

TLC densitometric method for screening of lycopsamine in comfrey root (*Symphytum officinale* L.) extracts using retrorsine as a reference compound

DAMJAN JANEŠ^{*a}
SAMO KREFT

Faculty of Pharmacy
University of Ljubljana
SI-1000 Ljubljana, Slovenia

Due to severe toxicity of pyrrolizidine alkaloids, their quantification in medicinal products is very important. The idea of this research was to use retrorsine as a surrogate reference compound instead of lycopsamine reference or lycopsamine isolated from comfrey. A method for the analysis of lycopsamine in extracts of comfrey roots was developed and validated, employing thin layer chromatography, derivatisation with Dann-Mattocks reagent followed by densitometric analysis. The new method showed linearity within 0.70 to 7.0 µg of lycopsamine per application of 10 µL of a solution. It has also been proven to be specific and precise (repeatability RSD 2–4 % within the plate). The method was successfully employed for quantification of lycopsamine in comfrey root and comfrey root medicinal products such as ointments.

Keywords: comfrey, lycopsamine, retrorsine, TLC, densitometry

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Extracts of comfrey roots (*Symphytum officinale* L., *Boraginaceae*) have been used topically for treating inflammatory disorders and internally for treating diarrhoea in Europe (1, 2). Apart from therapeutically effective allantoin and other active constituents, comfrey also contains toxic pyrrolizidine alkaloids, which are most frequently represented by lycopsamine, intermedine, their acetylated derivatives and symphitine. Pyrrolizidine alkaloids are metabolised in humans to hepatotoxic and pulmotoxic pyrrole derivatives (2–4) in a reaction catalysed by CYP 3A4 (5, 6). Because of their high toxicity, it is of interest to determine these alkaloids in medicinal products even at low concentrations (7). Several analytical methods have been used for the analysis of pyrrolizidine alkaloids in comfrey, employing thin layer chromatography (TLC) (8, 9), high performance liquid chromatography (HPLC) (10–15) and gas chromatography (GC) (4, 7, 12, 16–18).

* Correspondence; e-mail: damjan.janes@ffa.uni-lj.si

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Unfortunately, many reference compounds of pyrrolizidine alkaloids are not commercially available and should therefore be isolated individually from natural materials by expensive and time consuming procedures (16).

The aim of this work was to develop an analytical method to overcome the availability problem of pyrrolizidine alkaloids reference compounds and to avoid their isolation by employing readily commercially available alkaloid retrorsine as a surrogate reference compound for lycopsamine, which is actually present in the plant.

EXPERIMENTAL

Plant material, medicinal products and reagents

Comfrey roots were supplied by Farmex (Slovenia). The country of origin of plant material was Hungary. The identity of dry and comminuted comfrey roots was confirmed by morphological analysis (19). A voucher specimen has been deposited at the Faculty of Pharmacy in Ljubljana, Slovenia. Comfrey root ointment (Favn, Slovenia) was purchased from a local pharmacy. Retrorsine reference compound (97 % purity according to the certificate of analysis), solvents and reagents were purchased from Sigma-Aldrich (Germany). TLC silica gel 60 plates (20 × 10 cm, layer thickness 100 μm) were from Merck (Germany). Lycopsamine was isolated from comfrey roots according to the published method (20) by an improved ion-exchange chromatography method employing non-aqueous solutions, followed by preparative HPLC. It was identified by MS, IR and NMR spectra and the purity was 95 % according to the relative area in the chromatogram.

Instruments

The following equipment was used: laboratory blender (Waring Laboratory, USA), ultrasonic bath Sonorex Digitec DT 103 H (Bandelin, Germany), centrifuge 5804 R (Eppendorf, Germany), Camag chromatogram immersion device (Camag, Switzerland), TLC plate heater III, twin trough chamber, 20 × 10 cm, Linomat IV, TLC scanner 3, Reprostar 3 (Camag).

Preparation of comfrey root extracts

Five grams of finely ground comfrey root or 5 g of ointment were extracted with 10 mL of methanol for 30 minutes in an ultrasonic bath at room temperature. The sample was then centrifuged 10 min at 2935xg and clear solution was used for analysis. The procedure was repeated to check the extraction efficiency. Selection of the extraction method was based on published data (21) and modified to facilitate the extraction procedure.

Solutions of reference compounds

Solutions of retrorsine commercial compound (1.00 and 0.100 mg mL⁻¹) and lycopsamine trifluoroacetate (0.946 and 0.0946 mg mL⁻¹, corresponding to 0.684 and 0.0684 mg mL⁻¹ lycopsamine, respectively) were prepared by dissolving them in methanol and subsequently diluting with methanol.

Thin layer chromatography

Samples were applied to TLC plates using a Linomat IV at a speed of $5 \mu\text{L s}^{-1}$ in the form of 10-mm bands. Applied volumes were from 3 to 10 μL and the respective masses of both were 3.0, 5.0, 7.0 and 10.0 μg . Plates were developed in a mixture of methanol, dichloromethane and diethylamine (26:70:4, V/V/V) in a saturated chamber until the solvent front reached 85 mm. Plates were dried and derivatised with Dann-Mattocks reagent: step 1 – evenly sprayed with 3 mL of 30 % hydrogen peroxide, step 2 – evenly sprayed with 3 mL of a mixture of acetic anhydride, petroleum ether and toluene (1:4:5, V/V/V) and step 3 – dipped in Ehrlich's reagent, consisting of a mixture of 4-(dimethylamino)benzaldehyde, HCl 37 % and ethanol 96 % (0.3:5.4:94.6, m/V/V) using a Camag chromatogram immersion device. At each step, after the application of the reagent, every plate was heated at 120 °C for 20 minutes and then allowed to cool to room temperature in a desiccator.

Densitometry

Derivatised plates were scanned with a Camag TLC Scanner 3 under following conditions: slit dimension 8.00×0.45 mm, optimised optical system for maximal resolution, scanning speed 5 mm s^{-1} , data resolution 100 mm per step, wavelength 550 nm, second order optical filter, automatic detector mode, analog offset 10 %, automatic sensitivity, Savitzky-Golay 9 filter factor and lowest slope baseline correction. Data was processed with the Camag WinCats 1.2.2. programme. Each plate was measured four times.

Method validation

The method was validated through the following analytical parameters: linearity, sensitivity, specificity, precision, accuracy, detection limit and quantitation limit according to ICH-Q2(R1) guidelines (22). Calibration curves for retrorsine and lycopsamine were plotted with five different quantities applied on the plate, from 0.70 to 7.0 μg and from 0.48 to 4.8 μg , respectively. Specificity of the method was determined by the analysis of lycopsamine reference compound and comfrey root extracts. Lycopsamine peak was identified by comparing its retardation factor and UV spectrum with those of the reference compound.

RESULTS AND DISCUSSION

Extraction efficiency

Apart from the alkaloids, allantoin was the main compound extracted (19), but it did not give any colour reaction, since Dann-Mattocks reagent is specific to pyrrolizidine alkaloids (21).

Spectral characteristics

High similarity of the spectra of lycopsamine reference compound peak and the peak with the same retardation factor (0.69) from comfrey sample (Fig. 1a) and that of retrorsine reference compound (Fig. 1b) was observed.

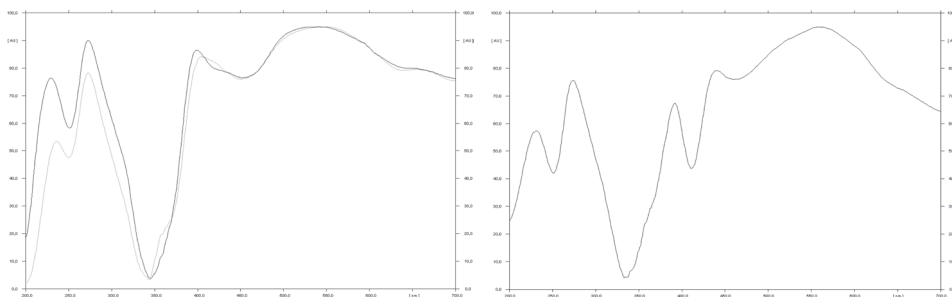


Fig. 1. UV spectra of: a) lycopsamine reference compound (black line) and lycopsamine in comfrey sample (grey line), b) retrorsine reference compound.

Repeatability within the TLC plate

Repeatability within the TLC plate was assessed by seven replicate analyses of standard solutions of the analytes (5.0 μg of retrorsine, 4.3 μg of lycopsamine). RSD of the peak area was found to be 4.0 % for retrorsine and 4.2 % for lycopsamine. Retardation factors of retrorsine and lycopsamine were 0.71 (RSD 4.7 %) and 0.69 (RSD 4.0 %), respectively.

Linearity and sensitivity

Response of the detector was linear in the range 0.70–7.0 μg for retrorsine and 0.48–4.8 μg for lycopsamine, with respective R^2 values of 0.999 and 0.991. For limits of detection (LOD) and quantitation (LOQ), a signal-to-noise ratio (S/N) of 3 and 10 was considered, respectively. The LOD for lycopsamine was 0.22 μg per application of 10 μL and the LOQ for the same analyte was 0.73 μg per application of 10 μL of solution, indicating sufficient sensitivity for lycopsamine quantification.

Precision and accuracy

For retrorsine (0.50–5.00 μg) and lycopsamine (0.34–3.42 μg), the intra- and inter-day RSDs ranged from 7–18 and 9–27 %, respectively. High imprecision is a common problem in TLC as an open system. When the retrorsine reference compound was applied to the same plate as the comfrey sample, RSD dropped to 2.8–4.0 %. Relative response factor for lycopsamine/retrorsine was calculated from the calibration curves and was found to be 0.98.

Since the signals of lycopsamine and retrorsine are not completely separated and are not additive, retrorsine cannot be used as an internal reference standard. It was, however, successfully used as an external surrogate reference compound.

Analysis of lycopsamine

The presence of lycopsamine in comfrey roots and ointment was confirmed by comparison of its retardation factor and overlaying of UV spectra with those of the reference

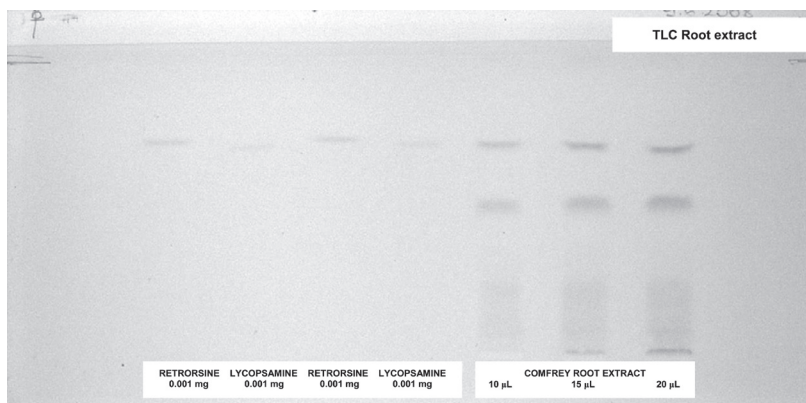


Fig. 2. Typical TLC chromatogram of retrorsine, lycopsamine and comfrey root extract.

compound. Quantification of lycopsamine was done using retrorsine as an external surrogate reference compound as well lycopsamine reference compound and was selective (see typical TLC chromatogram in Fig. 2). The concentration of lycopsamine in comfrey roots was found to be 710 mg kg^{-1} , which is in accord with literature data (2, 3). Lycopsamine in comfrey root ointment was found in a concentration of 4.6 mg kg^{-1} .

CONCLUSIONS

A simple TLC method for lycopsamine screening in comfrey roots and common comfrey medicinal products such as ointments was developed. The method is simple and fast and uses an external surrogate reference compound (retrorsine) instead of the generally unavailable and expensive lycopsamine. Several factors indicated suitability of retrorsine as a surrogate reference compound in the same range of concentrations as lycopsamine: comparison of UV spectra of high similarity, close retardation factors, very similar responses of derivatised alkaloids. However, due to its low precision, the method is suitable only for preliminary estimation of lycopsamine.

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