Determination of Hyoscyamine and Scopolamine in *Datura innoxia* Plants by High Performance Liquid Chromatography

Karin-Heide Plank and Karl G. Wagner

Gesellschaft für Biotechnologische Forschung mbH, Arbeitsgruppe Enzymologie, Mascheroder Weg 1, D-3300 Braunschweig, Bundesrepublik Deutschland

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For the determination of the hyoscyamine-scopolamine content in small quantities of fresh plant tissue an HPLC assay was developed; with a sensitivity of about 20 ng alkaloid per run. Analysis of the different organs of *Datura innoxia* plants are in accord with the concept that hyoscyamine is synthesized in the roots, whereas transformation to scopolamine starts in these organs and proceeds in the aerial parts of the plant.

Introduction

The main alkaloids of the tropane route are hyoscyamine and scopolamine; the latter is derived from the former by the incorporation of an epoxide oxygen [1]. Although there is agreement on the assumption that the root is the main locus of biosynthesis, there are questions concerning the contribution of the aerial parts, the translocation of the alkaloids and especially on the locus of the tranformation of hyoscyamine into scopolamine in the different *Datura* species [2].

Our interest in the biochemistry of this transformation initiated the present determination of the hyoscyamine-scopolamine ratio within the growing plant. We have developed an HPLC assay suitable for small quantities of fresh plant material. To date published data on the content of tropane alkaloids have been obtained by different methods, such as classical chromatography, paper and thin layer chromatography with colorimetric determination and gas-liquid chromatography. Furthermore a potentiometric determination, a radioimmune assay and even NMR spectroscopy have been reported [3–11]; these procedures have mostly used dried plant material. Our procedure is a modification of an extraction protocol for *Datura* cell suspension cultures [12] in combination with a HPLC protocol applied for the determination of nicotine from tobacco [13]. After completion of this work, an HPLC assay was described [14] based on a reversed phase matrix.

Reprint requests to Karl G. Wagner.

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Materials and Methods

100 to 400 mg fresh plant material was used for the assay; the volumes indicated below apply to 400 mg. The plant material was grounded with liquid N_2 in a morter and thereafter treated with 1.85 ml ethanol –25% aqueous NH_3 (20:1) for 30 min at ambient temperature. After centrifugation the extract was evaporated under N_2 at 45 °C. The residue was treated twice with 0.7 ml 0.1 N HCl; the combined extract was filtered through a Millipore filter (0.45 µ). After addition of 0.4 ml of 0.7 M Na_2CO_3 (pH 9.8) the solution was extracted twice with 3.0 ml $CHCl_3$. The combined $CHCl_3$ phases were evaporated and the residue dissolved in 185 µl 50% aqueous methanol. 20 µl were used for the HPLC determination.

When necessary a further purification step, using disposable columns filled with a RP 18 bonded-phase, was applied. 120 µl of the above extract were mixed with 880 µl 40% methanol and 20 µl 1 M Na_2CO_3 and applied to a 500 µl pipette RP 18 (from J. T. Baker Chemicals) which had previously been washed with 1 ml methanol and thereafter with 1 ml water. After application the pipette was washed with 1 ml 40% methanol (2 mM Na_2CO_3) and with 1 ml 30% methanol. The alkaloids were eluted with 1 ml acidic 100% methanol (10 mM HCl). After evaporation the residue was dissolved in 120 µl 50% methanol.

The HPLC system consisted of a LDC Consta Metric III pump, Rheodyne probe injector and Kontron Uvikon 725 monochromator. The alkaloids were separated on a 5 µm Merck Lichrosorb RP 18 reversed-phase column (25 cm × 4 mm) and eluted with an isocratic mobile phase composed of 60 parts of an aqueous solution of 0.2% phosphoric acid ad-

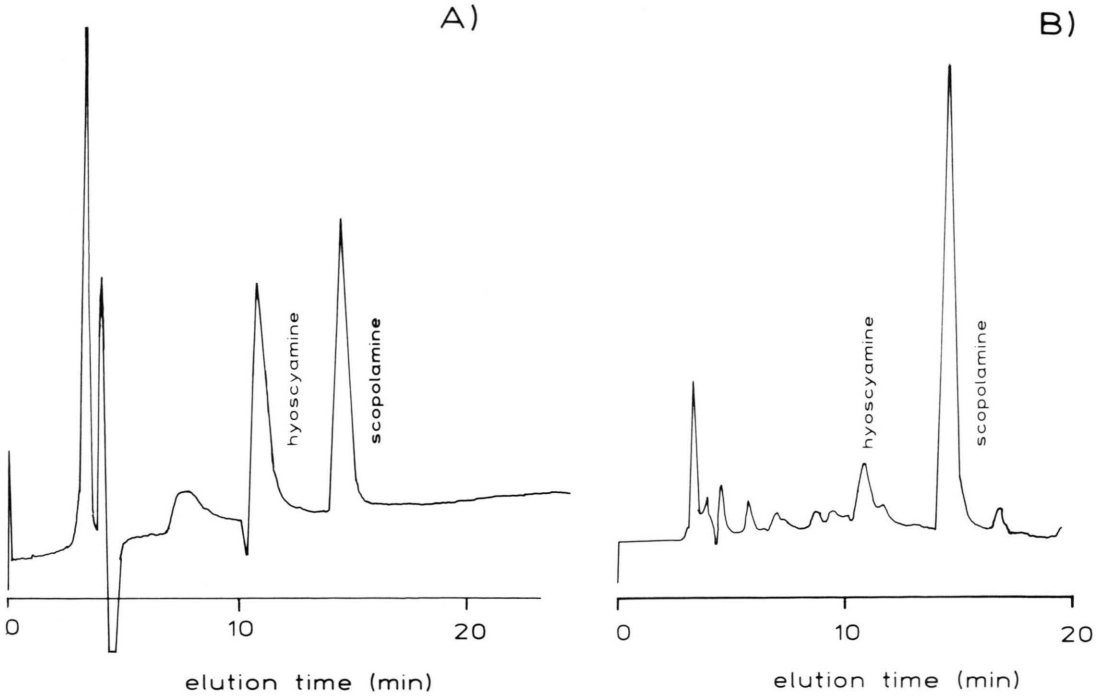

Fig. 1. Separation of hyoscyamine and scopolamine by reversed phase HPLC. The conditions are described in Materials and Methods. A) Separation of a standard mixture of 400 ng of each alkaloid. B) Separation of an extract of a root fraction (*cf.* Table II, plant 4, fraction 8–9 mm); 20 µl of the extract were injected.

justed to pH 7.25 with triethylamine and 40 parts methanol. The flow-rate was 0.5 ml/min and the detection was at 204 nm; the peaks were integrated with a Shimadzu printerplotter integrator and compared with authentic standards.

Results and Discussions

The extraction procedure was developed from a method described for the extraction of tropane alkaloids from cell suspension cultures [12]; the authors used thin layer chromatography for analysis. We applied a HPLC method which was developed for nicotine determination in fresh plant tissues [13]. The resolution of hyoscyamine and scopolamine was satisfying when a 5 µm RP 18 column was used. The sensitivity of detection was strongly enhanced by measurement at 204 nm, as the molar extinction coefficient of hyoscyamine and scopolamine in the near UV region around 260 nm is rather low. The detection limit was about 20 ng which is sufficient for the determination of hyoscyamine and scopolamine from differentiated plant tissues. The peaks were assigned by their elution times and by coelution (spiking) with reference compounds. The extraction of fresh plant material with ethanol-ammonia was performed at ambient temperature for 30 min; this procedure was also probed at elevated temperatures and increasing incubation times. Within the error of estimation, however, the same results were obtained as those from the standard conditions. The whole sample procedure was controlled with an artificial mixture of hyoscyamine and scopolamine. The recovery was within the range of the error of the estimation. Most of the data presented are from dublicate, in some cases triplicate, experiments, *i.e.* sample preparation and HPLC separation.

Datura innoxia plants were grown in a greenhouse; three plants about 4 months old which were just at the onset of blooming and one plant of a slightly younger cultivar were used for analysis. Although the data vary slightly from plant to plant and also from cultivar to cultivar the qualitative results are the same and consistent. In the tables the total alkaloid content (hyoscyamine plus scopolamine) in µg per g fresh weight and the percentage of hyoscyamine is listed.

Table I. Hyoscyamine-scopolamine content of different organs of *Datura innoxia* plants. The leaves are counted from the bottom. The main veins of the leaves were removed before analysis.

Plant organ	Plant 1 (leaf 6) Total content [µg/gFW]	Hyoscyamine [%]	Plant 2 (leaf 9) Total content [µg/gFW]	Hyoscyamine [%]
root	620	43	630	38
stem	240	15	640	8
leaf lamina	14	< 5	490	0

Table II. Hyoscyamine-scopolamine content of the roots of *Datura innoxia*.

Root fraction	Plant 3 Total content [µg/gFW]	Hyoscyamine [%]	Plant 4 Total content [µg/gFW]	Hyoscyamine [%]
hairy fraction	720	51	160	42
1–3 mm	1135	22	1080	26
2–4 mm	1310	21	420	27
8–9 mm	780	19	280	11

Table I shows the alkaloid content of the roots, the stem, and one leaf of two plants. Although the two plants differ in the ratio of the total alkaloid content of the root to the stem and the tested leaf, they both reveal that the percentage of hyoscyamine strongly decreases from the root to the aerial parts with a rather small content in the leaf lamina. These data confirm the results obtained from earlier work with classical methods [20, 24, 25]. Table II shows data for the roots of two plants which were divided into four fractions according to their diameter. Thus the four fractions represent sections of the roots from their tips (hairy fraction) to the thicker parts adjacent to the stem (8–9 mm). On a fresh weight basis the total alkaloid content is highest in the medium fractions (1–3 mm or 2–4 mm), whereas the hyoscyamine content decreases from about 50% in the tips to almost 10% in the parts near the stem.

It is generally expected that the tropane alkaloid biosynthesis takes place in the roots [1, 2]; the first product is hyoscyamine formed by condensation of tropine with tropic acid [1]. For the transformation of hyoscyamine into scopolamine different organs of the plant including the aerial parts have been discussed and the actual loci may vary with the different datura species [15–25]. The present data on alkaloid content and percentage of hyoscyamine in the different root fractions of *Datura innoxia* (Table II) reveal that about 50% of the hyoscyamine were already transformed in the thin hairy roots. As the hyoscyamine content decreases further in the thicker root parts, is obviously that about 80% of the hyoscyamine was already transformed into scopolamine in the roots. This transformation continues in the stem (Table I) and only a very small hyoscyamine content was found in the leaf lamina.

In order to elucidate the transport and deposition of the alkaloids in the aerial parts of the plants an analysis of the alkaloid content of whole leaves along a *Datura innoxia* plant was performed (Table III). The data clearly show that the total alkaloid content increases significantly from the older leaves to the younger ones by a factor of up to 40. Romeike [18,

Table III. Hyoscyamine-scopolamine content of whole leaves of *Datura innoxia*. The leaves are counted from the bottom; analysis were made with plant 4.

Leave position	5	6	7	8	10	11	12	13
total content								
µg/gFW	30	40	80	100	420	770	1060	1220
hyoscyamine %	12	11	9	18	3.5	4	5	3

Table IV. Hyoscyamine-scopolamine content of different leaf fractions of *Datura innoxia*. The leaves were counted from the bottom.

Leaf fraction	Leaf 7 (plant 3)		Leaf 9 (plant 4)	
	Total content [μg/gFW]	Hyoscyamine [%]	Total content [μg/gFW]	Hyoscyamine [%]
petiol	1125	10	270	5
main vein	1070	12	670	2
lamina	180	< 1	40	<5

20] has also reported a lower content in older leaves. Our data further show that the percentage of hyoscyamine decreases from the older leaves to the younger ones. Thus the youngest leaves have a rather high alkaloid content which is mostly scopolamine. With two plants (plant 3 and 4) an analysis of stem sections was performed (data not shown). These results also revealed that the total alkaloid content increases significantly towards the apex; however, the decrease in the hyoscyamine percentage towards the apex is less apparent.

Table IV shows data on the distribution of the alkaloids in the different parts of the leaf. On a fresh weight basis the concentration of the total alkaloids is high in the vascular parts, *i.e.* the petiol and the main vein, and rather low in the blade of the leaf. Furthermore, the percentage of hyoscyamine is rather low, at the limit of detection, in the lamina, whereas in the vascular parts it is as high as 12%. A similar distribution of the total alkaloid content in the various parts of the leaf has also been reported by Romeike [18]. However, when grafting *Cyphomandra* scions onto *D. innoxia* stocks the distribution was found to be reversed, with the highest alkaloid content in the lamina parts. These grafts also showed an inverse dependence on the leaf age [18] as found for the *D. innoxia* plants. Furthermore, the distribution of nicotine in the leaf of tobacco plants (*N. tabacum* Xanthi) is in striking contrast to the alkaloid distribution in the leaves of *D. innoxia*. We found a much higher concentration in the leaf lamina (760 to 850 μg/gFW) relative to that in the petiol and the main vein (120 to 240 μg/gFW). Furthermore, in contrast to *Datura innoxia* (Table III) the nicotine content in the leaves of the tobacco plant increases slightly with leaf senescence (data not shown).

It is possible to remove the bark portion from the stem of the *Datura innoxia* plant. An examination under the microscope showed that the bark contains parts of the phloem but no xylem. An analysis of bark and core revealed a slightly higher alkaloid concentration of the core (220 relative to 180 μgFW of the bark), whereas the percentage of hyoscyamine in the core was 24 relative to 11 in the bark. This may indicate a lower hyoscyamine ratio in the phloem relative to that of the xylem.

Conclusion

There are several open questions regarding the locus of the different biosynthetic activities, on the deposition and turnover of the tropane alkaloids. With the assumption that the epoxide oxygen of scopolamine is introduced into the already completed alkaloid hyoscyamine and that significant transport is only in the apical direction the present data are consistent with the following concept: synthesis of hyoscyamine occurs in the roots and transformation into scopolamine is performed to a large extent in the roots but also in the aerial vascular parts where the alkaloids are transported.

A second alternative would be based on the assumption that not only apical but also basipedal transport in the phloem could occur. In a very extreme case one could assume that transformation into scopolamine occurs mainly in the photosynthetic mesophyll cells of the leaves. In the case of *Datura ferox* there is strong evidence that the hyoscyamine-scopolamine transformation occurs mainly in the aerial parts of the plant [17, 19, 20, 22, 23]. With *D. innoxia* the situation may be similar but less stringent. In this case, one could interprete the data of the present work as indicating transport of newly synthesized hyoscyamine from the roots in the xylem and transport of transformed scopolamine from the leaves in the phloem back towards the roots. This view is supported by the findings of the low alkaloid concentrations in the leaf lamina, the high concentra-

tions in the vein and the lower hyoscyamine percentage of the phloem containing bark relative to the xylem containing core of the stem. The findings of the very low concentrations of alkaloids in the older leaves and the very high ones in the growing young leaves could also be reconciled with the assumption of an extensive transport of these alkaloids not only in the xylem but also in the phloem of the plant.

The quite different distribution of nicotine in the tobacco plant is in accord with the assumption that large amounts of this alkaloid are stored in the vacuole [26] and an extensive back-transport in the phloem does not occur. For the tropane alkaloids storage and hence transport may be different as shown by the values in the different parts of the leaves and the dependence on the leaf age. One should, however, also consider the results from histoautoradiography [27] which showed that tropane alkaloids are found in intercellular spaces of phloem and pit parenchyma and in glandular hairs of the epidermis. This indicates some sort of excretion mechanism. Furthermore, one should take into account the turnover of these alkaloids, although the results of Romeike [18] indicate that it may be rather low. Nevertheless, different turnover rates, *e.g.* higher rates for hyoscyamine than for scopolamine would also result in different hyoscyamine to scopolamine ratios as measured in the present work.

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