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Short Communication

Simultaneous analysis of *l*-hyoscyamine, *l*-scopolamine and *dl*-tropic acid in plant material by reversed-phase high-performance liquid chromatography

Marc-André Fliniaux*, Françoise Manceau and Annie Jacquin-Dubreuil

Laboratoire de Pharmacognosie et Phytotechnologie, Faculté de Pharmacie, 3 Rue des Louvels, 80000 Amiens (France)

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ABSTRACT

A sensitive reversed-phase high-performance liquid chromatographic (HPLC) procedure for the analysis of the main parasympatholytic tropane alkaloids in plant material is described. It uses an acidic aqueous acetonitrile mobile phase and UV detection at 204 nm. It allows a good simultaneous separation of *l*-hyoscyamine, *l*-scopolamine and tropic acid, their acidic precursor. The detection limits are 20 ng for the alkaloids and 5 ng for tropic acid. A simple and rapid method, very convenient for HPLC analysis, is also described for the preparation of purified alkaloid extracts. The procedure was applied to the evaluation of the alkaloid content of *Datura* leaves. The results are in good correlation with those obtained with a tropic acid derivatives-specific enzyme immunoassay.

INTRODUCTION

There are numerous reports in the literature about the analysis tropane alkaloids in plant material or for pharmacological studies. Various chromatographic methods have been used: thin-layer chromatography (TLC) [1,2], and other more sensitive methods such as gas chromatography (GC) using flame ionization detection (FID) or nitrogen–phosphorus detection (NPD) [3,4], or coupled with mass spectrometry (MS) [5–7]. High-performance liquid chromatography (HPLC) has also proved to be very useful for the analysis of tropane alkaloids [6–13]. These methods generally allow a good separation and a sensitive estimation of the components of plant extracts, but the extracts have to be purified prior to injection onto the HPLC column, resulting in a long assay time for a large number of samples.

Immunoassays, radioimmunoassay (RIA) and enzyme immunoassay (EIA) have also been developed specifically for atropine [14], hyoscyamine [15], scopolamine [16] or for all the tropic acid derivatives simultaneously [17]. These methods are selective and more rapid as purification of the plant extracts is unnecessary. The one drawback is that only one alkaloid or all the tropic acid derivatives together can be estimated, depending on the kind of antibodies used.

The present paper describes a new rapid and efficient reversed-phase HPLC procedure for the determination of the main tropane alkaloids, *l*-

* Corresponding author.

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hyoscyamine and *l*-scopolamine, as well as *dl*-tropic acid, one of their biogenetic precursors, which was not analysed by GC or by HPLC as previously reported. The method has been applied to the analysis of tropane alkaloids in *Datura stramonium* L. leaves.

EXPERIMENTAL

Apparatus and chromatographic conditions

The chromatographic system consisted of a Waters Model 510 pump operated at a flow-rate of 0.8 ml/min and a Rheodyne injector with 20 μl injection volume. The UV detector was a Waters 481 Lamba-Max spectrophotometer set at 204 nm wavelength. The column was a 150 mm × 4 mm Novapack C_{18} with 4-μm packing (Waters-Millipore, Saint Quentin en Yvelines, France) maintained at room temperature.

The mobile phase was a mixture of 12.5% aqueous acetonitrile supplemented with 0.3% (v/v) phosphoric acid adjusted to pH 2.2 with triethylamine. The mobile phase was filtered through an 0.45-μm nylon-66 Millipore filter and degassed prior to use.

The data were generated using a Shimadzu C-R6A integrator, which automatically integrated peaks areas and compared them with those of authentic standards, *l*-hyoscyamine, *l*-scopolamine and tropic acid obtained from Serva Fine Biochemicals (Saint Germain en Laye, France). All chemicals used for HPLC analysis were HPLC grade.

Extraction of tropane alkaloids

Several extraction conditions were compared: leaves of *Datura stramonium* L. were freeze-dried and powdered, and samples (100 mg each) were extracted with 25 ml of either 0.2 *M* aqueous sulphuric acid, methanol–1 *M* hydrochloric acid (24:1), methanol–27% ammonium hydroxide (24:1), methanol–chloroform–27% ammonium hydroxide (10:14:1) or chloroform–27% ammonium hydroxide (24:1). In each system, the alkaloids were extracted twice under reflux for 1 h. After filtration, each crude extract was evaporated to dryness under reduced pressure.

Purification of tropane alkaloids

Three purification processes were compared:

(1) A conventional process according to Kitamura *et al.* [18]. The crude extract was treated with 3 × 10 ml of 0.1 *M* sulphuric acid. The aqueous solutions were collected, filtered and the filtrate was basified to pH 9 with 27% ammonium hydroxide, then extracted by shaking with 3 × 20 ml of chloroform. Pooled extracts were dried (sodium sulphate) and evaporated to dryness under vacuum.

(2) Purification on a self-packed silica gel column. The crude alkaloid extract was resuspended in 10 ml of water at 40°C for 15 min. A 1-ml aliquot of this suspension adjusted to pH 7 was put on an 11 cm × 0.5 cm self-packed silica gel column made with 6 ml of silica gel, particle size 30 μm, obtained from Serva (France). The silica gel was degassed for 10 min under vacuum prior to packing. The column was connected to a peristaltic pump. Elution was successively performed with 10 ml of water and 10 ml of HPLC mobile phase supplemented with methanol (20%, v/v), first degassed. This last eluate fraction was kept for alkaloid analysis.

(3) Extraction of alkaloids from the crude extract with mobile phase. The extract was directly treated with 10 ml of the HPLC mobile phase at 40°C for 15 min and filtered before chromatographic analysis. This simplified procedure could be applied to the alkaline chloroformic extract but not to the methanolic one because too many polar compounds in this last extract would be reextracted with the mobile phase, inducing possible interferences during the chromatographic analysis.

Enzyme immunoassay of tropane alkaloids

Tropane alkaloids were also analysed by a competitive indirect enzyme immunoassay (EIA) using anti *dl*-tropic acid antibodies, as previously described [17]. This method allowed the simultaneous detection of all the alkaloids derived from *dl*-tropic acid and could be performed on crude or purified alkaloids as well as directly on dry-powdered plant material.

Capillary gas chromatography

The apparatus was a Girdel Series 30 chromatograph (Delsi Instruments, Suresnes, France) fitted with a 30 m × 0.32 mm I.D. capillary SE 30 basic column (0.25 μm film) (Supelco-Serlabo, Paris, France) and a flame ionization detector. Helium was used as carrier gas at an inlet pressure of 0.8 bar. Injector and detector temperature was 250°C. Oven temperature was 230°C. The results were given by Shimadzu C-R6A integrator.

RESULTS AND DISCUSSION

The HPLC method using a Novapack C_{18} column, an acidic aqueous acetonitrile mobile phase and UV detection at 204 nm as described in the Experimental section was applied to a standard solution of tropane alkaloids, *l*-hyoscyamine and *l*-scopolamine and *dl*-tropic acid, a precursor of the alkaloids (10^{-2} mg/ml of each). It allowed a good separation of the compounds: the retention times were 5.35 min for *l*-scopolamine, 7.35 min for *dl*-tropic acid and 11.7 min for *l*-hyoscyamine, as shown in Fig. 1. Detection at 204 nm increased the sensitivity 50-fold compared with that at 254 nm used by several authors [8–10]. Under these conditions, the working range of the assays was 20 ng to 5 μg for *l*-hyoscyamine and *l*-scopolamine and 5 ng to 1 μg for *dl*-tropic acid. The injection variability did not exceed 1–2% and the variations between several analyses of a same plant powder sample did not exceed 5%.

This procedure was applied to the analysis of plant material, which necessitated extraction and purification of alkaloids prior to HPLC analysis. Conditions for these preliminary steps were studied. To check that extraction under reflux did not modify the chemical structure of alkaloids, controls were performed: internal standard alkaloids added to plant powder sample indicated that these compounds were not converted to apoalkaloids or to dimer forms, since 98% of alkaloids added were recovered as original compounds. To determine the efficiency of various solvents in the extraction of tropane alkaloids, the freeze-dried powdered leaves of

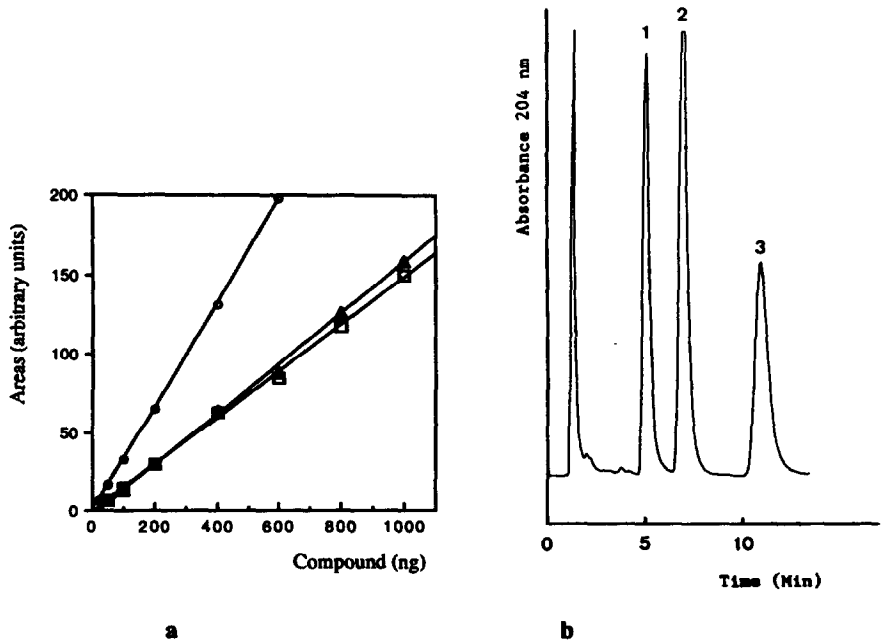

Fig. 1. HPLC analysis of a $5 \cdot 10^{-3}$ g/l standard solution of (1, □) *l*-scopolamine, (2, ○) *dl*-tropic acid and (3, ▲) *l*-hyoscyamine. (a) Calibration curves; (b) retention times: 1 = 5.35 min; 2 = 7.35 min; 3 = 11.7. Column packing: Novapack C_{18} 4 μm; mobile phase: 12.5% aqueous acetonitrile supplemented with 0.3% (v/v) phosphoric acid, pH 2.2 with triethylamine; flow-rate 0.8 ml/min; detection: UV 204 nm.

TABLE I

INFLUENCE OF THE SOLVENTS USED FOR EXTRACTION ON THE QUANTITY OF ALKALOID EXTRACTED (EXPRESSED AS mg PER g DRY MASS) FROM POWDERED LEAVES OF *DATURA STRAMONIUM*

Crude extracts and residues were analysed by ELISA. Each value represents the mean of five replicates. For details, see Experimental section.

Solvent	Alkaloids (mg per g dry mass)	
	Crude extract	Residue
Aqueous 0.1 *M* sulphuric acid	2.5 ± 0.17	0.11 ± 0.01
Methanol	2.3 ± 0.17	0.15 ± 0.01
Methanol–hydrochloric acid	2.4 ± 0.18	0.10 ± 0.01
Methanol–ammonium hydroxide	2.4 ± 0.17	0.11 ± 0.01
Methanol–chloroform–ammonium hydroxide	2.3 ± 0.17	0.10 ± 0.01
Chloroform–ammonium hydroxide	2.6 ± 0.15	0.12 ± 0.01

Datura stramonium were subjected to several extraction systems varying in pH and polarity (see Experimental section). Each extract and the corresponding residue, as well as the *Datura* powder, were submitted to EIA analysis. The alkaloid content of plant material was 2.9 ± 0.1 mg per g dry mass. The differences between the yield of alkaloids recovered with the various extraction conditions (2.3–2.6 mg/g dry mass) were not significant (Table I). The amounts of alkaloids remaining in the residues after two extraction steps were low (4–7% of the alkaloids detected in the extracts).

In order to control purification efficacy, an alkaline chloroform extract was purified by three different processes: (1) the conventional process, which used the amphoteric properties of the alkaloids [18]; (2) an elution from a silica gel column; and (3) a simple reextraction of the alkaloids from the dried crude extract with the polar HPLC mobile phase. Table II shows that the last process was quite efficient; it was also the quickest and easiest to perform. Thus, alkaloid extracts submitted to HPLC analysis were finally obtained by an alkaline chloroform extraction and a further reextraction of alkaloids from crude extract using HPLC mobile phase. Under these conditions, 50 μg per g dry mass was the detection limit of alkaloids in plant material.

One application of this HPLC procedure is the estimation of alkaloid accumulation in roots and leaves of *in vitro*-regenerated *Datura innoxia* [19]. Forty samples were analysed simultaneously by HPLC, GC and EIA. The results were compared. Mean values were 0.275 mg per g dry mass for HPLC, 0.2 mg per g dry mass for GC and 0.32 mg per g dry mass for EIA. For GC

TABLE II

COMPARISON OF EFFICIENCY OF THREE PURIFICATION PROCEDURES OF A CRUDE ALKALOID EXTRACT (PREPARED WITH ALKALINE CHLOROFORM) OF POWDERED LEAVES OF *DATURA STRAMONIUM*

Samples were analysed by HPLC. For details, see Experimental section. Each value represents the mean of four replicates.

	Conventional process	Purification on silica gel	Mobile phase extraction
Alkaloid content (mg per g dry mass)	2.6 ± 0.15	2.6 ± 0.12	2.8 ± 0.12

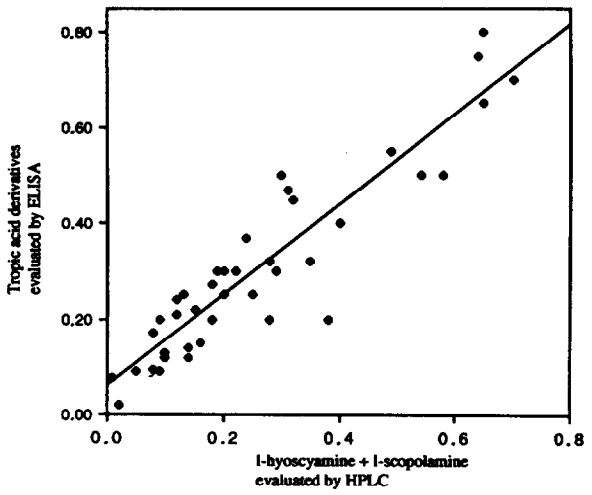

Fig. 2. Determination of the alkaloid content of *Datura innoxia* leaves by both HPLC and enzyme-linked immunosorbent assay. Comparison of the results. Units: mg/g dry mass. $y = 0.0581 + 0.9467x$; $R = 0.92$.

analysis, alkaloid extracts were necessarily prepared by the conventional process. This might partially explain the lower mean value found in this case. The results obtained with HPLC and EIA were more similar, and correlation of the results was determined and is presented in Fig. 2. The slightly higher values obtained by EIA (+16%) than by HPLC could be because tropic acid derivatives evaluated by EIA were not always only hyoscyamine and scopolamine.

CONCLUSIONS

The HPLC procedure presented in this paper is sensitive and allows a good simultaneous separation of hyoscyamine, scopolamine and one of their precursors, tropic acid. The procedure described for the preparation of the alkaloid extracts is easy to use and rapid. It has also previously been found to be efficient for the tobacco alkaloids [20], and could be of general use for the HPLC analysis of plant material containing alkaloids.

The method was applied to the evaluation of the tropane alkaloid content of *Datura* leaves and gave reliable results in good correction with those obtained by EIA. Thus the complementarity of the two methods is evident, EIA allowing a quick estimate of the total tropic acid derivatives in large series of plant samples, and HPLC giving the yield of each main tropane alkaloid in the plant material.

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