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Communication

Analysis of gypsogenin saponins in homeopathic tinctures★

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A relatively simple and short procedure for the quantitative determination of gypsogenin saponins was performed to evaluate homeopathic tinctures in which those compounds can be regarded as one of the active constituents. This method comprises partial hydrolysis of saponins, subsequent extraction of liberated prosaponin (gypsogenin 3-*O*-glucuronide) and its analysis by high performance liquid chromatography. Glycyrrhizic acid was used as an internal standard. This method was successfully applied to the analysis of mother tinctures obtained from *Saponaria officinalis*. Thus, the determination of triterpenoid saponins can be used as a convenient and sufficient method of standardization of selected homeopathic tinctures.

Keywords: gypsogenin, homeopathic tinctures, saponins

INTRODUCTION

Isoprenoids occurring in higher plants are known for their many biological activities (Sparg *et al*., 2004). Triterpenoid saponins are widespread in medicinal plants and very often are responsible for their pharmacological effects. There are many plant-derived products containing significant amounts of saponins, for example herbal infusions or tinctures (Ansel *et al*., 1995; Lyakina, 2004). Tinctures, traditionally obtained from dried, or occasionally fresh, plant material with the use of ethanol solutions (Bilia *et al*., 2002a; 2002b), are considered as one of the best liquid forms of plant extracts due to their stability and purity. Many tinctures made of various plants are commonly used in homeopathic therapies (Frye, 2003). Since homeopathy has become very popular as alternative or additional treatment in many diseases, homeopathic tinctures need to be characterised following the regulations of the European Agency for the Evaluation of Medicinal Products (Human Medicines Evaluation Unit, E.M.E.A. http://www.eudra.org.emea.html) and the European Parliament (directives 2001/83/EC and 2004/27/EC; Lyakina, 2004; Satti, 2005). Many homeopathic tinctures, e.g. *Bellis erecta*, *Clematis erecta, Cyclamen europeum*, *Saponaria officinalis* contain various amounts of saponins which can be regarded as the active constituents of the tincture. However, saponins are rather rarely considered to be suitable as marker constituents in evaluation of plant extracts. Obviously, a direct quantification of those compounds is difficult not only due to their relatively small amounts (often less than 1% d.w.), but also due to their occurrence in very numerous molecular structures. In one plant more than twenty saponins can occur with aglycones as oleanolic acid, hederagenin, gypsogenin or quillaic acid. Thus, qualitative and quantitative determination of saponins in plant material is still a challenge and there is no one single method that can be recommended as a routine procedure for analysis of complex saponin mixtures (Oleszek, 2002). Nevertheless, the develop-

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Abbreviations: EtOAc, ethyl acetate; EtOH, ethanol; G3Og, gypsogenin 3-*O*-glucuronide; HPLC, high-performance liquid chromatography; MeOH, methanol; PTFE, polytetrafluoroethylene; RP, reversed phase.

ment of convenient procedures characterizing saponins could be of interest in the case of medicinal products where those compounds are recognized as responsible for pharmacological activity. Therefore, as a first step, in the present work we tried to quantify gypsogenin saponins by means of their common component: gypsogenin 3-*O*-glucuronide (Fig. 1) present in chosen homeopathic tinctures, in accordance with the E.M.E.A. instructions.

MATERIAL AND METHODS

Acetonitrile, EtOAc, MeOH, EtOH were HPLC grade from Carlo Erba (Milan, Italy); *ortho*-phosphoric acid (85%), analytical grade was from Prolabo (Geney, France); glycyrrhizic acid, monoammonium salt trihydrate (98% purity) was from Aldrich Chemical Company, Inc (Milwaukee, WI, USA). Ultra-pure water used throughout the study was purified by a Purelab UHQ system from ELGA (Bucks, England). Solid phase Bond Elut® columns (6 ml, 1 g of C_{18} reversed phase silica gel per column) were purchased from Varian (Middelburg, the Netherlands). *Saponaria officinalis* mother tinctures, prepared following the recommendations of European Pharmacopoeia 5 (directives 01/2005:1038 and 01/2005:2029), were provided by a French pharmaceutical company involved in homeopathy. Generally, whole dried plants or individual parts of the plant were put in 70% (v/v) EtOH and left at room temperature for at least three weeks before use as a mother tincture for allopathic purposes or before dilution to obtain homeopathic remedies.

Homeopathic tinctures (5 ml) were filtered, evaporated to dryness and solubilised in 1 ml of water. Samples (500 µl) of this solution as well as control samples prepared with 1 mg of glycyrrhizic acid added as an internal standard, were subjected to acid hydrolysis (0.57 M H_2SO_4, 4 h, 95–100°C). Subsequently, obtained hydrolysates were submitted to neutralization with $NaHCO_3$ (powder), extraction with EtOAc and evaporation of organic solvent leading to aqueous phase submitted to a purification on RP-18 small column and elution with 1 ml pure MeOH. Before HPLC analysis, samples were filtered through a cartridge-type filtration unit with a polytetrafluoroethylene (PTFE) membrane. HPLC of 20 µl samples was performed on a Shimadzu instrument (LC-10AT VP pumps) at 40°C on a LiChrosorb RP-18e column (5 µm, 250 × 4.6 mm i.d.; Merck, Darmstadt, Germany) equipped with a LiChrosorb RP-18 precolumn (5 µm, 10 × 4 mm i.d.; Merck, Darmstadt, Germany) and a two-wavelength detector Model SPD-10A set at 210 and 254 nm. The mobile phase (flow-rate 1.0 ml/min) was a linear 30–50% CH_3CN gradient in H_2O with 0.1% H_3PO_4 during 10 min followed by a plateau at 50% CH_3CN for 5 min.

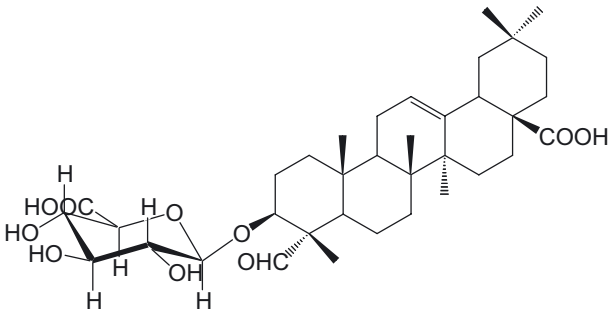

Figure 1. The structure of gypsogenin 3–*O*-glucuronide.

RESULTS AND DISCUSSION

According to E.M.E.A. instructions on validation of analytical methods, the procedure being developed should be characterized with respect to its accuracy, specificity, precision, repeatability and linearity.

Specificity

Usually it is impossible to characterize saponins by a unique reaction or simple spectrophotometric measurement. Gypsogenin 3-*O*-glucuronide can be characterized by comparison of its absorbance at 210 and 254 nm (the wavelength close to the maximum of absorbance typical for compounds containing aromatic rings i.e. proteins, nucleic acids, phenolics including flavonoids). A high ratio of absorbance at these two wavelengths is a good proof of the purity of the sample ($R_{210/254} = 550 \times 10^6$ for gypsogenin 3-*O*-glucuronide, Fig. 2). Moreover, the ad-

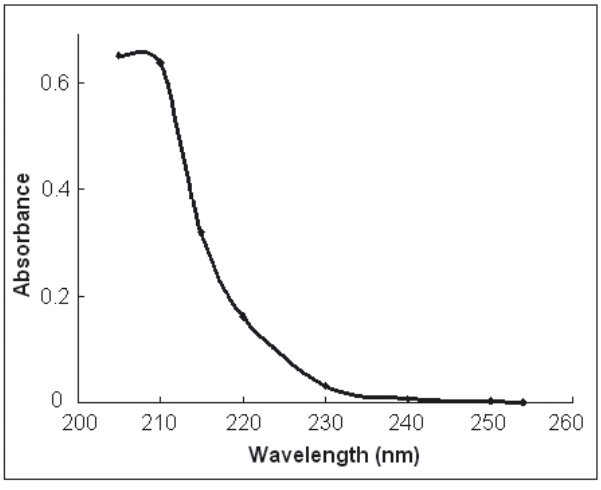

Figure 2. UV spectrum of G3Og.

Table 1. Quantitative HPLC assays of glycyrrhizic acid before and after hydrolysis

Number of the sample of glycyrrhizic acid (1 mg/ml)	Assay before hydrolysis	Assay after hydrolysis
1	0.99	0.93
2	1.05	1.01
3	1.01	0.97
4	1.09	1.01
5	0.98	0.93
6	0.87	0.96
7	0.99	1.00
Mean value ±S.D.	1 ± 0.07 mg/ml	0.97 ± 0.04 mg/ml

vantage of the presented method is the elimination of main by-products and possible spectrally overlapping compounds achieved as a result of hydrolysis and subsequent AcOEt extraction of liberated prosaponin (gypsogenin 3-*O*-glucuronide) at pH 7.4 after $NaHCO_3$ neutralization. To check the efficiency of the procedure, commercially available glycyrrhizic acid was specifically chosen as an internal standard for hydrolysis and quantitative determinations because of its absence in *Saponaria officinalis* tincture, spectral properties (high absorbance at 254 nm) and the presence of two glucuronic acid moieties in its molecule (thus resemblance to G3Og, a monoglucuronide). Very good extraction recoveries were obtained in performed experiments, moreover, it was demonstrated that in the applied mild conditions of acidic hydrolysis there is no cleavage of the glycosidic linkages either between the glucuronic acid moieties or between glucuronic acid and the aglycone, as it is shown in Table 1.

Identification of G3Og was performed by HPLC by comparison of the retention time of the peak in the extracts with that of the authentic reference sample. HPLC t_R value was 14.7 min for G3Og and 12.5 for glycyrrhizic acid. The G3Og contents were determined by comparison with genuine stand-

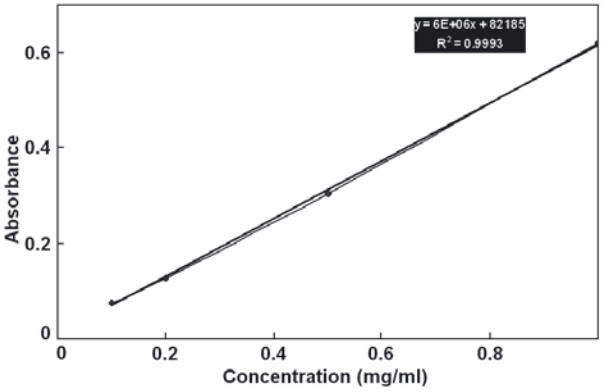

Figure 3. Calibration curve of quantitative HPLC determination of G3Og.

Table 2. Quantitative HPLC assays of gypsogenin saponins of *Saponaria officinalis* mother tincture by means of determination of G3Og after acidic hydrolysis and purification

Number of the sample of *Saponaria officinalis* mother tincture	G3Og content (mg/l)	Saponin content (μM)
1	7.46	11.54
2	9.32	14.39
3	7.45	11.43
4	8.17	12.55
5	7.34	11.33
6	7.90	12.15
Mean value	7.94	12.25
Standard deviation	0.75	1.12

ards previously purified from roots of *Gypsophila paniculata* (Henry *et al.*, 1989) and identified by NMR spectroscopy (Bouguet-Bonnet *et al.*, 2002).

Repeatability

To estimate the repeatability, six samples of *S. officinalis* mother tincture from the same batch were analysed by HPLC and their contents were evaluated to calculate the relative standard deviation (Table 2). The obtained average result was 7.94 mg/l of G3Og in the tincture with relative standard deviation of 5.4%. Taking into account the molecular weight of G3Og, the concentration of gypsogenin-saponins in the tested tincture could be estimated as 12.25 μM.

Reproducibility

To evaluate the reproducibility of injection, standard solutions of G3Og (1 μg/μl) and samples of the tested tincture were injected six times. The relative standard deviation was estimated as 0.8%. In addition, the stability of solutions of G3Og in MeOH at pH 6 during 20 days of storage at room temperature and during 1 h at 95°C was tested. The quantitative HPLC determinations in both cases gave exactly the same values and standard deviations as the control (results not shown). Thus, gypsogenin 3-*O*-glucuronide turned out to be a good marker compound in the developed method because of the stability of this molecule in the conditions of acidic hydrolysis applied and the excellent shelf-life at pH 6.0 at room temperature.

Linearity

The linearity of response was determined for five concentrations with three injections for each. Calibration graphs for HPLC (Fig. 3) were recorded with samples ranging from 1 μg/ml to 1 mg/ml ($r > 0.999$).

The obtained results show that the presented relatively simple and short procedure allows quantitative determination of small amounts of gypsogenin-saponins occurring in the analysed tincture with sufficient accuracy, specificity, repeatability (CD 5.4%) and reproducibility (0.8%).

The most popular methods of standardization of plant pharmaceutical products, including homeopathic tinctures, involve the determination of the content of flavonoids (Selivantchikova *et al.*, 2001; Bilia, 2002a; 2002b) and particularly alkaloids (Nandi, 1999). Saponins are not regarded as convenient marker compounds due to their structural diversity and a lack of a universal method of qualitative and quantitative determinations. However, despite the difficulties, it is worthwhile to develop unique procedures characterizing saponins as constituents of plant extracts even if practical applications of these methods are limited to appropriate products. The method proposed in the presented work is an example of such a procedure which can be applied to tinctures containing gypsogenin saponins.

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