [8]. Recently, it has been reported a case of TT induced hepatotoxicity, nephrotoxicity and neurotoxicity in a patient who used plant's extract to prevent kidney stone formation after consuming herbal water for only two days [9]. TT is used as nutritional supplement by athletes because of its claimed effect in increasing of testosterone anabolic and androgenic action through the activation of endrogenous testosterone production which has not been proven [10].

The TT is puncture vine or small caltrops, which belongs to the Zygophyllaceae family. It is a perennial, summer growing herb, 10-60 cm high, with long stems, pinnate leaves, small yellow flowers and large fruit [11, 12] known as "Chih-hsing" in China and "goat head" in USA. It can be found in arid climate regions around the world as: southern USA, Mexico, India, China, Vietnam, Spain, Macedonia, Bulgaria, Serbia, Greece, Turkey, Georgia and Iran [13, 14].

Publications on TT have reported the isolation of several steroid saponins of furostanol type [15-17], with protodioscin as the most dominant one [12], as well as alkaloids and flavonoids [18-22], and recently new feruloyl amide derivative named as tribulusamide C [23]. Obreshova *et al.* [15] identified protogracilin besides protodioscin as the dominate furostanol bisglycoside and, so these compounds are considered to be factor responsible for biological activity of products derived from this plant [14].

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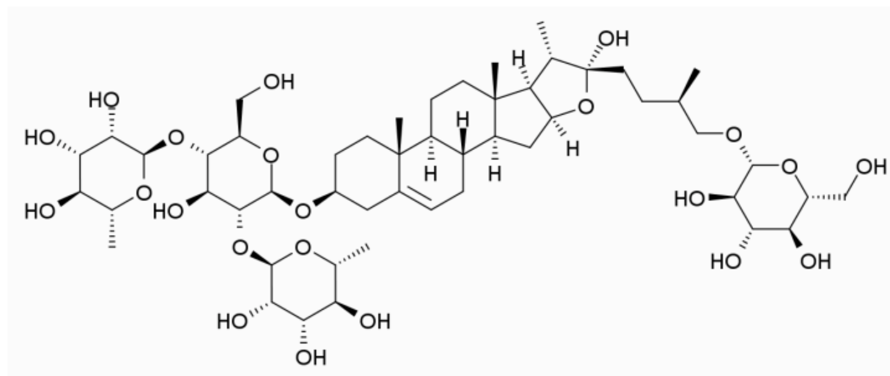

Figure 1: Chemical structure of protodioscin.

Protodioscin (Figure **1**) known as Saponin C according IUPAC is: [26-O-β-D-Glycopyranosyl-22-hydroxyfurost-5-ene-3β, 26-diol-3-O-β-diglucorhamnoside]. Besides *Tribulus,* it has been identified in a number of plant species such as *Trigonella* and *Dioscorea* families [16, 24, 25]. Thus, protodioscin has been isolated as ethanol extract from rhizomes of *Dioscorea panthaica* L. [25] and purified and identified from *Trigonella foenumgraecum* L. [24].

From the medical plant TT, non-hormonal natural products have been developed and available as dietary supplement [16]. The activity of its extract depends on the composition and the concentration of active saponins, which varies with many factors and so, are influenced by the geographical origin of plant material [14]. Protodioscin was used as a marker compound and quantified in samples of different origin as well as in several market products to demonstrate the utility of the new methods [12, 26]. For protodioscin determination in TT and this plant containing tablet formulations, there are analytical methods using Photometry [27], Nuclear Magnet Resonance (NMR) [24] and High Performance Liquid Chromatography (HPLC) with Evaporate Light Scattering Detector (ELSD) [12, 16] or with Mass Spectrometry Detection LC/ELSD/MS/MS [14], and Focused Microwave-Assisted Extraction coupled with GC/MS (FMAE/GC/MS) [28]. The photometric method reported by Gyulemetova *et al*. is not precise [27]. More lately, Ganzera *et al*. noticed that there is limited number of reports for this kind of analysis using HPLC because the saponins show very weak absorption, even in the short wavelength range, which renders a sensitive detection by ultraviolet (UV) detector impossible [26]. They solved the problem using ELSD technique. Similar, Lehmann *et al*. explained that reason for no procedure reported in the determination of protodioscin using UV detection is that protodioscin does not exhibit any absorption at 254 nm or 205 nm, and even at 190nm does not show any significant absorption [12].

Despite the reported suspicions [12, 14, 16, 24, 28, 29], the main objective of the study was to develop a rapid, simple and selective HPLC method with detection in UV region for determination of protodioscin in raw plant material using standard HPLC/DAD system by avoiding the special kind of equipment and detectors. Furthermore, the developed method should be applicable for routine analysis of raw plant material and medicines produced on base of *Tribulus terrestris* L.

2. EXPERIMENTAL

2.1. Solvents and Chemicals

Protodioscin standard substance (97.3 %, *m/m*) was purchased from ChromaDex™ (California, USA). The acetonitrile with HPLC LiChrosolv® grade was provided from Merck (Darmstadt, Germany). Purified water was obtained by TKA water purification system (Germany). All solvents were filtered through a 0.45µm nylon filter from Millipore Corporation (Billerica, MA, USA), before their usage.

2.2. Plant Material

Plant raw material was provided from three different locations in the mountain Belasica, near the village Novo Selo, southern Macedonia, harvested in 2014. After drying and powdering, the samples were stored in plastic bags at dark place at room temperature. The commercially available tablet sample was purchased from the market in the Republic of Macedonia.

2.3. Instrumentation and Measurements

UV spectra were scanned using instrument Lambda 12 Spectrometer produced by Perkin Elmer (USA).

Chromatography was performed on HPLC system 1200 series equipped with: binary pump SL, micro vacuum degasser, standard autosampler ALS SL, column compartment TCC SL, diode array detector DAD SL from Agilent Technologies (Germany). The separation was performed on HPLC column Purospher® RP-18e (150 x 4.6 mm i. d.; particle size 5 μm) coupled with guard column LiChrosorb® (4 mm x 4 mm i.d.; particle size 7 μm) from Merck KGaA (Darmstadt, Germany). This instrumentation is applied following the chromatographic system requirements of the United States Pharmacopeia [30].

The optimal chromatographic conditions are obtained by mobile phase composed of acetonitrile and water in a gradient mode (at first, it was used linear gradient with acetonitrile from 10% to 60% (*V/V*) for 15 min, followed by an isocratic mode with 60% (*V/V*) acetonitrile for 5 min), flow rate 1 mL/min, column temperature 40°C and detection at 200 nm.

2.4. Data Analysis

Chromatographic data were analyzed using ChemStation software from Agilent Technologies (Germany), following the requirements for chromatographic analysis [30]. Regression calculations were done with Microsoft Excel.

The linearity, LOD and LOQ values were presented using equation type:

$$y = a x + b$$

or

$$A = a c + b$$

In these equations A is absorbance, c is concentration, and a and b are coefficients.

2.5. Preparation of Solutions

The preparation of the solutions was carried on according to the ICH guidelines on validation of analytical methods [31-33].

All solutions were prepared using a mixture of equal volumes of acetonitrile and water as a solvent mixture. The stock standard solution was prepared by dissolving the standard substance protodioscin in volumetric flask. The solution was mixed in ultrasonic bath for 10 min.

For UV spectrum scan, 181.6 μg/mL protodioscin solution prepared in solvent mixture was used.

Ten working solutions in a concentration range of: 10.90-545.88 μg protodioscin/mL were prepared for linearity testing.

A working standard solution with protodioscin 54.49 μg/mL was used for precision testing.

Extracts for accuracy test were prepared using 240 mg raw plant material and 10 mL solvent. Extraction was carried out in ultrasonic bath for 20 min. Extracts were filtered through a quantitative filter paper (black ribbon). The prepared solutions were spiked with protodioscin stock standard solution (by adding 0.25 mL; 0.5 mL and 1.0 mL standard solution to 0.5 mL extracts, respectively).

Five working solutions were prepared in a concentration range near approximate limit of detection (10.90-54.49 μg/mL protodioscin) for estimation of LOD and LOQ. All solutions were filtered trough nylon 0.45 μm syringe Economy filter (Agilent Technologies) and then 10 μL of each solution were injected into HPLC system.

2.6. Sample Preparation

Aerial parts of the plant (flowers, leaves and stems), dried and finely powdered, were used for sample preparation. Sample solutions were prepared using 240 mg raw plant material and 10 mL solvent. Extraction was carried on in ultrasonic bath for 20 min, and then cooled at room temperature.

The tablet sample solutions were prepared using one tablet in 10 mL of solvent, in ultrasonic bath for 20 min, and then cooled at room temperature. All solutions were first filtered through a quantitative filter paper (black ribbon) and then trough nylon 0.45 μm syringe Economy filter (Agilent Technologies). Volumes of 10 μL of each solution were injected into HPLC system.

3. RESULTS AND DISCUSSION

The first reported method for TT analysis used photometry, based on a reaction of steroid saponins with a modified Ehrlich reagent [27]. A linear response has been shown between the amount of p-dimethylaminobenzaldehyde (p-DMAB) and the amount of protodioscin used. But, by that method, the results are influenced by the molarity of the acid used and by the temperature of the reagent. That method is not quite satisfactory because the results correspond to the sum of a group of compounds. Later, two similar HPLC methods were proposed to use ELS detector

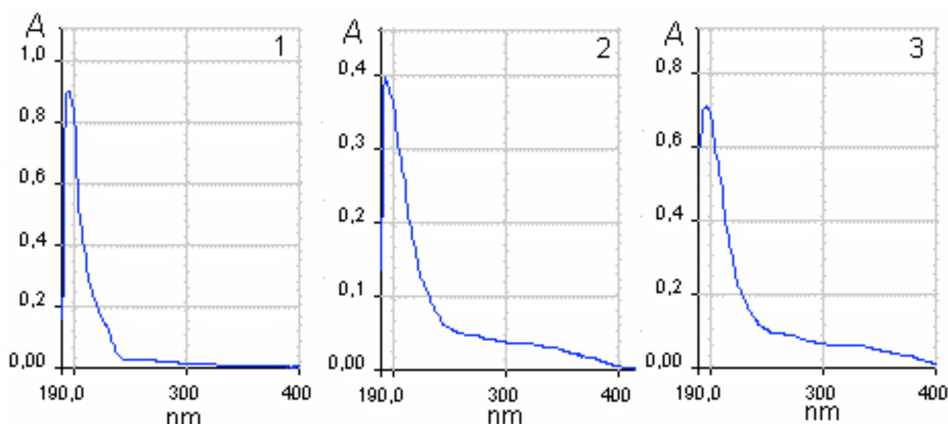

Figure 2: UV spectra of: (1) protodioscin standard solution prepared in aquatic 50% acetonitrile (181.6 μg/mL); (2) raw plant material extract prepared from 240 mg plant material in 10 mL 50% aquatic acetonitrile and diluted (3/10) with same solvent; (3) tablet solution prepared from 1 tablet in 10 mL 50% aquatic acetonitrile and further diluted (3/10) with same solvent.

[12, 16]. They compared their results with results obtained by photometric method in analyzing few samples of plant raw material and pharmaceuticals. They proved that photometric method shows false higher results and HPLC/ELSD method is more precise due to separation of the components. Ganzera *et al.* reported that the development of an analytical method for quantification of saponins is a challenge because most of these compounds have no chromofore and that is the reason why sensitive detection by UV is not possible and their ELS method has been proposed as the useful alternative [16].

The most precise method reported up to date is HPLC/ELCD/MS/MS proposed from Dinchev *et al.* [14]. Their detail study of TT samples from various regions indicated, besides protodioscin, the presence of: prototribestin, pseudoprotodioscin, dioscin, tribestin and tribulosin. Their results showed that the differences in content of these compounds depend on the region of sample collection, plant part and stage of plant development. According to these authors, the content of protodioscin and prototribestin are the main components in East South European region samples.

Photometric method is not precise enough and for applying the proposed HPLC methods special type of detector is needed. There is, also, a need of simple method for raw material quality control intended for pharmacy. Because of these reasons we used standard HPLC system and tested it for the same task.

3.1. Method Development

The methanol was avoided as solvent because it was reported that its protodioscin solutions are not stabile [16].

At first, UV spectrum of protodioscin dissolved in 50% aquatic acetonitrile was scanned. It was shown in Figure **2.1** that this compound has absorption band with maximum near 200 nm. During optimizing the HPLC working conditions, it has been observed chromatograms obtained parallel at two wavelengths: 200 nm and 205 nm for comparing the sensitivity of the response and quality of the chromatograms. The comparison of the results showed that during protodioscin analysis the signal obtained at 200 nm is about 1.60 times higher then signal at 205 nm. The results were obtained for protodioscin concentration range: 272.4-10.9 μg/mL for 15 samples (RSD is 5.13 % and SD is 0.08).

All chromatograms are with very similar quality. The UV spectrum scanned with plant TT extract (Figure **2.2**) and UV spectrum of tablet solution (containing this plant) (Figure **2.3**) are similar. They all show one band with maximum near 200 nm. This wavelength was used as optimal for UV detection.

Further, few types of C18 HPLC columns were tested and the best results were obtained with Purospher® because of the best protodioscin peak shape. Different mobile phase gradient modes were used during optimizing the method conditions.

3.2. Characteristics of the Method

Under the optimal chromatographic conditions, the retention time (t_r) of protodioscin is 9.7 min and the analysis run-time is approximately 20 min. The typical chromatogram of the standard substance solution is presented on Figure **3**(**1**). The important chromatographic data obtained by using these working

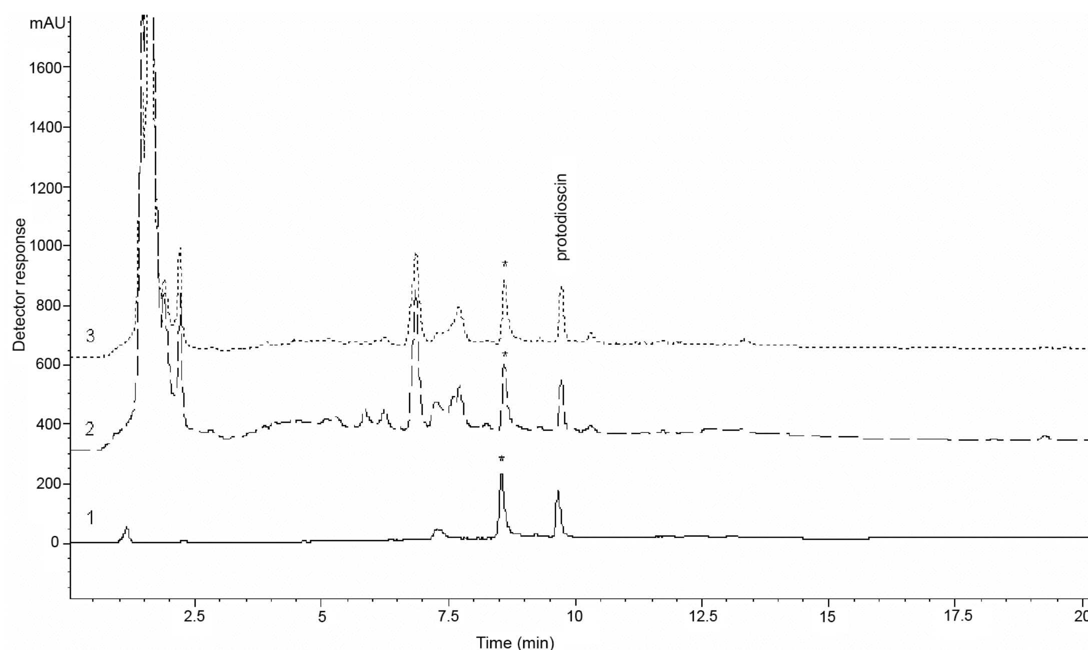

Figure 3: Typical chromatograms of: (1) protodioscin standard solution; (2) tablet solution containing *Tribulus terrestris* L.; (3) plant *Tribulus terrestris* L. raw material extract. The applied chromatographic conditions are: column Purospher® RP-18e (150 mm x 4.6 mm i.d., 5 µm), mobile phase composed with acetonitrile and water in gradient (a linear gradient with acetonitrile from 10 % to 60 % (*V/V*) for 15 min, then an isocratic mode with 60 % (*V/V*) acetonitrile for 5 min), flow rate 1 mL/min, column at 40°C, and UV detection at 200 nm.

*Unidentified component present in blank solution.

Table 1: Chromatographic Data Obtained by Proposed Method

Parameter	Value
Retention time of protodioscin (t_r /min)	9.7
Run-time of analysis (t/min)	20
Retention factor (k)	7.8
*Separation factor (α)	1.10
*Resolution (Rs)	3.54
Number of theoretical plates (N)	13031
Height of theoretical plate (H/µm)	11.51
Tailing (T)	1.1

*Compared data with peak from blank sample (t_r=8.5 min). Applied chromatographic conditions are: column Purospher® RP-18e (150 mm x 4.6 mm i.d., 5 µm), mobile phase composed with acetonitrile and water in gradient mode, flow rate 1 mL/min, column at 40°C, and detection at 200 nm.

conditions are shown at the Table **1**. Besides protodioscin, there is a component present in all chromatograms witch elutes at retention time 8.5 min (on Figure **3**, it is marked with asterix (*)). By applying blank solution (50 % aquatic acetonitrile) it was concluded that the unknown component comes from this solution. For preparing the blank we used acetonitrile with transmission characteristics: > 80 % at 195 nm and purity ≥ 99.9 % (given on the label) and water for HPLC produced from TKA water purification system. By injecting, eluting and analyzing the pure acetonitrile sample (Figure **4**) it was proved that this unknown component comes from the solvent. This component did not interfere or overlap with the protodioscin peak and did not influence the accuracy of the results.

3.3. Validation Results

The selectivity of the method was tested by comparing chromatogram of the protodioscin standard solution (Figure **3.1**) with the chromatogram of the raw material extract solution (Figure **3.2**). The presence of peak at 8.5 min in blank solution did not interfere with protodioscin because the resolution (Rs) between them is 3.54. In chromatogram of the extract solution, the

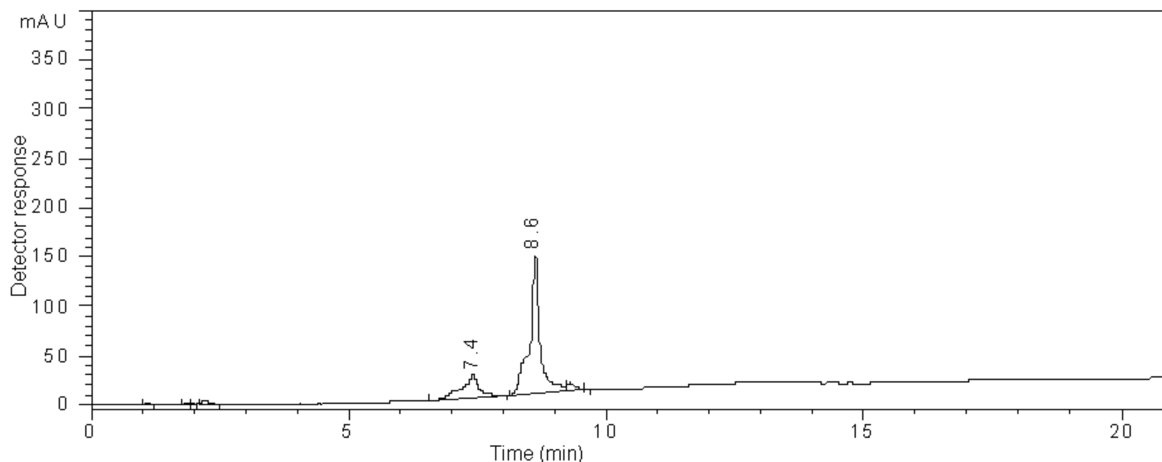

Figure 4: Chromatogram of acetonitrile using proposed method.

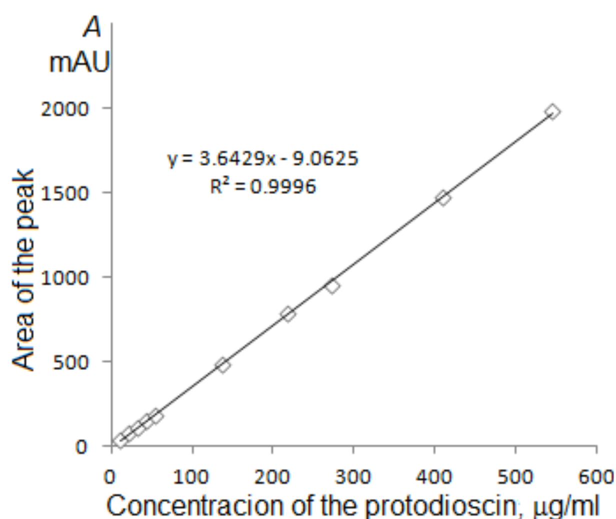

Figure 5: Linearity test: Protodioscin concentration against the area of the chromatographic peak.

presence of other components was obvious, but they, did not interfere or overlap with the protodioscin peak. Peak purity test showed that the protodioscin peak in extract at retention time (t_r) 9.5 min was attributable only to one component.

Linearity of the method has been tested in protodioscin concentration range: 10.90 μg/mL–544.88 μg/mL. The obtained results showed that there is linear relationship between peak area and quantity of protodioscin applied on column which is proven through the correlation coefficient value: R^2=0.9996 (Figure **5**). The obtained equation presented in y = a x + b form is: $A = 3.6429\ c - 9.0625$, where A is absorbance and c is concentration in μg/mL.

The precision of the method was tested by analyzing the area of protodioscin standard solution with concentration of 54.49 μg/mL, by injecting 10 μL six times. The evaluation was presented as RSD value, and it was found to be 1.11%, with SD value 2.05 (Table **2**).

Table 2: Precision Test: Results

Injection	Peak area (mAU)
1	187.5
2	186.6
3	184.9
4	184.2
5	181.6
6	184.7
<A>	184.92
SD	2.05
RSD	1.11 %

To confirm the accuracy of the proposed method, recovery experiments were carried out by standard addition technique to one of the samples of TT raw plant

Table 3: Accuracy Test: Results

Plant extract used (*V*, ml)	Standard solution added (*V*, ml)	Protodioscin added (%)	Protodioscin theoretically present (µg/ml)	Protodioscin found (µg/ml)	Recovery (%)
0.50	0.25	87.02	195.17	188.58	96.58
0.50	0.50	174.07	214.49	207.42	96.70
0.50	1.00	348.14	233.80	228.18	97.60
				Mean	96.96
				SD	0.5575
				RSD, %	0.5750

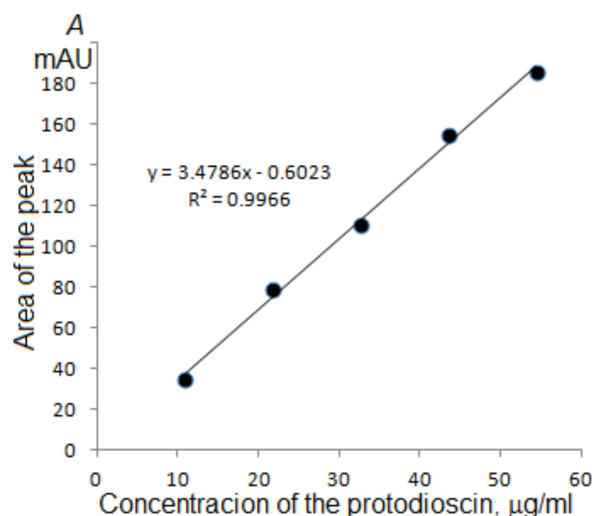

Figure 6: LOD and LOQ test: Protodioscin concentration against the area of the chromatographic peak at low concentration range.

material. Three different quantities of protodioscin standard solutions were added to prepared sample solution. The obtained recovery results are in range 96.58-97.60 % (RSD is 0.57%, and SD is 0.56, Table **3**).

For testing the limit of detection (LOD) and limit of quantification (LOQ) five solutions were prepared with lower concentration of protodioscin, in range: 10.90–54.49 µg/mL (Figure **6**). The LOD and LOQ were calculated by regression analysis. The SD-value of the line (in form: y = ax + b which corresponds, in this case, to: A = a c + b) obtained by analyzing five low-concentrated solutions and equations:

LOD=3.3 SD/a

and

LOQ=10 SD/a

In this case it was obtained: $A = 3.4786\ c - 0.6023$, $R^2 = 0.9966$ and SD = 4.068. Here, by injecting volume of 10 µL it was found for LOQ to be 11.70 ng and for LOD it is found to be 3.86 ng, which means presented at concentration units: 1.170 µg/mL (LOQ) and 0.386 µg/mL (LOD). Ganzera *et al.* detected protodioscin in concentration as low as 10 µg/mL [16]. Here the presented method allows us to detect a concentration which is 26 times lower. This advantage probably came from new generation instruments/detectors used and lower protodioscin concentration range applied in test. For comparison, they worked in a concentration range 31.2-1000.0 µg/mL protodioscin, probably in the same time for linearity and for limit tests. However, it is proved that UV detection is quite appropriate for this kind of analysis by detector set on 200 nm.

3.4. Determination of Protodioscin in Samples

The utility of the method has been tested for protodioscin analysis of three TT plant samples and one tablet formulation. Protodioscin peak from sample chromatograms was identified with retention time as compared with standard chromatogram (Figure **3.3**) and confirmed with its UV spectrum. Components

Table 4: Determination of Protodioscin in Samples: Results

Sample	Protodioscin Content ±SD (%)	RSD (%)	Number of Analyzed Samples	Technique
Raw plant material				
Raw plant material 1	0.714 ±0.002	0.32	3	HPLC/DAD
Raw plant material 2	0.730 ±0.003	0.43	3	HPLC/DAD
Raw plant material 3	0.649 ±0.003	0.49	3	HPLC/DAD
*[15]	0.1-57.0		12	Photometry
*[16]	0.024-1.337		7	HPLC/ELSD
*[12]	1.98		1	HPLC/ELSD
Medical products				
Tablets	0.3796 ± 0.010	0.61	6	HPLC/DAD
*[15]	0.0003-0.1189		22	Photometry
*[16]	0.176-6.49		3	HPLC/ELSD
*[12]	3.41		1	HPLC/ELSD

Applied chromatographic conditions are: column Purospher® RP-18e (150 mm x 4.6 mm i.d., 5 μm), mobile phase composed with acetonitrile and water in gradient mode, flow 1 mL/min, column at 40°C, and detection at 200 nm.

*Reported results.

present in plant extract and in tablet solution did not overlap or interfere with protodioscin peak. The purity of the peaks has been checked and proven.

The method worked in both cases, so for protodioscin has been identified and the assay results were obtained as it was expected (Table **4**). In samples of raw material it was found: 0.71–0.65% *m/m* protodioscin and in analyzed tablets it was 0.38% ± 0.01, with RSD=0.61%, for n=6.

For market products it was reported that they contain 0.17-6.49% protodioscin depending on the origin of the plant and plant parts used [16].

A comparative investigation in respect to qualitative and quantitative composition of raw materials from TT and a variety of preparations from different origin have been performed by Obreshova *et al*. [15]. Their results for furostanol saponins content were presented as summa of protodioscin and protogracilin. Their results for 12 analyzed raw plant material samples are in range: 57-0.1 % *m/m* furostanol saponins calculated with reference to protodioscin % and for analyzed 22 samples of furostanol saponins calculated with reference to protodioscin % in g per tablet or capsule results are: 0.3-118.9 mg/tablet or capsule. But, the majority of results were in range: 4-10 mg per tablet or capsule. Their results are unrealistically high because of the photometric method used.

In Table **4** we compared the data obtained in this study with the date from other authors obtained by using different techniques and methods. It was noticeable that for protodioscin content in raw plant material, our results are in range: 0.649-0.730 %, using HPLC/DAD; while other reported results are in range: 0.024-1.98 %, using HPLC/ELSD [12, 16], and 0.1-57.0 %, using Photometry [15]. Data obtained using chromatography techniques (HPLC/DAD and HPLC/ELSD) are compatible against data obtained using Photometry. Photometry gives false high results during multi-component solutions analysis, such as the plant extracts are. The reason for this unreal result is because absorbance is additive parameter.

4. CONCLUSIONS

We developed a rapid, simple and selective HPLC method with UV detection for determination of protodioscin in raw plant material of *Tribulus terrestris* L. The chromatographic conditions are as follows: RP C18 column (150 x 4.6 mm i. d.; particle size 5 μm) coupled with guard column (4 mm x 4 mm i. d.; particle size 7 μm); mobile phase composed of acetonitrile and water in a gradient mode; flow rate 1 mL/min; the controlled temperature at 40°C; and detection at 200 nm. The advantage is that the proposed method could be carried out using convenient HPLC/DAD instrumentation which could be found in almost every routine control laboratory. The sufficient sensitivity could be achieved without the employment of more sophisticated analytical equipment. Furthermore, according to the obtained validation results the method

was specific, precise, and accurate. Furthermore, it was proved that it is suitable for routine control of raw plant material and pharmaceuticals based on *Tribulus terrestris* L.

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