*Journal of Chromatography*, 609 (1992) 423–426
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 467

# Short Communication

# Determination of hypericin and pseudohypericin by thin-layer chromatography–densitometry

Th. Kartnig and I. Göbel

*Institute of Pharmacognosy, Karl-Franzens-University, Universitätsplatz 4/I, A-8010 Graz (Austria)*

(First received April 21st, 1992; revised manuscript received June 26th, 1992)

ABSTRACT

The accuracy and reproducibility of the determination of hypericin and pseudohypericin by measuring the emitted fluorescence *in situ* on thin-layer chromatography plates can be improved significantly by using appropriate dipping reagents. The effects of pyridine, polyethylene glycol 4000, paraffins, silicone oil and Triton X-100 in various solvents and combinations on the intensity and stability of emitted fluorescence are described. Using the test conditions established by this investigation, hypericin can be quantified exactly within the range 5–50 ng and pseudohypericin within the range 20–200 ng. The relative standard deviation for the quantification under the given conditions is less than 1.5% for both substances.

## INTRODUCTION

Thin-layer chromatography (TLC)–densitometry is still an appropriate method of determining hypericin and pseudohypericin in plant and plant tissue extracts as well as in pharmaceutical formulations [1]. Several TLC systems for the separation of hypericin and pseudohypericin are described [1–5].

The exact and reproducible quantification of TLC-separated hypericins by measuring the emitted fluorescence *in situ* depends on intensifying and stabilizing the fluorescence. This may be achieved by spraying or dipping the TLC plate with appropriate reagents after development. A review on the use of fluorescence enhancement reagents in TLC is given in ref. 6. In systematic investigations we tested substances reported to increase the intensity of fluorescence of various plant constituents [2,3,7–12]. Finally, we improved the conditions of quantification significantly so that an exact determination of hypericin within the range 5–50 ng and of pseudohypericin within the range 20–200 ng with a relative standard deviation of less than 1.5% was achieved.

*Correspondence to:* Universitäts-Professor Dr. Theodor Kartnig, Institute of Pharmakognosy, Karl-Franzens-University, Universitätsplatz 4/I, A-8010 Graz, Austria.

## EXPERIMENTAL

### *Chemicals*

Hypericin was obtained from Roth (Karlsruhe, Germany, product No. 7929 and was used as follows: 1 mg in 100 ml of methanol (solution A), 1 ml of solution A diluted with 3 ml of methanol (solution B) (2 $\mu$l of solution B = 5 ng of hypericin). Pseudohypericin was obtained from Hämosan (Graz, Austria) and was used as follows: 1 mg in 100 ml of methanol (2 $\mu$l = 20 ng of pseudohypericin). (Hypericin and pseudohypericin solutions

0021-9673/92/$05.00 

have to be kept in the dark and at low temperature.)

The other chemicals were as follows: pyridine (Merck, Darmstadt, Germany, product No. 9728), polyethylene glycol (PEG) 4000 (Merck, product No. 807490), viscous paraffin (Merck, product No. 7160), liquid paraffin (Merck, product No. 7162), highly liquid paraffin (Merck, product No. 7174), paraffin in block form (Merck, product No. 7150), silicone oil (Merck, product No. 7742), Triton X-100 (Merck, product No. 8603)

*Thin-layer chromatography*

The TLC plates were 10 × 20 cm TLC aluminium sheets coated with silica gel 60 (without fluorescent indicator) (Merck, product No. 5553) and were developed in unsaturated tanks (Desaga, Heidelberg, Germany, product No. 12 01 73). Test solutions and plant extracts were applied as bands of 6 mm (micropitette by HAACK, Vienna, Austria). The mobile phase was toluene–ethylformiate–formic acid (5:4:1) [3,4]. After development over 8 cm, solvent was quickly evaporated in a warm airstream and the plate dried for 10 min at 60°C.

Detection was with UV light at 366 nm. Hypericin ($R_F$ = 0.58) and pseudohypericin ($R_F$ = 0.63) are separated sufficiently and with sharp boundaries.

*Equipment*

A Shimadzu CS 9000 dual-wavelength flying-spot scanner was used with the following settings: beam size 0.4 × 10 mm; wavelength = 313 nm; delta "y" = 0.04 mm, filter 4, zero set mode at start.

*Sample preparation*

A 30- to 50-mg aliquot of powdered (sieve 180, European Pharmacopoeia) *Hypericum* leaves or herbs was extracted with 5 ml of methanol at room temperature using a magnetic stirrer for 1 h. Extraction of plant material was repeated twice with 5 ml of methanol for 5 min. The combined filtrates were evaporated to dryness and the residue dissolved in 2.00 ml of methanol. The solution was filtered again prior to application to the TLC plate.

*Preparation of the plate for fluorodensitometric determinations*

The developed and dried plate was first dipped into a solution of 10% pyridine in diethyl ether (so-

TABLE I

EFFECTS OF VARIOUS SUBSTANCES ON THE INTENSITY AND STABILITY OF THE FLUORESCENCE OF HYPERICIN AND PSEUDOHYPERICIN

The stabilizing effect is expressed as the decrease in fluorescence achieved within 30 min: − = >25%; (+) = 20–25%; + = <20%; + + + = <3%.

| Substance | Fold increase in intensity | Stabilizing effect | Concentration of substance; solvent |
|---|---|---|---|
| Pyridine [2,3] | 2–5 | − | 10%; diethyl ether, acetone |
| PEG 4000 [7] | 2–4 | (+) | 5%; ethanol |
| Pyridine + PEG 4000 | 2–5 | − | 5% pyridine, 5% PEG, ethanol |
| Highly liquid paraffin [7–11] | 3–10 | (+) | 20–70%; diethyl ether, *n*-hexane, toluene |
| Liquid paraffin [7–11] | 3–10 | + | 20–70%; diethyl ether, *n*-hexane, toluene |
| Viscous paraffin [7–11] | 3–10 | + + + | 20–70%; diethyl ether, *n*-hexane, toluene |
| Block form paraffin | 6–8 | − | 4–8%; toluene |
| Block form + viscous paraffin | 8–12 | + | 3–4% block form, 25–50% viscous paraffin; diethyl ether |
| Pyridine + highly liquid paraffin | 7–10 | − | 1–10% pyridine, 50–70% paraffin, diethyl ether, *n*-hexane |
| Pyridine + liquid paraffin | 7–12 | − | 1–10% pyridine, 50–70% paraffin, diethyl ether, *n*-hexane |
| Pyridine + viscous paraffin | 7–15 | (+) | 1–10% pyridine, 50–70% paraffin, diethyl ether, *n*-hexane |
| Silicone oil [7] | 2–10 | (+) | 30–70%; diethyl ether |
| Pyridine + silicone oil | 10–12 | (+) | 5% pyridine, 30–70% silicone oil, diethyl ether |
| Triton X-100 [9–12] | 2–3 | (+) | 1–33%; *n*-hexane, chloroform, toluene |

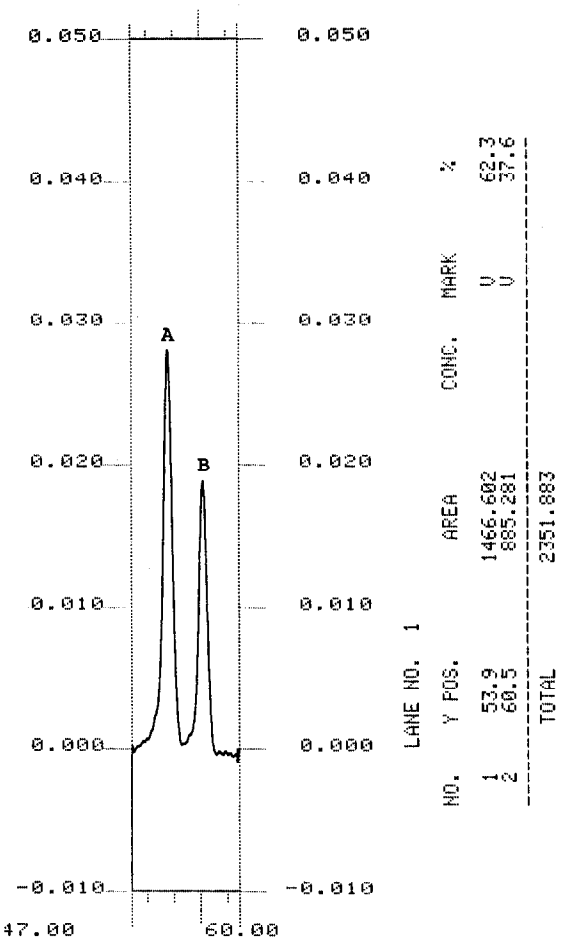


Fig. 1. Scanning profile of a methanolic extract of *Hypericum perforatum* herbs. A = Pseudohypericin; B = hypericin.

lution 1) in a dipping tank (Desaga, product No. 12 41 52). After the diethyl ether had evaporated (*ca.* 3 min) the plate was dipped into a homogeneous mixture of two volumes of viscous paraffin and one volume of diethyl ether (solution 2), also in a dipping tank.

Subsequently, the plate was placed vertically on a flat surface in the dark for 1 h and the emitted fluorescence was then measured.

*Calculations*

The contents of hypericin and pseudohypericin are calculated from the formulae:

$$\text{Hypericin (ng)} = \frac{45\,(A_t - A_1) + 5\,(A_2 - A_1)}{A_2 - A_1}$$

$$\text{Pseudohypericin (ng)} = \frac{180\,(A_t - A_1) + 20\,(A_2 - A_1)}{A_2 - A_1}$$

where $A_t$ is the area of the sample curve, $A_1$ is the area of the curve of 5 ng of hypericin and 20 ng of pseydohypericin, and $A_2$ is the area of the curve of 50 ng of hypericin and 200 ng of pseudohypericin.

Standards of hypericin and pseudohypericin were measured with every determination.

## RESULTS AND DISCUSSION

Fluorodensitometric quantification of hypericins *in situ* without application of fluorescence enhancing and stabilizing reagents produces only imprecise and non-reproducible results. The reason for this phenomenon is on the one hand a relatively weak fluorescence and on the other hand insufficient stability of fluorescence intensity [7].

To optimize the quantification of hypericin and pseudohypericin we checked all the steps of the procedure. Extracting plant material in the way described dissolves out the hypericins quantitatively.

TLC separation is better achieved by the suggested system [2,3] than by using RP-8 plates and acetonitrile [1]. The spots are separated well and exhibit sharp margins, as the scanning profile shows (Fig. 1).

To increase fluorescence intensity and improve stability, we tested several substances in various concentrations and combinations as well as in various solvents (see Table I).

Homogeneous application of the reagents to the plate is only guaranteed by dipping and not by spraying [7]. The best effect was achieved with 10% pyridine in diethyl ether as the first dipping solution. After removing diethyl ether from the plate in a warm airstream, other solutions were applied as second reagents (see Table II).

Finally, we found the best effect using consecutive applications of 10% pyridine in diethyl ether as the first dipping solution and 66% viscous paraffin in diethyl ether as the second dipping solution.

To conserve solvents, the dipping procedure should be carried out in dipping tanks. The TLC plate has to be held with forceps. Immediately after withdrawing the plate from the second solution, it

TABLE II

EFFECTS OF VARIOUS SUBSTANCES APPLIED AFTER A BASIC TREATMENT WITH 10% PYRIDINE IN METHANOL

The stabilizing effect is expressed as the decrease in fluorescence achieved within 30 min. − = >25%; (+) = 20–25%; + = <20%; + + + = <3%.

| Substance | Fold increase in intensity | Stabilizing effect | Concentration of substance; solvent |
|---|---|---|---|
| PEG 4000 | 2 | − | 5%; ethanol |
| Liquid paraffin | 12–14 | + | 70%; diethyl ether |
| Viscous paraffin | 6–15 | + + + | 50–70%; diethyl ether |
| Blijock form paraffin | 6–8 | − | 4–8%; toluene |
| Bock form + viscous paraffin | 10–12 | − | 3–4% block form, 25–50% viscous paraffin; diethyl ether |
| Silicone oil | 10 | − | 70%; diethyl ether |
| Triton X-100 | 2–3 | − | 1–33%; *n*-hexane, chloroform, toluene |

should be placed vertically on a flat surface, *e.g.* on the turned over metal cover of a TLC tank (Desaga, product No. 12 01 02), in the dark for 1 h. During this time the diethyl ether evaporates and the excess paraffin flows down the plate. After 1 h fluorescence is sufficiently stable for measurements to be made.

To maintain exact results with every TLC plate, 5 and 50 ng of hypericin as well as 20 and 200 ng of pseudohypericin have to be determined as calibration substances. In numerous determinations of identical samples we observed that, in spite of strict adherence to the protocol, there were small differences from experiment to experiment. These differences can be avoided by using hypericin and pseudohypericin standards on the same plate.

The accuracy of the fluorodensitomeric measurement of hypericin and pseudohypericin in the procedure described is expressed as the relative standard deviation and is less than 1.5%. The reproducibility of the determination of hypericin and pseudohypericin from plant material is also expressed as the relative standard deviation and is less than 3.5%.

ACKNOWLEDGEMENT

We thank Firma Hämosan (Graz, Austria) for generously providing pseudohypericin.

REFERENCES

1 M. Vanhaelen and R. Vanhaelen-Fastre, *J. Chromatogr.*, 281 (1983) 263.
2 H. Wagner, S. Bladt, and E.-M. Zgainski, *Drogenanalyse, Dünnschichtchromatographische Analyse von Arzneidrogen*, Springer, Berlin, 1983.
3 *Deutscher Arzneimittel-Codex (Ergänzung zum Arzneibuch)*, Govi-Verlag, 1979.
4 R. Berghöfer and J. Hölzl, *Dtsch. Apoth. Ztg.*, 126 (1986) 2569.
5 P. Pachaly, *Dtsch. Apoth. Ztg.*, 124 (1984) 2159.
6 C. F. Poole, S. K. Poole, T. A. Dean and N. M. Chirco, *J. Planar Chromatogr.*, 2 (1989) 180.
7 H. Jork, W. Funk, W. Fischer and H. Wimmer, *Dünnschichtchromatographie. Reagenzien und Nachweismethoden*, Band 1a, VCH Verlagsgesellschaft 1989.
8 S. Uchiyama and M. Uchiyama, *J. Chromatogr.*, 153 (1978) 135.
9 S. Uchiyama and M. Uchiyama, *J. Chromatogr.*, 262 (1983) 340.
10 B. Lin Ling, W. R. G. Baeyens, H. Marysael, K. Stragier and P. de Moerloose, *J. Liq. Chromatogr.*, 12 (1989) 3135.
11 S. Uchiyama and M. Uchiyama, *J. Liq. Chromatogr.*, 3 (1980) 681.
12 S. S. J. Ho, H. T. Butler and C. F. Poole, *J. Chromatogr.*, 281 (1983) 330.