

TLC-densitometric analysis of allantoin in *Symphytum officinale* L. roots

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A TLC-densitometric method for determination of allantoin in *Symphytum officinale* root was developed. Densitometric quantification of allantoin was carried out on TLC Si60 plates with butanol-50 % methanol/formic acid, 66.5:33.2:0.3 (V/V/V) as developing solvent, at a wavelength of 190 nm. The method was preliminarily validated in terms of specificity, linearity, precision, limit of detection, limit of quantification, recovery and robustness. The results of TLC quantification were compared with HPLC analysis carried out on a HILIC Luna NH₂ 100A column, with mobile phase consisting of acetonitrile/water 80:20 (V/V) and UV detection at 190 and 210 nm. Allantoin content was determined in two herbal products and it varied from 0.94 to 2.09 %, depending on the producer, and was in agreement with literature reports.

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Medical use of *Symphytum officinale* root (comfrey root) has a long-lasting tradition as a herbal medicine owing to its anti-inflammatory and analgesic properties. It has been used as a topical remedy for bone fractures, muscles and joint inflammations, distortions, blunt injuries and wounds, rheumatic disorders (1), as well as to treat gastritis, gastroduodenal ulcers, bronchitis and various allergies if taken *per os*. Nowadays, however, its oral use is not recommended due to the presence of hepatotoxic pyrrolizidine alkaloids (1).

Although its molecular mechanism of action is still unclear, the efficacy of comfrey root in topical treatment of pain, inflammation and swelling of muscles and joints has been confirmed in several clinical trials (2). Allantoin and rosmarinic acid are probably the main active compounds in roots (3).

Its biologically active constituents include allantoin (0.6–4.7 %), mucopolysaccharides composed of glucose and fructose units (about 30 %), phenolic acids (such as rosmarinic acid, chlorogenic acid, caffeic acid), tannins (4–6 %), triterpenoid saponins, glycopeptide consisting of 16 amino acids and toxic pyrrolizidine alkaloids (0.04–0.6 %) (1, 2).

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Pharmacological activities of allantoin include wound healing, anti-irritating, hydrating, stimulating the cell mitosis, removing the necrotic tissue, as well as analgesic action, keratolytic activity and promoting epithelization. It is suggested that the wound healing mechanism occurs *via* the modulation of inflammatory response, which leads to prevention of oxidative stress, as well as by stimulating the fibroblastic proliferation and extracellular matrix synthesis (4).

Several chromatographic methods have been developed for determination of allantoin in biological material, while only some of them refer to its quantification in complex plant material. Thus far, the analysis of allantoin in plant matrix was only performed by HPLC in *Zea mays* and rice (5–7) and high-performance capillary electrophoresis (HPCE) in comfrey root (8). Due to their simplicity, relatively low cost and short time of analysis, TLC methods are widely used for the separation of secondary metabolites in plant extracts. However, TLC-densitometric methods have so far been used only for quantitative analysis of allantoin in drug preparations (9) and bovine urine (10). When it comes to phytochemical analysis of allantoin, its only application is limited to qualitative analysis (11).

The aim of this work was to establish a fast and cheap TLC method for quantitative analysis of allantoin in the roots of *Symphytum officinale*.

EXPERIMENTAL

Materials

Methanol, *n*-butanol, hydrochloric acid and formic acid were obtained from P.O.Ch. (Poland). Ethanol was purchased from Polmos Lublin (Poland).

p-Dimethylaminobenzaldehyde of analytical-grade and the allantoin standard (according to *Eur. Pharm.*) were obtained from Merck KgaA (Germany).

Commercially available dried roots of *Symphytum officinale* were purchased from two different Polish sources (supplier 1 and supplier 2).

Optimization of allantoin extraction and sample preparation

Two methods of extraction were tested in preliminary studies: ultrasonic extraction (15 and 30 min) and solid-liquid extraction at elevated temperature under reflux (100 °C, 15 min). Based on the literature data suggesting solvent-sample ratio 10:1, 8–12 mL of solvent was used per 1 g of powdered plant material (9). Methanol, ethanol and their water dilutions (50 % methanol, 60 % ethanol) were used as solvents.

Finally, dried and pulverized plant material (1 g) was extracted under reflux with 95 % ethanol or methanol (25 mL, 15 min). The obtained extracts were filtered by filter paper (Whatman 1) and diluted to 25 mL with the same solvent.

Optimization of TLC separation

Several types of adsorbents and TLC plates were tested, namely, TLC Si60, HPTLC Si60 F₂₅₄ and TLC RP-18 W₂₅₄. Three different developing solvents described in the literature were tested: methyl ethyl ketone/acetone/formic acid/water 81.6:4.1:2:12.2 (V/V/V/V) (9,

11), methanol/formic acid/water 66:5.7:28.3 (V/V/V) and butanol/50 % methanol (75:25, V/V), though, as the result of performed modifications and optimization, the final developing solvent consisted of butanol-50 %/methanol/formic acid 66.5:33.2:0.3 (V/V/V). After development, the chromatograms were derivatized by spraying with *p*-dimethylaminobenzaldehyde (0.25 g dissolved in the mixture MeOH/37 % HCl, 76:24 (V/V, 50 mL) and heated at 100 °C for 5 min on a Thermoplate S plate heater (Desaga, Germany). Allantoin was observed as a light yellow zone (12).

Thin-layer chromatography

TLC analysis was performed on 20 × 10 cm (20 × 20 cm cut into two pieces) glass plates coated with Si60 silica gel (Merck). Allantoin standard and herbal sample solutions were applied to the plates as 5-mm bands, 10 mm from the bottom edge of the plate, using a Desaga AS-30 sample applicator. The application volume was 5 µL and the time for its application was 15 s. Plates were developed to a distance of 7.5 cm at room temperature and 47 % humidity in a Camag (Switzerland) ADC2 automated development chamber. Adjustment of humidity was carried out with potassium thiocyanate solution for 15 min, the preconditioning time was 2 min and the drying time 8 min. For qualitative analysis, the chromatograms were derivatized after development.

For quantitative analysis performed on TLC Si60 plates, densitograms were scanned without chromatogram derivatization, directly after the development and drying. Densitograms were obtained using a Desaga CD-60 densitometer at 190 nm. Linear scans were obtained with slit dimensions of 0.4 × 4.0 mm. The calibration plot was constructed by plotting the peak area against standard concentration. Differences in calibration curves in repeated experiments were eliminated in such a way that the standard solution was applied next to analysed samples in each TLC analysis and the new calibration curve was plotted for each separation.

For quantitative analysis, the allantoin standard (1 mg) was dissolved in 50 % methanol (1 mL). Calibration function for quantitative analysis was constructed using diluted standard solutions of allantoin to get 210–5000 ng per spot.

Method validation

Validation parameters were estimated according to ICH rules (13). They included specificity, precision, limits of detection (*LOD*) and quantification (*LOQ*), linearity, accuracy and robustness (Tables I, II and III).

Specificity was checked by performing two-dimensional separation of 5 µL of extract on TLC Si60 plates in the presence of the reference substance (1 µL). The chromatogram was developed in both dimensions with butanol/50 % methanol/formic acid 66.5:33.2:0.3 (V/V/V). Allantoin spot was visualized by *p*-dimethylaminobenzaldehyde (5 min, 100 °C).

The method was validated for precision by repeated scanning of the spot of allantoin standard (*n* = 7) at 190 nm (Table I). Intra-day precision was studied by analyzing three spots of analysed plant material per plate on three TLC plates for one day. Inter-day precision was tested by the same analyst and repeated on three consecutive days (Table I). Robustness of the method was tested by small variations in the developing solvent composition and changes in the development distance (Table II).

Table I. Some analytical performances of the proposed TLC-densitometric method

Validation data	Detection, $\lambda = 190$ nm
Specificity ($hR_f = R_f \times 100$)	46
Linear regression parameters (range: 210–5000 ng per spot) ^a	$y = ax + b$ $a = 8.4678 \pm 0.4033^b$ $b = -404.6982 \pm 119.9879^b$ $R^2 = 0.9971$
LOD (ng)	70
LOQ (ng)	210
Instrumental precision, RSD (%) ($n = 7$)	2.3 (1000 ng per spot of allantoin) 3.7 (4000 ng per spot of allantoin)
Repeatability, RSD (%)	5.2 (root extract) 3.0 (standard)
Intra-day precision, RSD (%)	7.7 (root extract)
Inter-day precision, RSD (%)	6.1 (root extract)

^a Seven concentration levels.^b Mean \pm SD, $n = 24$.

LOD – limit of detection, LOQ – limit of quantitation

Table II. Robustness testing of the videodensitometric TLC method

Factor	Level of changes	R_f variability (RSD, %)
Developing solvent composition (butanol/50 % methanol/formic acid (V/V/V))	59.8:39.9:0.3	5.2
	66.5:33.3:0.2	2.2
	66.4:33.2:0.3	2.2
Development distance (cm)	6.5	8.9
	8.5	7.2

Limits of detection (LOD) and quantification (LOQ) were determined by the analysis of allantoin standard solution (50–5000 ng per spot) using as criterion the signal-to-noise ratio 3:1 and 10:1, resp. (Table I).

The accuracy of the method was established by performing a recovery study by the standard addition method. Allantoin standard was added to the extracts from the roots of *Symphytum officinale* at two levels – 25 and 50 % of analyzed content in the sample (*i.e.*, 2.2 and 4.4 mg of allantoin for 1 g of raw material, res.) and each was analyzed as described above. The results are given in Table III.

Table III. Allantoin recovery by the proposed TLC method

Extract	Amount in sample (mg)	Amount added (mg)	Recovery (%) ^a
EtOH (<i>n</i> = 6)	8.9	4.4	93.1 ± 1.2
MeOH (<i>n</i> = 3)			86.8 ± 4.0
EtOH (<i>n</i> = 3)		2.2	104.5 ± 2.3
MeOH (<i>n</i> = 3)			100.3 ± 1.4

^a Mean ± SD.Table IV. Allantoin content in the extracts from *Symphytum officinale* roots of a different origin

Analysed extract/producer	Allantoin content in raw material (%)		
	TLC ^a	HPLC (190 nm) ^b	HPLC (210 nm) ^b
EtOH extract/supplier 1	0.94 ± 0.12	1.06 ± 0.05	1.05 ± 0.05
MeOH extract/supplier 1	0.95 ± 0.10		
EtOH extract/supplier 2	2.09 ± 0.15	1.84 ± 0.11*	1.84 ± 0.11*
MeOH extract/supplier 2	2.10 ± 0.15		

Mean ± SD, ^a *n* = 15, ^b *n* = 3.Statistically significant difference relative to TLC: * *p* < 0.05.

HPLC

HPLC analysis was performed using an LC system by Shimadzu (Japan) consisting of two pumps LC-20AD, a semi-micro mixer, CBM-20A system controller, CT0-20AC column thermostat, SIL 20AC_{XR} autosampler and UV-Vis detector (diode array detector) SPD-M20A. Data was acquired and processed by LabSolution software.

HPLC was performed on a HILIC Luna NH₂ 100A (15 cm × 1 mm, 3 μm) column (Phenomenex, USA), under isocratic elution with an acetonitrile/water 80:20 (V/V) mixture as mobile phase, temperature was 20 °C, injection volume 0.1 μL, flow rate 0.1 mL min⁻¹; UV detection was performed at 210 and 190 nm.

RESULTS AND DISCUSSION

Extraction

Comparing two extraction techniques, it was observed that ultrasonic extraction of comfrey root resulted in high viscosity of the extract, caused by the extraction of mucous polysaccharides, which impeded filtration and application onto TLC plates. The same problem occurred while using 50 % methanol and 60 % ethanol as extracting solvents. As

a result, solid-liquid extraction (60 °C, 15 min, under reflux) with methanol or ethanol as solvent was chosen as most effective. In further optimization, the amount of the solvent was increased from 10:1 to 25:1 ratio to reduce extract viscosity.

TLC separation conditions

In preliminary studies, TLC separation of the extract from analyzed plant material was performed on TLC silica gel 60 plates with the developing solvent methyl ethyl ketone/acetone/formic acid/water 81.6:4.1:2:12.2 (V/V/V/V) and resulted in the presence of an un-separated grey-colored band of unidentified compound(s) in the allantoin band.

The same developing solvent was used for determination of allantoin in *Symphytum asperum* roots (11) or in drug preparations (9) by TLC. However, the attempt to adapt it for quantitative analysis of allantoin in *Symphytum officinale* roots, using the recommended derivatization agent [solution of *p*-dimethylaminobenzaldehyde in methanol and 37 % hydrochloric acid (12)], resulted in wide and blurred yellow spots of allantoin overlapped with grey-colored spots of another compound. Replacement of TLC Si60 with HPTLC Si60 gave similar results. Changing the proportions of the developing solvent to 43:2:1:4 and later 42.8:2:1.5:4 and repeating the analysis on HPTLC Si60, F₂₅₄ plates did not improve the efficacy of separation. TLC RP-18 W₂₅₄ plates in the reverse phase system, with a mixture of methanol/formic acid/water 66:5.7:28.3 (V/V/V) were also tested, but no separation was achieved. While the obtained separation could be effective enough for detection of allantoin, like in the studies of Barbakadze *et al.* (11), it was not suitable for the planned densitometric analysis. In order to obtain acceptable separation and resolution of the allantoin band, different developing solvents were tested.

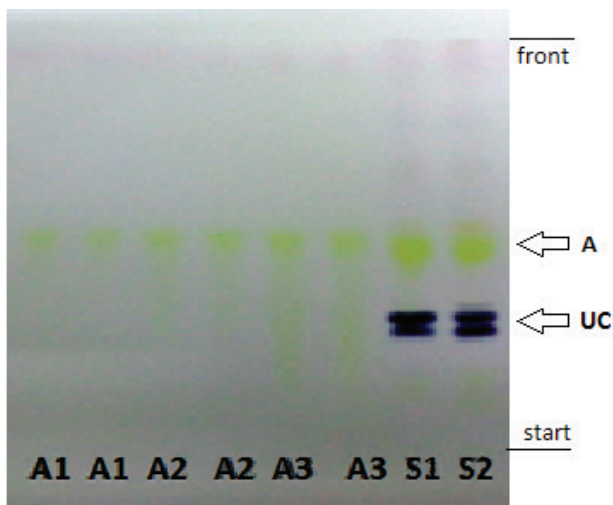


Fig. 1. Video-densitogram of TLC separation of ethanolic extracts from comfrey roots of a different origin (S1, S2) and allantoin standard solution (1 mg mL⁻¹): A1 – 5 µL, A2 – 10 µL, A3 – 20 µL; S1 – extract from supplier 1 sample; S2 – extract from supplier 2 sample; A – allantoin band; UC – unidentified compound. For details see Experimental.

The best resolution of the allantoin band was observed on TLC Si60 plates with butanol/50 % methanol (3:1, V/V). To improve the efficacy of separation, the composition of the developing solvent was modified by changing the proportions of solvents [1:1, 1:5, 2:1 (V/V)] and addition of different volumes of formic acid (0.16, 0.25, 0.5, 0.6 or 1 mL) to 60 mL of the butanol-50 % methanol mixture. As a result, the developing solvent butanol-50 % methanol-formic acid 66.5:33.2:0.3 (V/V/V) was chosen as the most effective in enabling the separation on TLC Si60 plates (Fig. 1).

Densitometric analysis

Densitograms were primarily obtained after derivatization. However, a rapid decrease in peak intensity was observed, due to the instability of color. To improve method repeatability, the chromatograms had to be scanned immediately after development and drying, without derivatization.

Although the literature mentions densitometric analysis of allantoin at 254 nm (10), a multiscan of the allantoin standard band in the range of 190–270 nm was performed. The highest peak areas were obtained in the range 190–210 nm, though at 210 nm the allantoin peak area of the sample extract was much higher than that of the allantoin standard solution, which may indicate the presence of interfering compound(s) in the allantoin band. As a result, the wavelength of 190 nm was chosen (Fig. 2), since it was proven to give better results than 254 nm, which was used in allantoin analysis in cow urine by Vlassa *et al.* (10).

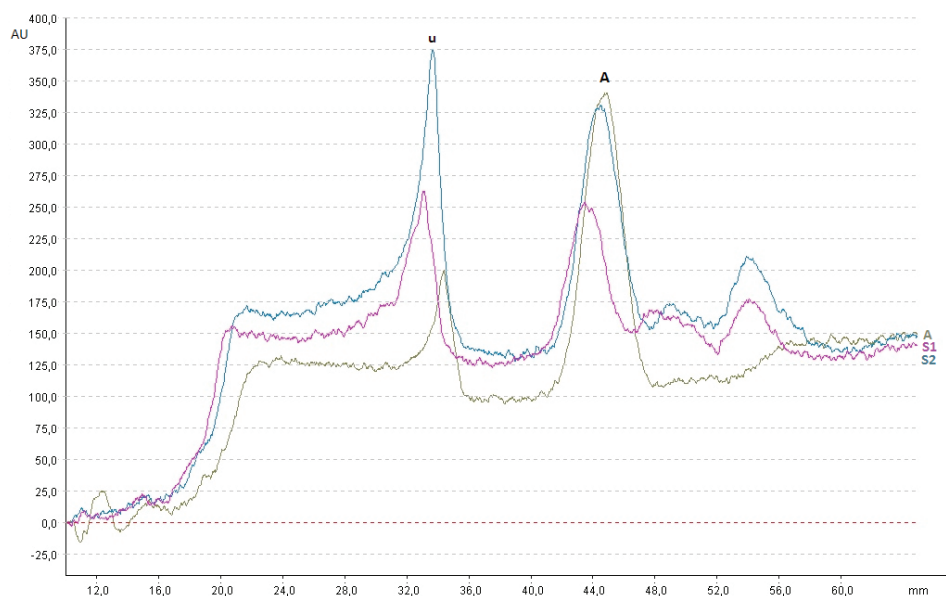


Fig. 2. Densitograms of the analyzed ethanolic extracts from comfrey roots of a different origin and allantoin: A – allantoin, S1 – extract from supplier 1 sample, S2 – extract from supplier 2 sample. For details see Experimental. Key to the peaks: A – allantoin, U – unidentified peak.

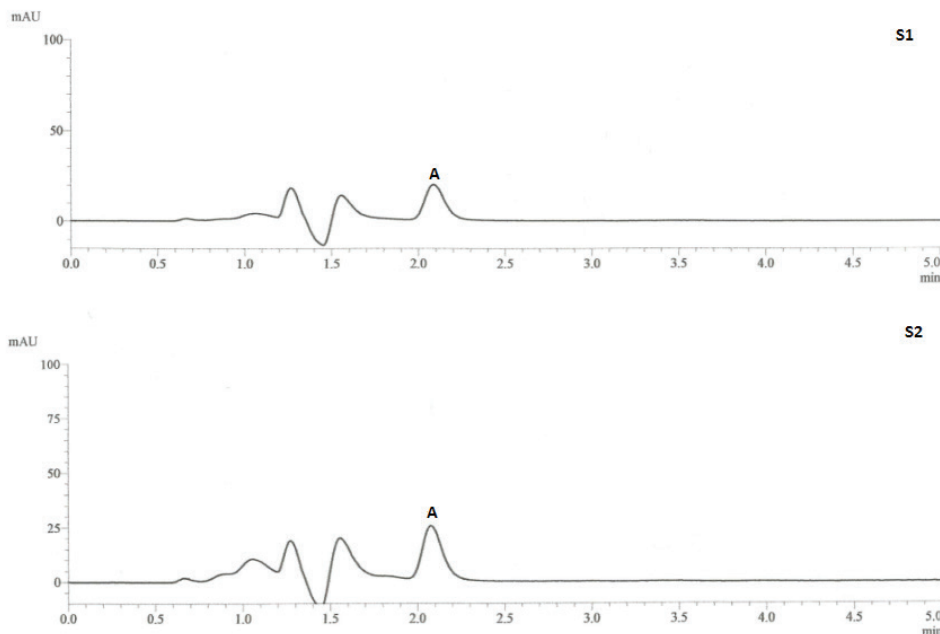


Fig. 3. HPLC chromatograms of the ethanolic extracts from analyzed comfrey roots of a different origin: S1 – supplier 1 sample, $\lambda = 210$ nm; S2 – supplier 2 sample, $\lambda = 190$ nm. For details see Experimental. Key to the peaks: A – allantoin.

This is probably due to the difference in composition of analyzed samples and the TLC plates used (silica gel $-\text{NH}_2$, $F_{254\text{nm}}$).

TLC method validation

Linearity of the method was good, with the correlation coefficient of $R^2 > 0.9971$. The method was specific, precise and reproducible (Table I). Lower recovery in samples with addition of 50 % of the standard was probably caused by low solubility of allantoin in 95 % ethanol and methanol. According to *Polish Pharmacopeia IX* (12), for 1 g of allantoin, 1000–10000 g of solvent are needed. Addition of 4.4 mg of allantoin probably exceeds its solubility in alcohols (Table III). It was observed that allantoin was fairly soluble in 50 % methanol, although it could not be used for extraction of the samples due to co-extraction of mucous polysaccharides and increased viscosity of extracts.

HPLC separation conditions

Simultaneously with TLC analysis, HPLC analysis of ethanolic extracts from the samples was performed. Separation was initially carried out on a Discovery HS C18 column with the eluent consisting of a buffer solution of $(\text{NH}_4)_2\text{HPO}_4$ 0.05 mol L^{-1} , pH 7.78, following the parameters used by Vlassa *et al.* (10). Due to insufficient separation of the allantoin

peak from interfering compounds, the method was optimized on the HILIC Luna NH₂ 100A column with eluent consisting of acetonitrile/water 80:20 (V/V) (14); flow rate 0.1 mL min⁻¹, UV detection at 190 and 210 nm (Fig. 3). Similar conditions had been previously used for determination of allantoin in cosmetic samples by Kajimura *et al.* (COSMOSIL HILIC triazol-bonded silica column, eluent consisting of acetonitrile/water 90:10, UV detection at 210 nm) (14).

Validation of the HPLC method and quantification

The HPLC method was validated in terms of specificity, linearity and limit of quantification (LOQ). Calibration plot was constructed by plotting the peak area against the standard concentration. Results from linear regression, from the calibration plot constructed by plotting the peak area against the standard concentration, were: $y = 0.00105x + 0.263$ ($R^2 > 0.999$, LOQ 40 µg mL⁻¹) at 190 nm and $y = 0.0035x + 4.061$ ($R^2 > 0.999$, LOQ 35 µg mL⁻¹) at 210 nm.

The presence of allantoin was determined in both analyzed herbal products. The evaluated concentration of allantoin in *Symphytum officinale* roots from supplier 1 was 0.94 % and twice as high in the supplier 2 product, 2.09 % (Table IV). This is in agreement with the literature data reporting the content of allantoin in comfrey roots in the range of 0.6–4.7 % (2). Preliminary comparison of TLC-densitometric and HPLC data may indicate the possible replacement of HPLC by TLC-densitometry for allantoin analysis in *Symphytum officinale* roots. However, more experimental work is necessary to prove that TLC-densitometric and HPLC methods are interchangeable.

CONCLUSIONS

The established TLC-densitometric method may possibly be applied for preliminary screening and quantitative evaluation of allantoin in comfrey roots and other plant materials, for example in comfrey leaves.

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