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Note

Comparison between thin-layer chromatography–densitometry and high-performance liquid chromatography for the determination of hyoscyamine and hyoscyne in leaves, fruit and seeds of *Datura* (*Datura* spp.)

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For the assay of *Datura* alkaloids, both in pharmaceutical preparations and in plant extracts, a number of methods have been proposed, including titrimetry[1], spectrometry[2–4], biological procedures[5,6] and chromatographic techniques including gas–liquid chromatography[3,7–9], high-performance liquid chromatography (HPLC)[10–25] and paper[26] and thin-layer chromatographic (TLC) photodensitometry[27,28].

A survey of the available literature shows that none of the various HPLC conditions described proves really satisfactory for the analysis of plant extracts; indeed, only a very few of these methods deal with such material[16,25].

TLC or paper densitometry, as previously described, resulted in poor sensitivity (UV, Dragendorff reagents) or high instability of the colours produced (iodine reagents).

This paper describes two new analytical procedures for the assay of *Datura* alkaloids: HPLC on a trimethylsilyl column, and TLC photodensitometry with 4-(dimethylamino)benzaldehyde as chromogenic reagent.

EXPERIMENTAL

The Perkin-Elmer chromatograph was equipped with a pump (Model 601), a sample loop (Rheodyne 7105), a UV detector (Model LC-55 equipped with scanner LC-55S) operating at a wavelength of 259 nm, a 150 × 4.6 mm I.D. Zorbax TMS 5-μm column (DuPont, U.S.A.) and a 125 × 4 mm I.D. Hibar column prepacked with LiChrosorb RP-2 5 μm (Merck, Darmstadt, F.R.G.).

The mobile phase was 0.2 *M* NaH_2PO_4 plus $5 \cdot 10^{-3}$ *M* *d*-10-camphorsulphonic acid in water: 0.2 *M* H_3PO_4 plus $5 \cdot 10^{-3}$ *M* *d*-10-camphorsulphonic acid in water: acetonitrile: propylamine (34:60:6:0.6) with a final pH of 2.5 ± 0.05. The flow-rate was 1 ml/min and the column was maintained at 40°C.

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Standards for HPLC were prepared by dissolving 10 mg of atropine sulphate and 10 mg of scopolamine bromide (Merck) in 10 ml of the mobile phase.

Standards for TLC were 2:10 to 8:10 dilutions in methanol of a stock solution prepared by dissolving 60 mg of atropine sulphate and 60 mg of scopolamine bromide (Merck) in 25 ml of methanol.

A 5-g amount of *Datura* leaves or fruit 315-μm powder or of ground seeds was weighed in an 80-ml glass-stoppered centrifuging tube, mixed with 300 mg of calcium hydroxide, 0.2 ml of water and 30 ml of deperoxidised ether (prepared by overnight contact with ferrous sulphate heptahydrate and further distillation) and left for 1 h at 4°C; this suspension was added with 30 ml of deperoxidised ether, shaken for 15 min and centrifuged at 2000 *g*, these last steps being repeated thrice. The supernatants were combined, reduced to *ca.* 10 ml, and then applied to an 8 mm I.D. glass column filled with 1 g of either diatomaceous earth or Extrelut® packing (Merck) supporting 0.5 ml of 5 *N* sulphuric acid. The non-alkaloidal material was discarded by column elution with deperoxidised ether until the eluate became decoloured (*ca.* 80 ml) then with 20 ml of chloroform. Alkaloids were eluted with *ca.* 90 ml of ammonia-saturated chloroform (prepared by shaking chloroform with aqueous 25% ammonium hydroxide and filtering through a Whatman silicone-treated paper filter); the chloroform eluent was then filtered through anhydrous sodium sulphate and evaporated under reduced pressure to dryness. The residue was dissolved in 12 ml of chloroform and separated into two portions of 5 ml, which were evaporated under reduced pressure to dryness. The first residue was dissolved in 1 ml of methanol and used for the TLC–densitometric determinations; the second dissolved in 1 ml of the HPLC mobile phase, was used for HPLC assays after filtration through a Millipore HV-4 filter.

TLC plates pre-coated with silica gel 60F 254 (10 × 20 cm) were obtained from Merck. The solutions (1-μl standards or crude extracts) were applied 15 mm from the lower edge of the plates and then developed with 1,1,1-trichloroethane–diethylamine (90:10); this mobile phase was allowed to travel, in the unsaturated tank, a distance of 100 mm. After development and drying at 105°C for 2 h, the plates were sprayed with 200 mg of 4-(dimethylamino)-benzaldehyde, dissolved just before use in 40 ml of ethanol–8 *N* sulphuric acid (1:1), then heated at 105°C for 1 h. The reddish spots were measured with a Shimadzu high speed TLC scanner CS-920 using the following settings: λ(absorption) = 495 nm; zig-zag stroke width = 9 mm; beam size = 1.2 × 1.2 mm; linearizer 1; AZS off. The mean values were calculated from the integration 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

A comparison between different packings, reversed phases, using both ionic and non-ionic mobile phases, and underivatized silica gel showed that, although it was possible to reproduce chromatographic conditions described in literature, none of these conditions gave sufficient separation and resolution of hyoscyne and hyoscyamine in plant extracts; however, a method recently developed for the assay of belladonna[25] showed a suitable separation of these alkaloids, but the pH of the

mobile phase (pH 12) was out of the pH range commonly allowed by the column manufacturers and the flow-rate had to be modified during the chromatographic runs.

The procedure described here included the use of a trimethylsilyl bonded phase and *d*-10-camphorsulphonic acid added to the mobile phase (Figs. 1 and 2); this counter-ion was initially selected with the aim of separating hyoscyamine from its biologically inactive isomer[28] but without avail. A low pH of the mobile phase, the addition of propylamine and the increase of the column temperature to 40°C were necessary to reduce the tailing of the hyoscyamine peak. It was essential to inject both standards and samples dissolved in a mixture such that the proportion of the organic solvent was not greater than that of the mobile phase in order to avoid band distortions. The purity of peaks was tested by UV spectroscopy (absorbance ratios at three wavelengths).

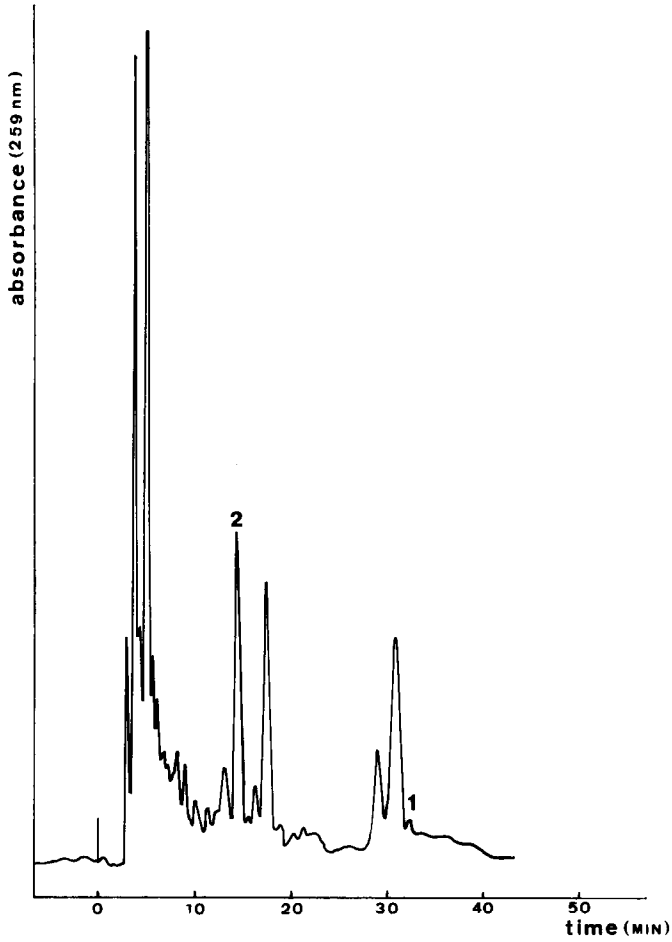

Fig. 1. HPLC chromatogram of a *Datura innoxia* leaves extract. Column packing, Zorbax TMS 5 μm; mobile phase, 0.2 *M* NaH_2PO_4 plus $5 \cdot 10^{-3}$ *M* *d*-10-camphorsulphonic acid–0.2 *M* H_3PO_4 plus $5 \cdot 10^{-3}$ *M* *d*-10-camphorsulphonic acid–acetonitrile–propylamine (34:60:6:0.6) at a flow-rate of 1 ml/min; column temperature, 40°C; detection, UV 259 nm; peaks, 1 = hyoscyamine; 2 = hyoscyne.

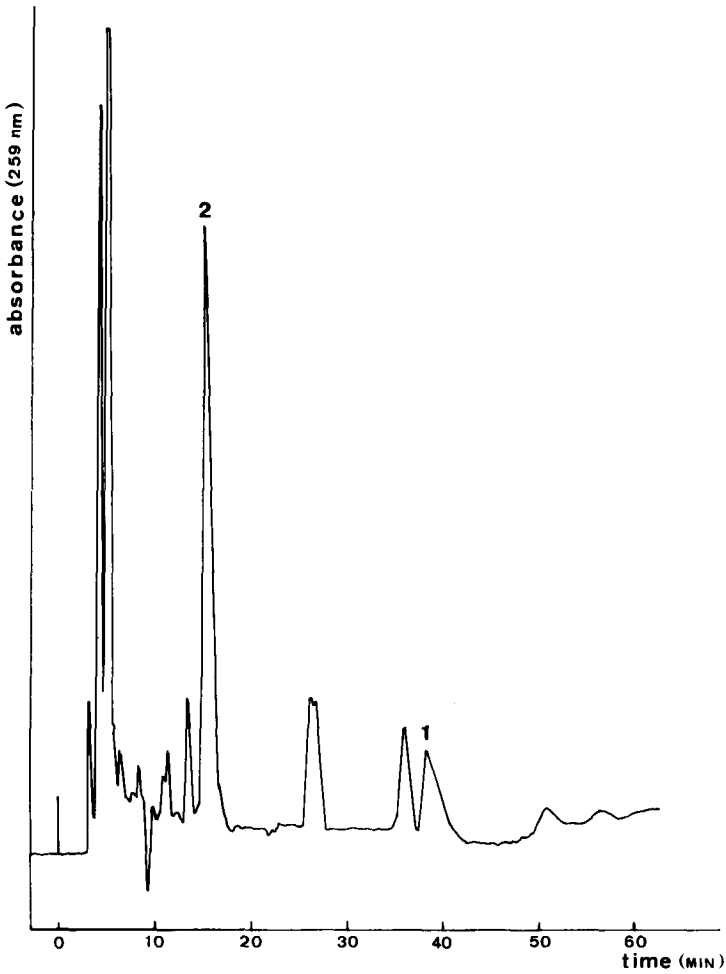

Fig. 2. HPLC chromatogram of a *Datura innoxia* fruit extract. Column packing, Zorbax TMS 5 μm; mobile phase, 0.2 *M* NaH_2PO_4 plus $5 \cdot 10^{-3}$ *M d*-10-camphorsulphonic acid–0.2 *M* H_3PO_4 plus $5 \cdot 10^{-3}$ *M d*-10-camphorsulphonic acid–acetonitrile–propylamine (38:58:4:0.6) at a flow-rate of 1 ml/min; column temperature, 40°C; detection, UV 259 nm; peaks as in Fig. 1.

A decrease of the column resolution and separation was observed with both the LiChrosorb RP-2 and the Zorbax TMS packings so that an adjustment of the acetonitrile proportion in the mobile phase was necessary during column life in order to conserve the initial chromatographic performances.

TLC–densitometry

More than 100 TLC systems have been described for the separation of tropine alkaloids[30]; one of these classical mobile phases (chloroform–diethylamine, 90:10) was slightly modified (1,1,1-trichloroethane–diethylamine, 90:10) to obtain well-defined circular spots with rather similar hR_F values (22 for hyoscyamine and 36 for hyoscyne) (Fig. 3).

Fig. 3. Scanning profile of an TLC chromatogram of an extract of *Datura innoxia* seeds. Adsorbent, silica gel 60 F 254; mobile phase, 1,1,1-trichlorethane–diethylamine (90:10); absorption wavelength, 495 nm; peaks as in Fig. 1.

Because the previously described detection conditions resulted in either poor sensitivity (excessive colouration of the background by the sprayed reagents; low absorption in UV) or lack of stability of the colours produced, a highly sensitive method was proposed for the detection of these alkaloïds. It involved a modified Van Urk's reagent, used previously by Karawya *et al.*[3] for *in vitro* colourimetric determinations of hyoscyamine and hyoscyne, the solvents being adapted to obtain a reproducible spraying of the TLC plates. The heating temperature (105°C) was found optimal for the colouration development without any charring of the alkaloids.

The reddish derivatives, well-contrasted on a colourless background, were found to present an absorption maximum at 495 nm (determined on TLC plates).

TABLE I

DETERMINATION OF HYOSCYAMINE AND HYOSCYNE IN *DATURA INNOXIA* AND *DATURA STRAMONIUM* EXTRACTS: COMPARISON OF THE DENSITOMETRIC AND HPLC RESULTS

Alkaloid	*Sample**	*TLC–densitometry*		*HPLC*	
		Mean alkaloid (%) (dried powder)	*Relative standard deviation (%)*	*Mean alkaloid (%) (dried powder)*	*Relative standard deviation*
Hyoscyamine	Leaves 1 (D.i.)	Traces	–	Traces	–
	Leaves 2 (D.i.)	Traces	–	Traces	–
	Leaves 3 (D.i.)	Traces	–	Traces	–
	Fruit (D.i.)	0.003	8	0.002	6
	Seeds (D.i.)	0.12	8	0.14	7
	Seeds (D.s.)	0.19	4	0.18	2
Hyoscyne	Leaves 1 (D.i.)	0.010	2	0.012	7
	Leaves 2 (D.i.)	0.028	2	0.028	1
	Leaves 3 (D.i.)	0.059	6	0.077	1
	Fruit (D.i.)	0.018	8	0.019	3
	Seeds (D.i.)	0.082	4	0.071	5
	Seeds (D.s.)	Traces	–	Traces	–

* D.i. = *Datura innoxia*; D.s. = *Datura stramonium*.

Concentrations of 0.2–2 μg of alkaloid base per microlitre spotted afforded a linear calibration graph with an r value (correlation coefficient) typically greater than 0.995; the detection limit was *ca.* 50 ng.

Extraction

The extraction procedure, adapted to the analytical scale from a method described by Evans and Partridge[31], had the advantage of avoiding liquid–liquid extractions, thus simplifying the experimental conditions. The recovery of the alkaloids was at least 95%.

Comparison of TLC–densitometry with HPLC

The proposed densitometric TLC method was applied to the determination of hyoscyamine and hyoscyne in leaves, fruit and seeds from different *Datura* species, mainly from *D. innoxia* and *D. stramonium*; the data were compared with those obtained by HPLC and were found to be similar (Table I). Therefore, because of the non-availability of suitable HPLC methods, the proposed densitometric TLC assay is of interest for the rapid determination of these tropine alkaloids in various plant tissues.

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