Talanta 192 (2019) 263–269

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

A validated ^{1}H qNMR method for direct and simultaneous quantification of esculin, fraxin and (–)-epicatechin in *Hippocastani cortex*

Aleksandra Owczarek[a,*], Arkadiusz Kłys[b], Monika A. Olszewska[a]

[a] Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Lodz, Muszynskiego 1, 90-151 Lodz, Poland
[b] Department of Chemistry, University of Lodz, Tamka 12, 91-403 Lodz, Poland

ARTICLE INFO

Keywords:
Aesculus hippocastanum
Horse chestnut bark
Esculin
Fraxin
Epicatechin
qNMR

ABSTRACT

A fast and precise qNMR method was developed for quantification of major bioactive constituents in the bark of horse chestnut and dry extracts prepared thereof. The method was optimised using 600 MHz spectrometer, and the final acquisition parameters (90°-pulse, acquisition time – 3.0 s, relaxation delay – 27 s, number of transients – 16) allowed for performing of quantitative experiments in under 15 min. The contents of three analytes were determined using specific ^{1}H resonances at δ7.45 ppm for esculin, δ5.00 ppm for fraxin, and δ5.94 ppm for (–)-epicatechin. The validation showed good precision (RSD < 1.5%) and accuracy (95–103%), and adequate sensitivity (LODs in the range of 3.3–5.9 μg) of the measurements. The determined levels in commercial samples of *Hippocastani cortex* were in the range of 25.89–38.94 mg/g dry weight (dw) of the bark for esculin, 12.58–17.13 mg/g dw for fraxin and 10.42–13.96 mg/g dw for (–)-epicatechin, and in the dry extracts prepared thereof 97.02–143.51 mg/g, 45.78–58.92 mg/g and 28.07–46.29 mg/g, respectively. The obtained results were cross-validated by a HPLC-PDA method with the use of a fused-core column, and no statistical differences were found between the results obtained by both methodologies, but with the advantage of higher precision of the qNMR assay. The relevant variability in quantitative composition of the commercial samples emphasise the need to introduce quality control studies in production of preparations containing horse chestnut bark and the developed method was proved suitable for this purpose.

1. Introduction

Aesculus hippocastanum L. (horse chestnut) is a large deciduous tree originating from Balkans, specifically from small areas in northern Greece, Albania, the Republic of Macedonia, Serbia, and Bulgaria. Today, due to its large, beautiful flower clusters and excellent resistance to environmental conditions it is widely cultivated in the temperate regions throughout the world as an ornamental tree [1]. Horse chestnut is also a well-known medicinal plant with different plant parts having been used both in traditional and official medicine. The bark of the tree (*Hippocastani cortex*) has found its application in the symptomatic treatment of functional disorders of cutaneous capillary fragility, subjective signs of venous insufficiency, symptoms associated with haemorrhoids, as well as oedemas, small bruises, and limited skin and subcutaneous tissue inflammations [2]. The plant material might be used in form of tees and infusions, and extracts prepared thereof are constituents of multi-ingredient preparations, such as creams, ointments or suppositories. Its pharmacological efficiency is considered to be based mainly on esculin and fraxin – simple hydroxycoumarin glycosides possessing a wide range of beneficial biological properties. For example, esculin has been found to be a selective inhibitor of lipooxygenase [3] and its anti-inflammatory potential has been confirmed in numerous in vivo studies [4–6]. Both compounds have been also demonstrated to alleviate the negative results of oxidative stress. These activities are in accordance with strong antioxidant and anti-inflammatory properties of the plant material itself [7–9].

Since the proper standardisation of plant materials is recognised as the major prerequisite of safe and effective application of herbal remedies, the development of quick and reliable standardisation procedures is an important area of research, that in case of *Hippocastani cortex* seems rather underdeveloped. While for other important coumarin plant materials, such as ash leaf (*Fraxini folium*), numerous methodologies have been proposed, including UV-spectroscopy [10], HPLC-PDA-ESI-MS [11], micellar electrokinetic chromatography (MEKC) [12], HPTLC [13,14], or capillary electrophoresis (CE) [15–17], there is almost a complete lack of validated procedures dedicated for the horse-chestnut bark. Single HPLC and TLC evaluations carried out back in the 90's [18,19] have shown that the content of both

* Corresponding author.
E-mail address: aleksandra.owczarek@umed.lodz.pl (A. Owczarek).

https://doi.org/10.1016/j.talanta.2018.09.036
Received 27 April 2018; Received in revised form 7 September 2018; Accepted 11 September 2018
Available online 15 September 2018
0039-9140/

coumarins in the bark is very variable depending on the age of the branches it was obtained from, bark layer, as well as the collection season; the results ranged from 0.4% to 7.9% for esculin and 0.2–3.1% for fraxin. Since then, despite huge advances in analytical technologies, no reliable and validated methodology was reported for quality control of the title plant material. Moreover, no study so far has taken into account other compounds that may accompany the coumarins and have influence on activity and support authentication of horse chestnut bark preparations.

In the last years, the use of NMR spectroscopy as a quantitative tool (qNMR) has received considerable attention. Due to direct proportionality between the signal integral and the number of protons giving rise to it, it allows for quantification of multiple constituents using single reference standard, and thus offers certain advantages over other commonly applied methods, that require compound-specific calibration [20]. As to date, qNMR has been successfully applied for standardisation of herbal drugs containing phytochemicals from various chemical groups, such as terpenoids, flavonoids and alkaloids [21–25], but no coumarin-based plant material has been investigated so far.

The aim of the present study was to develop, optimise and validate the first qNMR methodology for quality control of *Hippocastani cortex* and extracts thereof. Apart from dominating coumarins, (–)-epicatechin was quantified as the main representative of flavan-3-ols, and additional marker of plant material quality.

2. Experimental

2.1. General

Methanol-d_4 and other deuterated solvents (used for solvent optimisation) were purchased from Armar (Döttingen, Switzerland). Certified qNMR standard dimethyl terephthalate (TraceCERT®) and reference standards of esculin monohydrate, fraxin, and (–)-epicatechin of HPLC purity were purchased from Sigma-Aldrich (Seelze, Germany/St. Louis, MO, USA). HPLC grade acetonitrile and all other solvents of analytical grade were obtained from Avantor (Gliwice, Poland). In all analyses, redistilled water was used.

2.2. Plant material

The plant material used in the current study were commercial *Hippocastani cortex* samples bought from four different local Polish distributors (Table 1). Prior to the analyses the plant material was grounded using electrical grinder and sieved (0.315 mm).

2.3. Optimisation of extraction procedure

Samples of the dried plant material (100 mg) were extracted twice with 30 mL of various solvents: chloroform, chloroform-methanol (1:1, *v/v*), methanol, methanol-water (1:1, *v/v*), and water. The obtained extracts were evaporated to dryness and weighted. The residue was resolved in methanol-water (7:3, *v/v*, 100 mL) and injected into the HPLC system.

Table 1
Characteristics of the analysed commercial samples of horse chestnut bark.

Sample	Producer/Distributor	Extract yield [%][a]
S1	Flos (Mokrsko, Poland)	27.2 ± 0.2
S2	Herbapol Kraków (Kraków, Poland)	32.4 ± 0.5
S3	Zioła z Kurpii (Jednorożec, Poland)	25.8 ± 0.4
S4	Farmvit (Żeromin, Poland)	26.6 ± 0.2

[a] Data presented as means ± SE ($n = 3$).

2.4. HPLC-PDA analysis

Dried plant material (100 mg) was extracted exhaustively with methanol (3 × 30 mL) and the combined extracts were diluted to 100 mL and injected into the HPLC system.

The HPLC-PDA assays were carried out on a Waters 600E Multisolvent Delivery System (Waters, Milford, MA, USA) with a PDA detector (Waters 2998) and a manual 7725 sample injection valve with a 5 µL injection loop (Rheodyne, Pittsburgh, PA, USA). A C18 Ascentis® Express column (2.7 µm, 75 mm × 4.6 mm i.d.; Supelco, Bellefonte, PA, USA) with a C18 Ascentis® C18 Supelguard guard column (3 µm, 20 mm × 4 mm i.d.; Supelco) was maintained at 30 °C using a Jetstream Plus 5480 thermostat (Thermotechnic Products, Langenzersdorf, Austria). The elution system consisted of solvent A (0.5% water solution of orthophosphoric acid, *w/v*) and solvent B (acetonitrile) with the elution profile as follows: 0–1 min, 6% B (*v/v*); 1–6 min, 6–16% B; 6–11 min, 16–35% B; 11–12 min, 35% B; 12–13 min, 35–6% B; 13–18 min, 6% B (equilibration). All gradients were linear. The flow rate was 1.4 mL/min. The peaks of esculin, fraxin and (–)-epicatechin were identified by comparison with standards and their levels were quantified using six-point calibration curves prepared in the relevant range of concentrations.

2.5. Sample preparation for qNMR analysis

Dried plant material (100 mg) was extracted exhaustively with methanol (3 × 30 mL), the combined extracts were evaporated under reduced pressure to dryness, then weighted and resolved in methanol-d_4 (1 mL). An aliquot of the concentrated extract (500 µL) was transferred to NMR-probe and the solution of standard (dimethyl terephthalate) was added (100 µL, 1 mg/mL). The extraction was performed in triplicate for every plant material and the NMR analysis was run in triplicate for every extract.

2.6. NMR spectroscopy

NMR measurements were recorded at 600.26 MHz on a Bruker AVANCE III spectrometer (Bruker BioSpin Co., Billerica, MA, USA) equipped with a 5 mm TBI probe. All experiments were recorded at 298 K (25 °C) without sample spinning. The ^{1}H chemical shifts (δ) are expressed in parts per million (ppm) with reference to the residual solvent signal, and coupling constants (J) are given in Hertz (Hz).

The 1D ^{1}H NMR spectra were acquired under quantitative conditions using a 90° single-pulse experiment with inverse gated decoupling (Bruker pulprog: zgig). The 90° pulse width for each sample was determined by prorating the measured 360° pulse width (p_{90} = ¼ x p_{360}). The spectral width and the acquisition time were set to 12,500 Hz, and frequency tuned and impedance matched before each sample run. The receiver gain was set to 45.2 for all ^{1}H NMR measurements. A total of 16 transients were acquired with an acquisition time of 3.0 s and a relaxation delay of 27 s, which is more than 5 times the longest T_1 observed. The total accumulation time per sample in quantitative experiments was 15 min.

Offline 1D NMR data processing was performed using TopSpin software (v.3.5 pl 6, Bruker BioSpin Co.). For qHNMR analysis, the following processing scheme was used: a window function using exponential multiplication (line broadening = 0.5 Hz) was applied, followed by zero filling to 128k acquired data points before Fourier transformation. The digital resolution after zero-filling was 0.095 Hz/pt (0.16 ppb/pt at 600.26 MHz). After manual phasing, a fifth-order polynomial baseline correction was applied.

T1 values were calculated using the T1 relaxation routine (TopSpin 3.5).