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A sensitive TLC method to identify Echinaceae pallidae radix

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In this work a fast, simple and sensitive qualitative TLC method was developed to identify Echinaceae pallidae radix and to distinguish this drug from similar ones. The TLC method is based on the lipophilic compounds of *E. pallida*. Three mobile phases provided good separation, e.g. toluene/ethylacetate 7 + 3 (v/v). A marker substance was found which shows a blue fluorescence at an excitation wavelength of 366 nm after detection with a spray agent containing 95 volume parts ethanol 96%, 5 parts trifluoroacetic acid 99% and zinc ions in 0.15 molar concentration. After spraying the chromatogram was heated at 110 °C for 7 min. This method is superior to HPLC methods to characterise mixtures of *Echinacea* extracts in terms of selectivity due to this post-chromatographic derivatisation and subsequent fluorescence detection.

1. Introduction

Preparations of *Echinacea* plant parts are used for preventing and treating cold, influenza like and upper respiratory tract infections. They are also used to increase the general immune system functions. The therapeutic effect has been attributed to polar components such as polysaccharides, glycoproteins and polar caffeic acid derivates as well as to lipophilic ones containing alkylamides and polyacetylenes. Especially polyacetylenes are typical for *E. pallida* (Bauer et al).

In previous examinations of *Echinacea* roots erroneous determinations occurred when *Echinacea* species should be distinguished, especially *E. pallida* and *E. angustifolia*. Object of this study was to find a plant specific standardisation substance (PSSS), which enables to determine *E. pallida* among *E. purpurea*, *E. angustifolia* and other drugs which are often combined to *Echinacea* drugs, such as Baptisiae tinctoriae radix and Thujae occidentalis herba. Chromatographic methods are commonly used for the qualitative and quantitative determination of plant extracts. In this case GC methods are not suitable due to the instability of the compounds under investigation. Preliminary TLC tests using post chromatographic derivatisation showed, that a straightforward, selective, sensitive and fast method should be obtainable as routine analysis. The value of HPLC is restricted due to the very high complexity of the extracts. Whereas sufficient selectivity was in reach for TLC using post chromatographic derivatisation and subsequent fluorescence detection, the same strategy for HPLC would have required a highly sophisticated device and a loss of resolution.

2. Investigations, results and discussion

2.1. Development of the TLC-System

The effects of *Echinacea pallida* extracts are mainly ascribed to lipophilic components (Bauer et al. 1988a, 1989). Among others, ketoalkenines and polyacetylens are of high interest (Remiger 1988; Mueller et al. 1994). Using previously described TLC methods for *Echinacea* species, good separations were achieved (Bauer and Remiger 1989; Bauer et al 1988b). However, the detection reagents used here (e.g. anisaldehyde/sulfuric acid, diphenyl-boric acid-ethanolamine complex) were unselective. Thus various reagents for carbonyl groups and unsaturated hydrocarbons were tested (Jork et al. 1989). Thereby a blue fluorescent band was found, which was object of the following investigations (Schicke 2002).

First different extracting agents were tested concerning total extract amount and relative intensity of the blue fluorescent compound, tested by TLC. Cyclohexane and hexane showed the best results. Cyclohexane showed a nearly complete extraction of the component of interest after only three extraction cycles.

Less capable were xylene, petroleum ether and more polar agents such as methanol or ethanol.

The mobile phase of the TLC-System was improved with regard to the blue fluorescent compound. Adjacent bands had to be separated. Further, the Rf value should be reduced from 0.8 to about 0.5 in order to achieve optimal separation efficiency. Thereby the fluorescent band was divided into two. Three constitutions of the mobile phase are well suited: Using hexane/acetone/toluene at the rate of 7 + 3 + 3 (v/v/v), the blue fluorescent bands had retention factors of 0.48 and 0.53, using toluene/ethylacetate 7 + 3 (v/v/v), the blue fluorescent bands had the retention factors 0.68 and 0.72, and using petroleum ether/toluene/ethylacetate 5 + 1 + 1 (v/v/v), the blue fluorescent bands had retention factors of 0.65 and 0.68.

The separating efficiency as well as the sensitivity were improved by using HPTLC-Plates. Immersion is superior to spraying. It is particularly suitable for quantitation, because the detection agent is more evenly distributed. It also improves sensitivity.

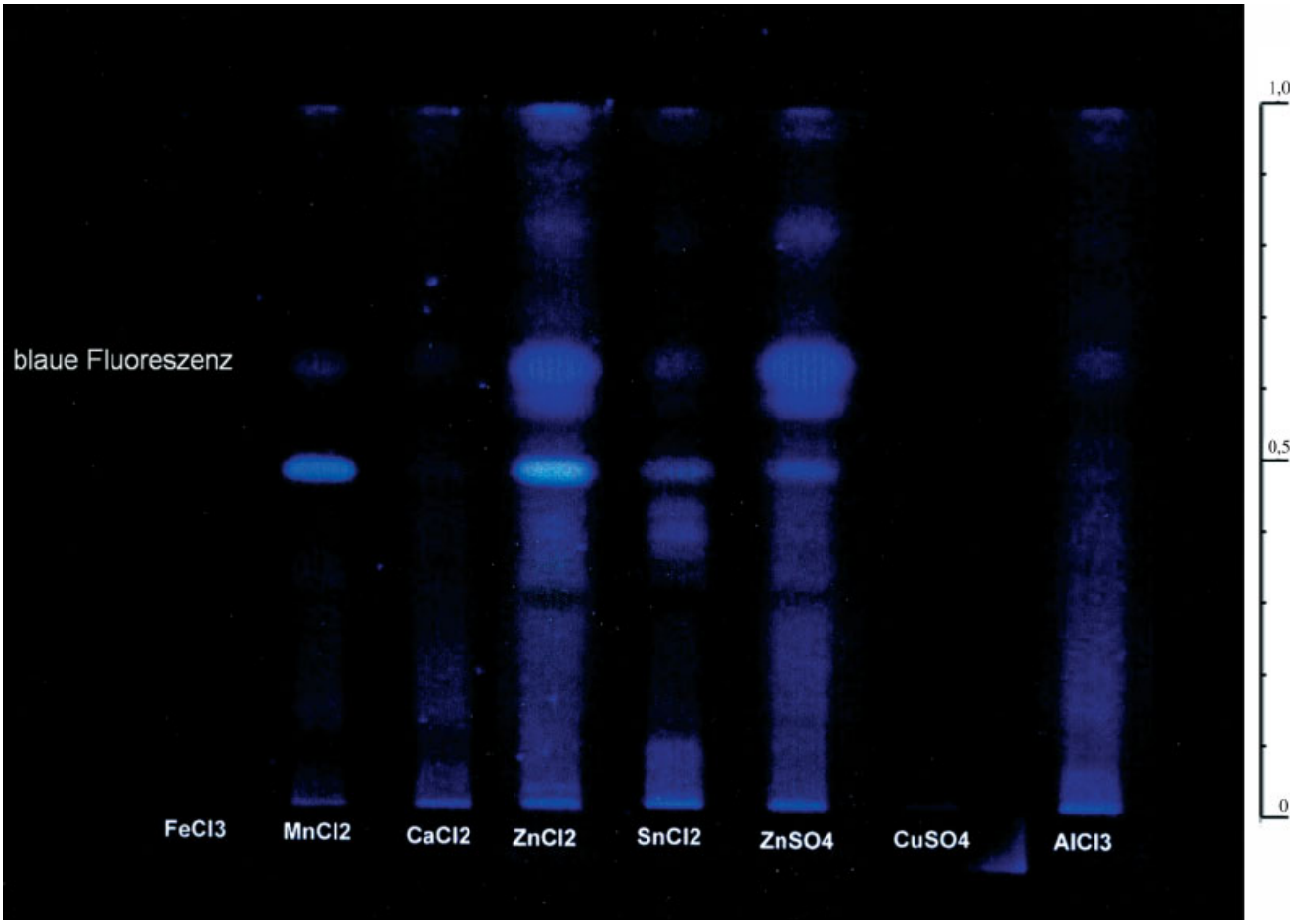

Fig. 1: Different metal ion-containing solutions as detection agent. The blue fluorescent bands only appear in the presence of zinc ions.

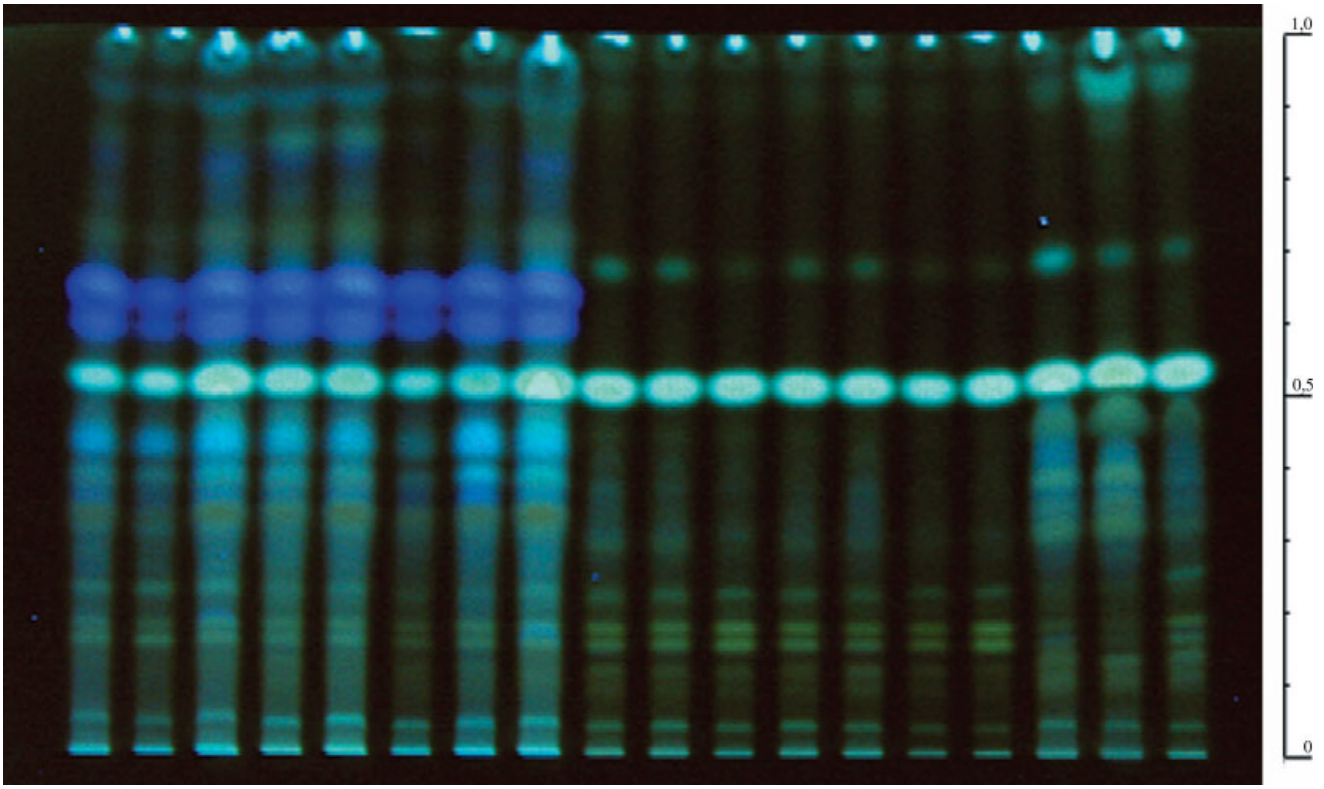

Fig. 2: Selectivity of derivatisation and detection with regard to the blue fluorescent bands from *Echinacea pallida*. 1st 8 lanes: extracts from different *E. pallida* drugs; lanes 9 to 15: extracts from *E. purpurea*; lanes 16 to 18: extracts from *E. angustifolia*. The blue fluorescent bands are visible at every *E. pallida* extract but nowhere else. The light green band in every lane at the retention factor of 0,54 is β-sitosterol.

In the following investigations the detection agent had to be optimised. It was found that the blue fluorescent band only appeared on plates with fluorescence indicator containing manganese-activated zinc silicate. Thereupon the composition of the spray agent was varied and it appeared that zinc ions were necessary to induce the fluorescence. Different metal ions were tested but only zinc ions containing solutions showed the blue fluorescent band on plates without fluorescence indicator (Fig. 1).

Furthermore the presence of an acidic component was essential. Diverse acids were tested, including perchloric acid, sulfuric acid, phosphoric acid, trifluoroacetic acid, hydrochloric acid, formic acid and acetic acid. Best results were achieved by the spray agent containing trifluoroacetic acid. Here the blue fluorescent band of interest was most intensive compared to the other bands. Hydrochloric acid and phosphoric acid were acceptable as well, though other bands were also blue fluorescing.

The next step was to verify the selectivity for *Echinacea pallida*. Fig. 2 shows the chromatogram of diverse extracts from different *Echinacea* roots. The volume in every lane was the same, but the drugs were of different age and from various batches. In lane 1–8 extracts of different *Echinacea pallida* roots are analysed. The blue fluorescent bands were found in every extract. Lanes 9–15 show extracts of *Echinacea purpurea* and lanes 16–18 of *Echinacea angustifolia*. The blue bands are not visible in any of these lanes, even in fivefold higher concentrated extracts these bands do not occur. The selectivity was also verified with regard to Thujae occidentalis herba and Baptisiae tinctoriae radix.

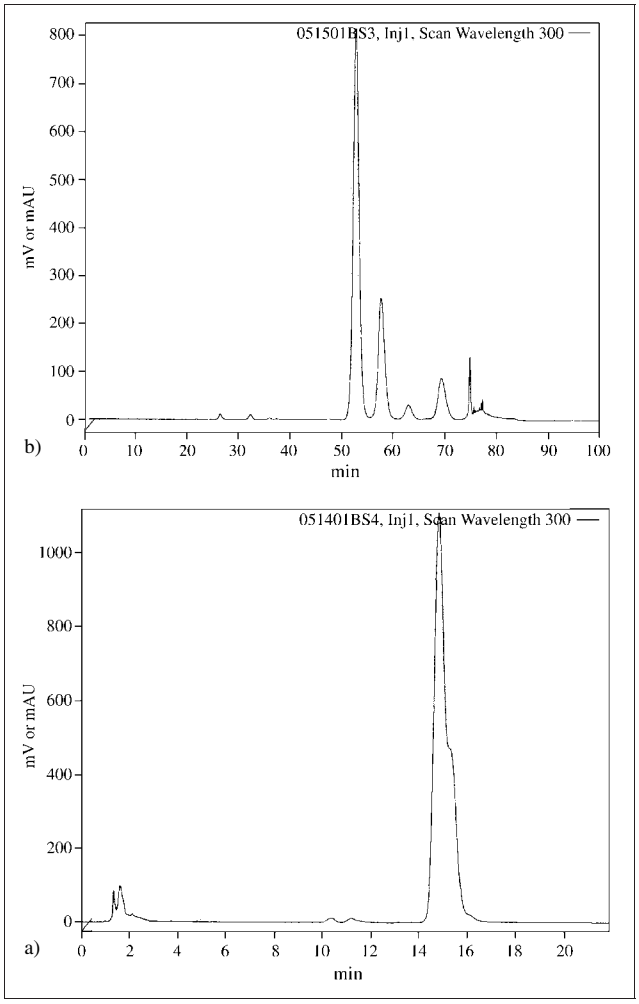

Fig. 3: Analytical HPLC chromatograms, fraction from 38 to 46 min from preparative HPLC, system a) acetonitrile/water 45 : 55 (v/v), system b) methanol/water 45 : 55 (v/v), $\lambda = 300$ nm.

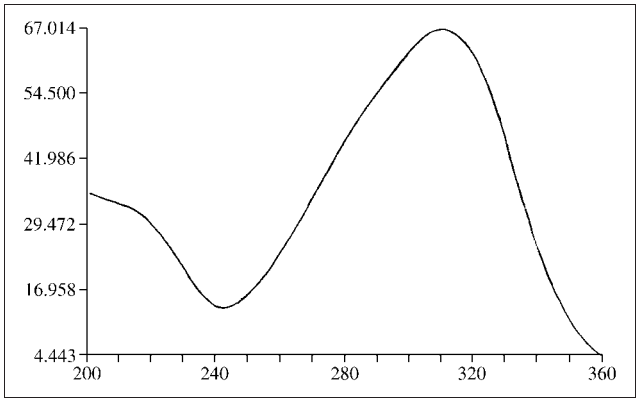

Fig. 4: UV spectrum of the substance from *E. pallida* that is responsible for the blue fluorescence.

2.2. Quantitative tests

Quantitation was achieved using TLC densitometry. The optimal excitation wavelength for a most intensive blue fluorescence is 360 nm, but the more common 366 nm excitation is also well suitable. The use of a commercial available narrow range spectral filter could further increase the signal/noise value.

2.3. Process of isolation

In order to isolate the blue fluorescent compound the drug was extracted with petroleum ether for 5 days. After concentration, the extract was separated by column chromatography, using silica gel as stationary phase. Tests with Sephadex™ showed insufficient separation results. The blue fluorescent compound-containing fractions, detected by TLC, were unified and concentrated. The subsequent HPLC isolation was progressed in two steps. The first separation was carried out with acetonitrile/water 45 : 55 (v/v) (System A). The relevant fractions appeared between 38 and 46 min in preparative HPLC, corresponding to 14 to 16 min in analytical HPLC (Fig. 3A). These fractions were unified, and a second step was progressed with methanol/water 45 : 55 (v/v) (System B) in order to achieve a better separation. The relevant fractions eluted between 141 and 147 min, corresponding to 51 to 54 min for analytical HPLC (Fig. 3B).

Using this approach, about 1 mg was isolated. The concentration in the original extract (starting from 15 L) was therefore below 1 ppm. The UV spectrum of the blue fluorescent substance was determined (Fig. 4). The isolation of amounts suitable for NMR and mass spectral analysis is possible using this method, but the starting volume of original extract should be at least 50 L.

3. Experimental

3.1. Plant material

The drug material was obtained between 1992 and 1999 from different suppliers and the identity was confirmed by TLC testing. The root fragments were between 15 and 40 mm long and 7 mm of diameter. They were milled to powder with a particle size smaller than 1 mm before extracting.

3.2. Chemicals

All chemicals for the extraction of the plant material were pure grade. For the mobile phase of the TLC and the preparative HPLC solvents of analytical grade were used.

The solvents for the analytical HPLC were of chromatographic grade. In particular, cyclohexane and acetone (Roth, Karlsruhe, Ger), petroleum ether and xylene (Baker, Groß-Gerau, Ger), methanol and toluene (Merck, Darmstadt, Ger) were used.

3.3. Extraction

The milled samples were extracted by suspending the drug in cyclohexane for 25 min at room temperature using an ultrasonic bath. After centrifugation the supernatant was separated and collected. Subsequently, the extract was concentrated by rotary evaporation.

3.4. Thin layer chromatography

Different silica gel plates (Merck, Darmstadt, Germany) were used:
TLC aluminium foil Silica gel 60 F254 Art. 1.05554 (layer width 0.2 mm), TLC aluminium foil Silica gel 60 Art. 5553 (layer width 0.2 mm), PSC plates Silica gel 60 without fluorescent indicator 2 mm Art. 5745.
Samples were applied to the plates using a Camag Linomat IV automated applicator (Camag, Muttenz, CH) with a distance of 2 cm to lateral and 1,5 cm to the bottom edge of the plates. The spray rate varied between 4–7 s/ml depending on the extract viscosity and intended bandwidth. The mobile phase constituents were put into the chamber and mixed therein 20 min before development. The plates were developed without complete chamber saturation.
After the development the mobile phase was removed from the TLC plates. The chromatogram was developed either by spraying or preferably by immersion. An immersion time of 6 s was best suited processed with CAMAG Chromatogramm-Tauchvorrichtung. Afterwards the chromatogram was heated at 110 °C for 7 min in a cabinet drier. The chromatogram was evaluated under UV radiation at 366 nm and recorded with the DC-Fotodokumentationsystem CAMAG Reprostar 3. Quantitative analysis was carried out with a CAMAG TLC Scanner 3.
Three different mobile phases provided good results:
Flow agent 1: hexane/acetone/toluene at the rate of 7 + 3 + 3 (v/v/v),
Flow agent 2: toluene/ethylacetate at the rate of 7 + 3 (v/v),
Flow agent 3: petroleum ether/toluene/ethylacetate at the rate of 5 + 1 + 1 (v/v/v)
Spray reagent: 95% volume share ethanol 96% (v/v) and 5% volume share trifluoroacetic acid 99% are mixed. Then 0.15 mol/L zinc sulfate are added.

3.5. HPLC

3.5.1. Analytical HPLC

The analysed fractions were diluted with acetonitrile approximately 1 : 2 and then filtered through a 2.5 µm micro filter. The elution was isocratic at the complete runtime. To avoid too much pressure when using methanol containing mobile liquid, the column temperature was attuned to 40 °C at a flow of 1 ml/min. Using acetonitrile-containing mobile phases, the column temperature was 20 °C. Following components were part of the HPLC-system: Autosampler: Spectra System AS 3000 by Thermo Separation Products; Pumps: Consta Metric 3500 and 3200 Solvent delivery system by LDC Analytical; Degasser: Membrane Degasser® by LDC Analytical; Mixer: HPLC Mixing Chamber Stirrer; Interface: SN 4000, Spectra System; Column: Hypersil® RP-18 5µm; 240 mm × 4.6 mm; ODS loaded by Knauer (Berlin, Ger); Detector: Spectro Monitor 5000 Photodiode array detector; Software: PC 1000 by Thermo Separation Products; Mobile phases: System A: acetonitrile/water 45 : 55 (v/v); System B: methanol/water 45 : 55 (v/v). All mobile phases were degassed for 10 min using ultrasound prior to use.

3.5.2. Preparative HPLC

The sample loop had a volume of 2 ml. Instead of a degasser the mobile phase was degassed for 30 min in an ultrasonic bath (Sonorex super RK 514). Using methanol-containing mobile phases the column was tempered to 40 °C, while acetonitrile-containing mobile phases were run at room temperature. Pump: HPLC Pump 64 by Knauer, Berlin, Ger); Mixer: HPLC Mixing Chambers Stirrer (static-dynamic); Column: Hypersil ODS 5 µm; 240 × 32 mm; Flow: 35–37.5 ml/min.; UV-Detector: Uvicard SD by Pharmacia LKB (Freiburg, Ger; wavelength 254 nm) with pen recorder (Knauer, Berlin, Ger.); Fraction collector: Foxy (Lincoln, Nebraska, US), tube-change every minute.
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