Analytica Chimica Acta 633 (2009) 188–196

Contents lists available at ScienceDirect

Analytica Chimica Acta

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

Pressurized liquid extraction and anticholinesterase activity-based thin-layer chromatography with bioautography of *Amaryllidaceae* alkaloids

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ARTICLE INFO

Article history:
Received 18 July 2008
Received in revised form
19 November 2008
Accepted 22 November 2008
Available online 30 November 2008

Keywords:
Lycorine
Galanthamine
Pressurized liquid extraction
Thin-layer chromatography
High-performance liquid chromatography
Bioautography

ABSTRACT

Modern extraction technique-pressurized liquid extraction (PLE) was optimised for extraction of lycorine and galanthamine (*Amaryllidaceae* alkaloids) from *Narcissus jonquilla* 'Pipit'. Crude extracts were purified on Oasis MCX cartridges, and the alkaloids eluted with 80–100% recoveries using methanol–10% ammonia solution (3:1, v/v). Quantitative results were obtained by both HPTLC–densitometry on silica gel plates and RP-HPLC with diode array (DAD) on XTerra C_{18} stationary phase. Both methods were fully validated in terms of specificity, precision (including *intra*- and *inter*-day measurements), LOD and LOQ values, correlation of UV spectra and linearity of calibration curves. The methods were also well correlated each other with correlation coefficients (r) 0.98823 and 0.99081, respectively, for the mean values of galanthamine and lycorine.

Among the investigated solvents methanol and 1% tartaric acid methanolic solution at default conditions (120 °C, p = 60 bar, time: 10 min, one static cycle) permit the highest yields of the total sum of the alkaloids, whereas for toluene the lowest amounts were measured. Lycorine to galanthamine mean ratios were dependant on the type of solvent used, and in toluene galanthamine and related alkaloids were preferably extracted.

In temperature experiments for galanthamine, the levels of this compound increased from the temperature of 20 till 150 °C in the investigated solvent systems, then decreased with slight increase from the temperature of 175 to 200 °C in 1% tartaric acid methanolic solution. When lycorine was analysed, similar trends were observed, however the maximum of the concentration was measured at a temperature about 125 °C. The ratios of the mean values of these two compounds differed in temperature-dependant experiments in both solvent systems.

Further more, two TLC with bioautography approaches were used in screening for anticholinesterese properties of the extracts. No qualitative differences were found among the different solvent extracts, and AChE inhibition was correlated with galanthamine and related compounds.

In conclusion, optimised PLE was much more effective than previously applied hot-solvent extraction, microwave-assisted extraction (MAE) or ultrasound-assisted extraction (USAE).

1. Introduction

Amaryllidaceae alkaloids represent a kind of phenylalanine and tyrosine derivatives restricted to the only *Amaryllidaceae* plant family. There are some types of structure involved in this group, e.g. lycorine, galanthamine, crinamine, tazettine, homolycorine, mesembrenone and others [1,2]. These compounds exhibited several types of pharmacological activities including antiviral [3], antimalarial [4], on central-nervous system (c.n.s.) [5] as well as antineoplastic [6] one. Galanthamine is widely applied in Alzheimer disease, due to the ability of enhancement of cholinergic activity in c.n.s. by blocking the enzyme–acetylcholinesterase [7].

For extraction of pharmacologically active *Amaryllidaceae* alkaloids methanolic or ethanolic maceration (using solvents at ambient temperature) or hot-solvent extraction (HSE, utilizing boiling solvents in normal extraction flasks under reflux) were used [8–10]. However, there are long-lasting procedures with possible drawbacks of artifacts creation. A percolation [11] and Soxhlet's extraction [12] can do also that.

There are only few reports on other extraction techniques of *Amaryllidaceae* alkaloids, such as: ultrasound-assisted extraction (USAE) [13] using methanol at a temperature of 25 °C, 3 times during 40 min, microwave-assisted extraction (MAE) with methanol also and the microwave power at 450 W [14] or supercritical fluid extraction (SFE) with the optimisation of a pressure and type of a modifier. [15]. However, the USAE method [13] is also rather time-consuming, MAE [14] was not optimised for lycorine-type alkaloids, whereas SFE [15] was not validated for galanthamine-like alkaloids.

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doi:10.1016/j.aca.2008.11.053

Pressurized liquid extraction (PLE), so-called accelerated solvent extraction (ASE) in the previous literature, is a modern extraction technique, which uses an organic solvent at high pressures and temperature above the boiling point (default conditions). It ensures the higher solubility of analytes in solvent at higher temperatures, higher diffusion rate as a result of higher temperatures, and disruption of the strong solute–matrix interaction caused by van der Waals forces, hydrogen bonding and dipole–dipole attractions between solute molecules and active sites on the matrix [16]. Also, changing the size of the extraction cells it can be also simply scale-up. Although, it was applied for extraction of other types of alkaloids, such as cocaine [17] or berberine [18], etc. (with the optimisation of a type of solvent, a temperature and time of the extraction process), until now it was not used for extraction of lycorine, galanthamine and related compounds. Therefore, the aim was to develop a new accelerated extraction methodology of potent acetylcholinesterase inhibitors from plant material, and clearly indicate its advantages over existing extraction methods.

Crude alkaloidal extracts were typically purified by liquid–liquid partitioning of their basic forms in organic medium (chloroform or toluene) [12,19]. But this process is rather time- and solvent-consuming with poor efficiency. On the other hand, solid-phase extraction (SPE) on C_{18} RP cartridges was sometimes used [20]. However, methanolic–water extracts thereafter obtained, contain usually polar co-extractives, which influence the separation and background absorbance in HPLC. For these reasons, in the procedure presented below, alkaloids were introduced as protonated molecules onto Oasis mixed-mode cation-exchanger (MCX) cartridges, and after elution of co-extractive phenolics, the alkaloids were extracted as free bases at basic medium (chloroform–10% ammonia). Such the procedure enabled recovering of pure fractions, which could be further analysed by chromatographic methods.

Analysis of complex mixtures of the *Amaryllidaceae* alkaloids was the most often performed by HPLC methods on RP C_{18} column without [21] or with [22] ion-pair reagents to improve a peak shape, as well as on silica gel 60 stationary phase in NP mode [10]. GC methods were also used [23,24], however there are usually not suited for *N*-oxides analysis. In the present paper complex of the alkaloids was efficiently separated and analysed on a *charge-transfer* RP C_{18} sorbent with a mobile phase elaborating at high pH (9.5), therefore omitting ion-pair reagent additives. High separation efficiency, relative high sensitivities in diode-array detection system and high precision of both qualitative and quantitative (for *intra-* and *inter-*day) results were the main detectable advantages of the elaborated HPLC procedure. Also, to achieve the highest standards of the quantitative results, additionally, quantitative HPTLC with densitometry on silica gel plates was evaluated and validated. Both methods were well correlated each other with correlation coefficients of their mean values higher than 0.98.

While PLE is well established for analytical sample preparation, the ability of the technique to preserve biological activity of samples is not as understood. Therefore, TLC with bioautography was applied for evaluating of anticholinesterese activity of the components in the extracts. An influence of the PLE parameters (e.g. type of solvent) on the extraction of potential acetylcholinesterase inhibitors could be checked.

2. Experimental

2.1. Chemicals, reagents and plant material

The standards of galanthamine hydrobromide, hippeastrine hydrobromide and lycorine hydrochloride were obtained from Latoxan (Valence, France). Oasis MCX extraction cartridges (60 mg, d_p = 60 μm, 3 ml) and Waters XTerra C_{18} (4.6 mm × 150 mm, d_p = 3.5 μm) HPLC column were purchased from Waters (Milford, MA, USA). Acetylthiocholine iodide (ATCI), acetylcholinesterase from *Electrophorus electricus* (lyophilized powder type VI-S, 200–600 U mg^{-1} protein), 2-naphtyl acetate, Fast Blue B Salt, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), trizma hydrochloride and albumin from bovine serum (BSA) were from Sigma–Aldrich (St. Louis, MO, USA). HPTLC silica gel 60 F_{254} (20 cm × 10 cm, 0.25 mm thickness) coated plates and silica gel 60 F_{254} aluminium sheets (0.2 mm thickness) were bought in Merck (Darmstadt, Germany). Methanol, acetonitrile, tetrahydrofurane and 25% ammonia water solution (each solvent was of HPLC gradient grade) were obtained from J.T. Baker (Gross-Gerau, Germany). Chloroform, toluene, tartaric acid, ethyl acetate, 30% hydrochloric acid (each one of analytical grade) were from The Polish Reagents (POCh, Gliwice, Poland). Double-distilled water was obtained from Simplicity Ultrapure Water System (Millipore, NYSE, MIL). In SPE recoveries a mixture of three standards (galanthamine hydrobromide, hippeastrine hydrobromide and lycorine hydrochloride) in tetrahydrofurane–methanol–water (12:10:3, v/v/v) solution at 0.2052, 0.2148 and 0.1952 mg ml^{-1} concentrations, respectively, was used. The investigated plant materials: bulbs of *Narcissus jonquilla* 'Pipit', *Narcissus* c.v. 'Hawera' and *Narcissus* c.v. 'Tete a tete' containing suitable authenticity certificates were purchased from Florexpol (Lublin, Poland). Voucher specimens of the plant samples are deposited at the Medicinal Plants Laboratory Unit at the Department of Pharmacognosy, Medical University of Lublin, Poland.

2.2. Pressurized liquid extraction

2.2.1. Solvent optimisation

Some of 0.5 g samples (in triplicate) of plant material (*Narcissus jonquilla* 'Pipit'), stirred with an neutral material (ground glass) were extracted in a 22-ml extraction cells at default conditions (i.e. 120 °C, p = 60 bar, 1 static cycle, t = 10 min) subsequently with pure methanol (MeOH), 1% tartaric acid methanolic solution (TarMeOH), water (H_2O), 1% tartaric acid water solution ($TarH_2O$), toluene (Toluene) and ethyl acetate (EtOAc) by the use of Dionex 200 ASE apparatus (Dionex Corp., Sunnyvale, CA). After the extraction was done, the sample cell was flushed out with 60% volume of the extraction solvent and thereafter purged with nitrogen during 120 s. Obtained extracts (about 30 ml) were transferred into 50-ml volumetric flasks and diluted to the mark with the solvent for further SPE purification.

2.2.2. A temperature influence

The efficiencies of the extraction were evaluated for two solvent systems: MeOH and TarMeOH in the temperature ranges from 20 till 200 °C in similar way as it was described in Section 2.2.1.

2.3. Cation-exchange solid-phase extraction (CEx-SPE)

2.3.1. Method optimisation

In 50-ml round bottom flasks 1.0-ml, 0.8-ml or 1.2-ml aliquots of the mixture of three standards in tetrahydrofurane–methanol–water (Section 2.1) were placed together with 10-ml aliquots of the crude extracts from *Narcissus* c.v. 'Hawera' or *Narcissus* c.v. 'Tete a tete' and evaporated to the dryness under reduced pressure at 50 °C. Dry residues were dissolved in 6 ml of 0.05 mol l^{-1} hydrochloric acid (HCl) and elaborated thereafter. Two fortified samples were analysed simultaneously (it means that 2 SPE cartridges were utilized simultaneously for cleaning up of 2 fortified plant extracts. Using the SPE device up to 14 cartridges could be elaborated simultaneously). In SPE, a cartridge (Oasis MCX) was at first conditioned with 5 ml of 0.05 mol l^{-1} HCl under small vacuum (5 mm Hg) and a flow rate of 1 drop/2 s. About 2 mm layer of the solvent had to remain