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Note

Comparison between high-performance thin-layer chromatography–fluorometry and high-performance liquid chromatography for the determination of sennosides A and B in Senna (Cassia spp.) pods and leaves

PIERRE DUEZ*
Laboratoire de Chimie Analytique, Chimie Pharmaceutique Inorganique et Toxicologie, CP 205/1, Université Libre de Bruxelles, Campus Plaine, 1050 Brussels (Belgium)
M. VANHAELEN and R. VANHAELEN-FASTRE
Laboratoire de Pharmacognosie, CP 205/4, Institut de Pharmacie, Université Libre de Bruxelles, Campus Plaine, 1050 Brussels (Belgium)
and
M. HANOCQ and L. MOLLE
Laboratoire de Chimie Analytique, Chimie Pharmaceutique Inorganique et Toxicologie, CP 205/1, Université Libre de Bruxelles, Campus Plaine, 1050 Brussels (Belgium)
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Recent studies[1–6] on the biological activity and metabolism of the anthracenic derivatives of Senna have confirmed that the most active constituents of this drug are sennosides A and B. A number of spectrophotometric[7–9], high-performance liquid chromatographic (HPLC)[10–16] and thin-layer chromatographic (TLC) photodensitometric methods[1,2,17–19] have been proposed for the assay of sennosides A and B; however, most of them either require hydrolysis of the glycosides before determination of the total anthracenic content, or lack sensitivity.

In this paper a new densitometric HPTLC method is described for the separation of sennosides A and B from other constituents present in crude extracts and compared with a HPLC method.

EXPERIMENTAL

The Perkin-Elmer liquid chromatograph was equipped with a pump (Model 601), a sample loop (Rheodyne 7105), an UV detector (Model LC-55 and LC 55 S) operating at a wavelength of 275 nm and an 100 × 4.6 mm I.D. stainless-steel column packed with Nucleosil® 5 $N(CH_3)_2$ (Macherey Nagel, Düren, F.R.G.): wet packing was achieved with a Chromatem pump (Touzart et Matignon, France). The mobile phase was methanol–water–acetic acid (80:20:6.5) at a flow-rate of 1 ml/min and the column was maintained at 45°C.

HPTLC pre-coated plates of silica gel 60 F 254 (10 × 20 cm) were obtained from Merck (Darmstadt, F.R.G.) and buffered by homogeneous spraying with 10 ml of 0.05 *M* aqueous KH_2PO_4 solution adjusted to pH 7.5 by addition of 0.05 *M*

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aqueous NaOH solution. The plates were dried at ambient temperature for 2 h then heated at 90°C for 1 h.

A 2.5-mg amount of sennosides A and B obtained from Carl Roth (Karlsruhe, F.R.G.) was dissolved in 5 ml of acetone–water (7:3). This solution was diluted to 25 ml in methanol–water (7:3), and 1/10 to 8/10 dilutions of this stock solution in methanol–water (7:3) were used as standards for both HPLC and HPTLC methods.

A 100-mg amount of powdered Senna leaves and pods (315 mesh) was weighed into 10-ml glass-stoppered centrifuge tubes and extracted with 5 ml methanol–water (7:3) by successive shaking (10 min), sonication (30 min) and centrifugation at 2000 *g*; this step was repeated three times. The supernatants were combined, diluted to 25 ml with methanol–water (7:3) and finally filtered through a Millipore HV.4 filter before injection or application on the TLC plates. The solutions (1 μl, standards or crude extracts) were applied 15 mm from the lower edge of the plates, and then developed with ethylacetate–2-propanol–buffer solution, pH 7.5 (0.05 *M* KH_2PO_4 adjusted to pH 7.5 with 0.05 *M* NaOH) (36:36:28); this mobile phase was allowed to travel, in saturated tanks, a distance of 100 mm (90 min). After development and drying at 90°C for 30 min, the plates were sprayed with 400 mg sodium borohydride dissolved just before use in 10 ml of an aqueous solution of sodium stannite (2.5 g of $SnCl_2$ in 50 ml water treated with 20 ml of 20% aqueous NaOH then diluted to 100 ml in water). Immediately after spraying, the yellow-green fluorescent spots were measured with a Shimadzu high speed TLC scanner CS-920 using the following settings: λ (excitation) 365 nm; filter 3 (UV cut-off 480 nm); zigzag stroke width 12 mm; beam size 1.2 × 1.2 mm. The mean values were calculated from the integrations of nine spots corresponding to three different standard concentrations, each being analysed twice, and three spots of the solution of unknown concentration.

RESULTS AND DISCUSSION

HPLC

After comparison with several reversed-phase packings, using both non-ionic and ionic mobile phases, and weak and strong anion exchangers, it was evident that the N-dimethylamino packing proposed by Ohshima and Takahashi[16] exhibited the best performance for the HPLC separation of sennosides A and B; however, the tetrahydrofuran (THF) introduced in the mobile phase employed by these authors was replaced by methanol without interference with the separation (Fig. 1) and the column temperature was increased to 45°C in order to avoid peak tailing. It should be pointed out that the acetic acid concentration in the mobile phase is essential to this chromatographic separation and may be adjusted from packing to packing or during column "life". The purity of peaks was tested by UV spectroscopy.

HPTLC

The well known chromatographic conditions initially published by Hörhammer *et al.*[20] and chosen for the Senna monograph of the European Pharmacopoeia allowed a sufficient separation of sennosides A and B; however, distortions of the spots, in the case of sennoside B, were observed. The combination of a buffered silica gel and a mobile phase whose composition was derived according to Khafagy *et al.*[21] improved the separation of all constituents present in the crude extracts and com-

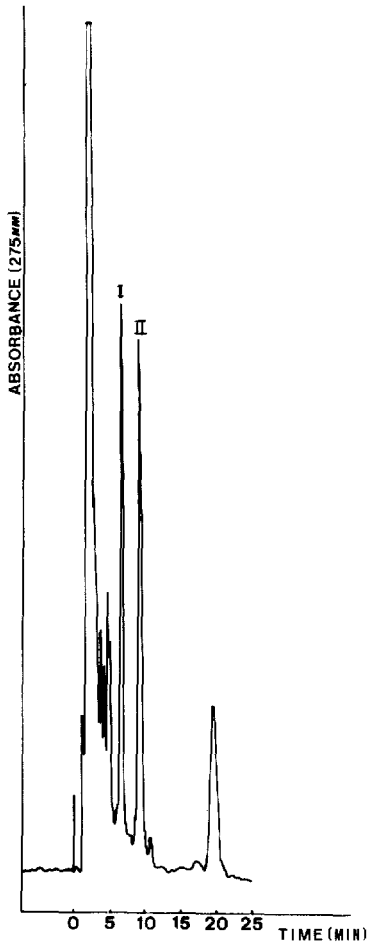

Fig. 1. HPLC chromatogram of a *Cassia italica* extract. Column packing: Nucleosil 5 $N(CH_3)_2$. Mobile phase: methanol–water–acetic acid (80:20:6.5) at a flow-rate of 1 ml/min. Column temperature: 45°C. Detection: UV, 275 nm. Peaks: I = sennoside A; II = sennoside B.

pletely suppressed the tailing effect observed for the dianthrone derivatives (Fig. 2).

Furthermore, the previously described detection conditions afforded a very poor sensitivity useless for densitometric measurements. Thus, a highly sensitive method for the detection of the dianthrone derivatives was proposed. It involved their *in situ* reduction by an aqueous solution of sodium borohydride in the presence of sodium stannite. This reaction produced yellow-green fluorescent derivatives which were too unstable for further chemical investigation but were probably related to the reduction products described by Criswell and Kandeman[22] for anthracenic monomers. It was obtained only on silica gel thin layers and not on other adsorbents such as alumina, polyamide or cellulose. The fluorescent spots must be measured immediately after spraying; indeed, a slow degradation of the reduction products occurs during the drying of the plates. The rate of this degradation, which probably corresponds to an oxidation, was strongly inhibited by addition of sodium stannite

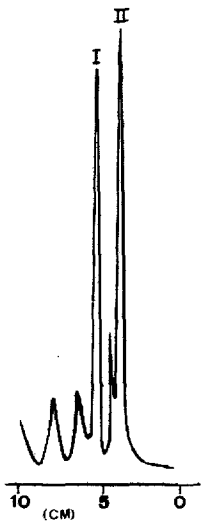

Fig. 2. Scanning profile of an HPTLC chromatogram of a *Cassia italica* extract. Adsorbant: silica gel 60 F 254 buffered at pH 7.5. Mobile phase: ethyl acetate–2-propanol–buffer, pH 7.5 (36:36:28). Excitation wavelength: 365 nm. Filter 3 (UV cut-off 480 nm). Peaks as in Fig. 1.

to the spraying agent: under these conditions, no change in the fluorescence intensities was detected within 25 min of spraying.

Fluorescence spectra of the reaction products prepared *in vitro* by addition of sodium borohydride to a methanolic solution of sennoside B exhibited two excitation and one emission maxima respectively at 245, 365 and 515 nm. However, the background noise was too high on silica gel 60 at the lower excitation wavelength and the conditions were finally dependent on the equipment characteristics.

TABLE I

COMPARISON OF THE HPTLC DENSITOMETRIC AND HPLC METHODS FOR THE DETERMINATION OF SENNOSIDES A AND B IN *CASSIA ITALICA* EXTRACTS

Sennoside	*Sample*	*HPTLC*			*HPLC*		
		Mean sennoside % (dried powder)	*Standard deviation*		*Mean sennoside % (dried powder)*	*Standard deviation*	
			Absolute	*Relative*		*Absolute*	*Relative*
A	1	0.69	0.05	8	0.72	0.04	6
	2	0.75	0.05	6	0.73	0.02	3
	3	0.56	0.05	9	0.51	0.01	2
	4	0.57	0.10	17	0.60	0.03	5
	5	0.16	0.01	6	0.16	0.01	6
B	1	0.81	0.16	19	0.78	0.03	4
	2	0.93	0.06	7	0.85	0.05	6
	3	0.71	0.05	7	0.61	0.03	5
	4	0.74	0.02	3	0.66	0.01	1
	5	0.18	0.02	10	0.20	0.01	7

Concentrations of 40–120 ng per μl spotted afforded a linear calibration graph with an r value (correlation coefficient) typically greater than 0.995; the detection limit was about 10 ng.

Extraction and comparison of HPTLC with HPLC

A four-step extraction procedure guaranteed that at least 99% of the total sennosides A and B were extracted. The proposed HPTLC method was applied to the determination of sennosides A and B from different Cassia species and mainly from *Cassia italica* leaves; the data were compared with those obtained by HPLC (Table I) and show little differences in the coefficients of variation (C.V.). Therefore, the HPTLC densitometric method is proposed as an interesting alternative to HPLC.

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