STUDY OF THE CHROMATOGRAPHIC BEHAVIOUR OF PROTODIOSCINE ON A C8 CORE-SHELL 2.6 µm SHORT (5 cm) COLUMN AND ITS DETERMINATION WITH UV/ELSD DETECTION

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ABSTRACT

The separation capabilities of a core-shell C₈ short (3x50mm) 2.6µm column's have been tested on protodioscin and a new method for its determination in plant extracts with UV detection is proposed. The higher efficiency of the core-shell column allows reduction of the separation time down to 10 - 15 min in isocratic mode (including column conditioning time), when SPE pre-cleaning of samples from stronger retained plant extract matrix components is applied. The sensitivity achieved was enhanced as well: LOD for protodioscin with UV detection reached to 2 ng injected amount, and 2 µg injected amount with ELSD detection. The application of gradient elution is more complicated because it requires very high purity solvents as mobile phase constituents. The column tested needs more than 20 min for conditioning and this hampers the significant reduction of the separation time in gradient elution.

The application of the column for analysis of plant extracts/preparations is effective when isocratic elution of protodioscin with 24 – 26 % acetonitrile/water mobile phase, modified with 1.3 - 1.6 % propanol for peak symmetry improvement, is exploited in combination with polymer-based RP SPE clean-up.

Keywords: protodioscin, Kinetex C8 2.6 µm column, HPLC/UV/ELSD.

INTRODUCTION

Protodioscin (PD), 3β,22α,25R)-26-(β-D-gluco-pyranosyloxy)-22-hydroxyfurost-5-en-3-ylO-6-deoxy-α-L-mannopyranosyl-(1→2)-O-[6-deoxy-α-L-mannopyranosyl-(1→4)]-β-D-glucopyranoside, CAS No 55056-80-9 belongs to the chemical class of furostanol saponins, with chemical structure shown on Fig. 1.

It is an active principal in some medicinal herbs: Dioscorea (dioscoreaceae) [1-3], Asparagus (asparagaceae) [4,5], Tribulus (zygophillaceae) [6-9]. Most of them have been used in traditional medicine and are used nowadays in modern phytopharmacy. PD, as their ingredient, is of particular interest not only for its therapeutic properties, but also because of its specific for Bulgaria higher content in the widespread plant Tribulus terrestris L. [6-10].

The curative properties of formulations prepared from the listed medicinal herbs in treatment and prophylaxis of cardiovascular diseases [3, 8, 10, 18] and reproductive system disturbances [8,16,18] are attributed to the steroidal saponins content [3, 8-11] and mostly to PD [21, 22]. Because of its leading contribution to the biological action of the extracts obtained from Tribulus terrestris, with its particular PD concentration, or furostanol saponins concentration in general represented as PD, is used for standardization of extracts and the raw materials used in their production [23]. That is why the interest in convenient, fast and sensitive methods for PD determination in plants and their extracts is growing. Such methods are also needed in the control of the technological processes for PD extraction and final preparations testing, as well as in biological media for pharmacokinetic studies [24, 25].

Spectrophotometric methods, used for group determination of saponins in plant extracts [26], are nonspecific and for this reason are being gradually abandoned [27].
A thermo luminescence method is described as a non-invasive technique for rapid plant diagnostics of the physiological status (energetics of photosystem II reaction) of Tribulus terrestris [27] that correlates with the optimal content of steroidal saponins in the plant. The poor quantitative information which this method proposes, however, makes it practically unsuitable for process control and precise raw materials evaluation.

The selective determination of PD in plant extracts and preparations mostly demands methods with a chromatographic separation step [1, 3 - 11, 16, 23 - 27, 30]. Gas chromatography is inappropriate for PD analysis because of its large molecule (Fig. 1).

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There are thin layer chromatographic methods [8, 29], that use [29] RP\textsubscript{18} HPTLC plates as stationary phase, 0.1M KH\textsubscript{2}PO\textsubscript{4}/acetonitrile/methanol/triethylamine 5:4:1:0.1 as a mobile phase, treatment with 0.1M H\textsubscript{2}SO\textsubscript{4} for developing the plates, and densitometry at 366 nm. Such methods do not need expensive equipment and show high sensitivity (LOQ 0.03 µg). In some cases these methods permit group differentiation of furostanol and spirostanol saponins [8], but they have limited selectivity and accuracy and can not distinguish individual representatives of these classes and quantitatively analyze them.

The application of instrumental column liquid chromatographic methods is also accompanied by problems in separation, as well as in detection. The big, flexible, with very different in polarity sectors and conformational forms, molecule of PD (Fig 1) compromises the optimization of the chromatographic system parameters. The shifted towards the far ultraviolet, nonspecific range of light absorbance maximum of PD (202-203 nm [1]), restricts the use of the most popular UV detector [6, 26].

The relatively low concentration of PD in plant extracts is yet outside the detectable concentration range of refractive index detectors. Therefore most HPLC methods for PD analysis realize separation with gradient elution and UV-transparent mobile phases, most often water/acetonitrile, and evaporative light scattering detection (ELSD) [1,7,8,22,24,27] or mass spectral detection [5, 8 - 11, 23, 26]. In these cases conventional C\textsubscript{18} analytical columns with gradient of 15-20% ACN to 35-100% CAN are used. Under these conditions, PD determination (along with other steroidal saponins and some flavonoids) takes up from 20 to 50 minutes (excluding column reconditioning time). A detection limit of about 10 - 100 µg/ml for PD is achieved [1, 6] using ELSD. LOD drops to 0.02 - 0.3 µg/ml [5,11,14,26] when a tandem mass spectral detection is employed.

A method for PD determination uses a core-shell (Kinetex) C\textsubscript{18} column in gradient elution mode and electrochemical (puls amperometric) detection, by which the time for separation of four saponins is shortened to less than 30 minutes with LOQ 0.1-0.3 ng (20-60 ng/ml) [24]. The method is used for PD determination in biological media for pharmacokinetic studies, but still has the disadvantage of long separation time, and electrochemical detectors are usually outside the conventional inventory of a HPLC laboratory.

The use of a core-shell C\textsubscript{18} chromatographic column for PD analysis is still not described.

As it is well known, the “core-shell” technology permits higher efficiency in comparison to porous silica-based HPLC columns used in the literature, and lower separation time as a result. That is why a reduction of separation time in PD analysis is expected as well.

The aim of our work is to study the behavior of a short core-shell C\textsubscript{8} 2.6 µm (Kinetex) HPLC column with UV detection on the HPLC analysis of PD.

**EXPERIMENTAL**

**Instruments**

A ser. 200 quaternary HPLC pump (PE) with mod. 785A UV-detector, 12 µl flow cell (ABS), mod LC-135, 4 µl flow cell (PE), mod. 500 evaporative light scattering detector, ELSD (Alltech) and mod. 112A oven/injector (ABS), with Kinetex C\textsubscript{8} 3 x 50 mm, 2.6 µm column (Phenomenex), were used in chromatographic experiments.
Chemicals
Protodioscin (PD) "reference substance" > 98 % purity (ChromaDex), "gradient grade" acetonitrile, ACN (Sigma), "suprapur" n-propanol (Fluka) and MilliRO/MilliQ “HPLC-grade” water, “HPLC-grade” tetrahydrofurane (Fluka) and 0.45µm nylon membrane filters (Merck-Millipore) were used for samples/standards and mobile phase preparation. Tribestan® powdered substance was examined as a preparation from Tribulus terrestris plant.

RESULTS AND DISCUSSION
The retention of PD on the C₈ “core-shell” Kinetex 3 x 50 mm 2.6 µm HPLC column was studied by obtaining the chromatograms of PD solutions at 0.8 ml/min flow rate and a mobile phase consisting of different water/acetonitrile concentrations.

A chromatogram obtained with 23 % ACN is shown on Fig. 2. Fig. 3 represents the relationship between PD retention time (RT) and the mobile phase composition.

The chromatogram on Fig. 2 shows fronting of PD peak when ACN/water only is used as mobile phase. Different modifiers of the mobile phase were tested and THF, n-propanol and i-propanol at concentrations of 1.3 – 2 % in the ACN/water mobile phase showed improvement of the PD peak symmetry. Propanol at concentration of 1.3 - 1.6 % is preferable on account of its lower UV cut-off. The chromatogram on Fig. 4 obtained at 0.8 ml/min with ACN/water/n-propanol 24:74.4:1.6, and double detection (detectors connected in series), shows excellent symmetry of the PD peak, and the difference between sensitivity with UV (204 nm) and ELSD detection is excellent as well.

The limit of detection (LOD) for PD with ELSD, as shown on Fig. 4B, with 1.2 µg injected amount. The LOD for PD with UV (204 nm) detection was tested by building of a standard graph with series of chromatograms of standard solutions with decreasing PD concentrations, as shown on Fig. 5.
The chromatogram of 5 µl 0.3 µg/ml PD, represented on Fig. 6, shows the LOD of 1.5 ng injected amount, reached with the LC-135 photodiode array (DAD) detector with 4 µl flow cell at 205 nm.

Fig. 7 presents the temperature dependence of PD retention on the C₈ Kinetex column. Contrary to literature data for C₁₈ Kinetex column [23], the RT on Kinetex C₈ decreases with temperature, together with the column back pressure. 35°C can be a convenient column temperature, keeping an acceptable back pressure of 32 MPa at 0.8 ml/min 24% ACN/water, and a lower risk of thermal decomposition for the sensitive sample components.

SPE was applied for sample clean-up from components stronger retained in the analytical column. Strata-X (a polymer based C₁₈ sorbent, Phenomenex Inc.) showed better results with a 94% recovery, after elution with 1 ml 50% ACN/water (Fig. 8). The cartridge was activated with 3 ml ACN and washed with 3 ml water before sample loading. Elution was carried out with 2 ml 18% ACN/water. Chromatograms obtained after washing, first elution with 1 ml 50% ACN/water and second elution to check the recovery, are presented on Fig. 8.

Separation of PD by gradient elution from a Kinetex C₈ column was tested as well. After column conditioning for 30-40 min with mobile phase ACN/water 15:85, a gradient of the ACN-concentration to 50:50 for 5 min was applied after injection of 5 µl 25 µg/ml PD in 25% ACN, and after injection of 5 µl 25% ACN only (Fig. 9). The PD peak symmetry and efficiency were improved with gradient elution without mobile phase modification, but a big peak of impurities from the mobile phase, trapped in the column at lower ACN concentration, was observed. Its area increases with the column conditioning time. The Kinetex column tested needs more than 30 min for conditioning after gradient and this leads to longer analysis time than for the isocratic analysis of PD after SPE clean-up.

CONCLUSIONS

The results obtained show a Kinetex (C₈ 3x50 mm 2.6 µm) column as a good possible alternative for PD determination in natural plant extracts. When an ACN/water mobile phase is used, a modifier as n-propanol, i-
propanol or tetrahydrofurane at concentration 1.3 – 2 % is required for peak symmetry improvement. A modified mobile phase with 23 – 26 % acetonitrile in water is optimal for isocratic separation. Acidification of the mobile phase, used in most of the published methods, does not affect PD retention with this column, but can be useful for improvement of its separation from sample matrix constituents. Use of UV detection is possible when high purity solvents with low UV cut-off, like gradient grade water and acetonitrile, are used. UV detection permits more than 50-fold lower LOD to be reached, in comparison to ELSD detection. On the other hand, because of the higher efficiency, a significantly higher increase of sensitivity can be accomplished with a core-shell C₈ column, in comparison with conventional analytical columns packed with fully porous silica-based stationary phases. Because of this higher efficiency and good selectivity towards polar compounds, as PD is, separation can be much faster, decreasing the analysis time more than 5 times. On account of the longer time for conditioning that this column demands, however, the gradient elution requires more time and very high purity solvents to be used. At gradient elution, starting from less than 20 % ACN, efficiency and sensitivity together could be increased for multicomponent analysis but at a price of longer reconditioning and thus longer analysis time. Sample clean-up with polymer-based RP-cartridges results in higher recovery in comparison.
with silica-based ones.

The isocratic analysis of PD with the core-shell C8 short Kinetex column is effective, because it is faster (less than 20 min total analysis time) when SPE is applied to clean the sample from strongly retained components.

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