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Simultaneous analysis of hyoscyamine, scopolamine, 6β-hydroxyhyoscyamine and apoatropine in Solanaceous hairy roots by reversed-phase high-performance liquid chromatography

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Abstract

A new high-performance liquid chromatographic method is described for tropane alkaloid analysis in genetically transformed root cultures of *Datura innoxia* Mill. and *Atropa belladonna* L. Sample preparation, tropane alkaloid extraction with chloroform–methanol–concentrated ammonia 15:5:1 (v/v/v), was followed by solid-phase extraction on Supelclean LC-18 cartridges. Optimized conditions and careful pH control resulted in high recovery and reproducibility. Simultaneous determination of apoatropine, 6β-hydroxyhyoscyamine, hyoscyamine and scopolamine was performed by HPLC on C18 (2) reversed-phase column. The application of Luna new-generation silica-based stationary phase resulted in excellent peak shapes using an ion-pair reagent and triethanolamine free mobile phase and allowed to exploit the full power of pH-dependent selectivity. Simplicity and improved selectivity make this method a preferred alternative of published ion-pair chromatographic methods. Validation studies proved that the global method has good repeatability and satisfactory recovery. Absolute limits of detection were 0.6, 0.6, and 0.8 ng for hyoscyamine, 6β-hydroxyhyoscyamine, and scopolamine respectively.

Keywords: *Datura innoxia*; *Atropa belladonna*; Hyoscyamine; Scopolamine; 6β-Hydroxyhyoscyamine

1. Introduction

Hyoscyamine and scopolamine are medicinally important tropane alkaloids. Because of possessing anticholinergic and central nervous system activities, they have well-established use in therapy (in ophthalmology, cardiology, gastroenterology, etc.). They are synthesized in several Solanaceous plants of the genera *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus* and *Scopolia*. Industrially, these natural substances are exclusively produced by plants and the demand for them is continuous [1]. This prompted intensive studies on alkaloid biosynthesis, accumulation and production in plant breeding programmes in order to obtain alternative production systems of hyoscyamine and scopolamine. Transgenic root cultures, so-called hairy roots, have a great value in the latter aspect. Obtained by transformation via *Agrobacterium rhizogenes*, these cultures provide plant material with high growth rate on hormone-free media and alkaloid yields similar to or higher than that of untransformed plants [2,3]. Therefore, genetically transformed cultures of *Atropa belladonna* L. and *Datura innoxia* L. were established as sources of hyoscyamine and scopolamine respectively. Phytochemical characterization of these Solanaceous hairy roots requires sophisticated analytical methods for isolation, identification, and determination.

An excellent review on the current methods for tropane alkaloid analysis has been published recently [4]. Several works that deal with HPLC separation and the determination of hyoscyamine, scopolamine and related alkaloids in plant materials including hairy roots have also been published. In these studies RP18 was the preferred stationary phase, while others (RP8, RP6, RP-cyano) were rarely used [5–7]. Hyoscyamine and scopolamine from roots and leaves

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of *Datura stramonium* were determined at 204 nm, using a RP18 HPLC column eluted by buffered methanol–water mobile phase (40:60, v/v; pH 7.25, triethyl ammonium phosphate 0.2%) [8]. This method was optimized later for the separation of tropic acid, tropinone, 6β-hydroxyhyoscyamine, littorine, scopolamine, hyoscyamine and 3β-tigloyloxytropane [9]; and was applied recently to field-grown *Duboisia* plants regenerated from hairy roots [10]. An isocratic mixture of acetonitrile–water–acetic acid–tetrahydrofuran (50:50:5:2, v/v/v/v) was used as an eluent on a RP18 column when measuring hyoscyamine production in transformed roots of *D. stramonium* [11]. HPLC analysis of hyoscyamine, scopolamine and tropic acid in plant samples was achieved on a RP18 column with a 12.5% aqueous acetonitrile mobile phase buffered at pH 2.2 with 0.3% (v/v) phosphoric acid and triethylamine [12]. The method was applied to micropropagated plants [13] and immobilised cells of *D. innoxia* [14]. Hyoscyamine and scopolamine in adventitious root cultures of *Scopolia parviflora* were determined after separation on a RP18 column eluted with an isocratic mixture of acetonitrile-50 mM potassium phosphate (22:78, v/v) adjusted to pH 3.0 with phosphoric acid [15]. Though triethylamine was frequently used, peak tailing remained unsolved, especially working at neutral pH to increase the retention and selectivity [8,9]. Tropane ester alkaloids are moderately strong bases and they remain partially/fully protonated in neutral buffers. Therefore, they can interact with dissociated silanol groups on the RP columns leading to excessive tailing. At low pH (2–3) where only the most acidic silanol groups are ionized peak tailing can be significantly reduced. In general, the silanol interactions with the basic analytes are considered to be the causes of the broad and tailing peaks that are often observed [16,17].

Ion-pair chromatography (IPC) on RP columns was successfully applied to improve peak shape and selectivity [18–20], allowing baseline separation of 7β-hydroxyhyoscyamine, 6β-hydroxyhyoscyamine, scopolamine, hyoscyamine and littorine from hairy roots [21,22]. However, IPC separations are more complicated to develop and use than reversed-phase ones [23], and can be incompatible with LC–MS analysis due to the ion-pair reagent [24]. Further, most of the reviewed RP HPLC methods used conventional C18 columns based on an old, less pure silica material. Metal impurities will increase the acidity of the free silanols, leaving them in a charged state even at low pH [25–27]. Most modern columns are made from high-purity silica with the absence of or low content of metals. Additionally, these new-generation columns can be endcapped to decrease the number of surface silanols and ensure low silanol activity [28]. Therefore, a new HPLC method was developed using Phenomenex Luna C8 column and reversed-phase conditions for the determination of apoatropine, scopolamine and hyoscyamine in *A. belladonna* hairy roots [29]. This new generation column is manufactured with high-purity silica, dense bonded phase coverage and exhaustive endcapping. The used bonding process produces a uniform, dense "hydrophobic shield" around the basic silica. Therefore, this column can provide good peak shape and chemical stability over a broad pH range and these properties were utilized for our samples working at optimized pH (6.20). Investigations also revealed that Luna C18(2) column provides additional selectivity for *D. innoxia* hairy root samples which are rich in components. Further optimization resulted in a new HPLC method for improved separation and determination of apoatropine, 6β-hydroxyhyoscyamine, scopolamine and hyoscyamine in both *A. belladonna* and *D. innoxia* hairy roots. This method is presented here including solid phase extraction (SPE) on C18 column, method development, validation and application.

2. Materials and methods

2.1. Chemicals

L-Hyoscyamine, scopolamine $HBr{\cdot}3H_2O$ and apoatropine were purchased from Merck (Darmstadt, Germany), 6β-hydroxyhyoscyamine (anisodamine) was obtained from Hangzhou Drug Inc. (Hangzhou, China). Acetonitrile and methanol were of HPLC grade and were obtained from Carlo Erba (Milan, Italy). All other chemicals were also of analytical quality and were supplied by Reanal (Budapest, Hungary). Water was purified with a Millipore Milli-Q^{TM} 5 equipment (Millipore, Billerica, MASS. USA).

2.2. Alkaloid extraction

Lyophilized and powdered plant material (200–500 mg) was extracted once with 25 ml and three times with 20 ml of chloroform–methanol–concentrated ammonium hydroxide (15:5:1, v/v/v) with sonication at $27 \pm 2\,^{\circ}C$ (Braun Labsonic U, Melsungen, Germany) for 4×10 min. Fractions were filtered under vacuum over a 3-cm Buchner funnel with a filter paper (Whatman No. 5). Combined filtrates were dried over anhydrous Na_2SO_4 (1.0 g) and were evaporated to dryness under reduced pressure (Büchi Rotavapor R-200, Flawil, Switzerland) at 50 °C.

2.3. SPE method

For the SPE procedure, Supelclean LC-18 columns (500 mg, 3 ml), supplied by Supelco Inc. (Bellefonte, PA, USA), were used. Extraction was performed on a 12-port vacuum manifold processor (Lichrolut extraction unit, Merck).

Samples were dissolved in 1.25 ml of methanol, diluted with 3.75 ml of phosphate buffer solution of pH 8.0 (0.053×30 mM/0.947×30 mM KH_2PO_4/K_2HPO_4) and were homogenized by sonication. After centrifugation at 6000 rpm for 10 min ($2500 \times g$), the supernatant was loaded onto octadecyl SPE microcolumn activated previously with methanol (5 ml), followed by bidistilled water (5 ml) and by 30 mM phosphate buffer, pH 8.0 (5 ml). The support was then

washed with 2.5 ml methanol–3 mM phosphate buffer, pH 8.0 (25:75, v/v) and 2.5 ml water to remove matrix. When the solution had passed through, the column was dried by maintaining the vacuum in the SPE manifold for 1 min. The tropane alkaloids were eluted with 1 ml of 0.2% TFA, followed by 7.5 ml of methanol 0.2% in TFA-water (98:2, v/v) at a flow rate of 0.5 ml/min. Samples were brought to a final volume of 10.00 ml with methanol.

2.4. Recovery test

2.4.1. Standard solution

A stock solution containing hyoscyamine, scopolamine, 6β-hydroxyhyoscyamine and apoatropine (each 400 μg/ml) in methanol was prepared. This solution was diluted four times with phosphate buffer (30 mM; pH 8.0) and 5.00 ml aliquots were submitted to previously described SPE procedure. For standard solution, recovery was calculated as percentage of initial standard amount applied on SPE column.

2.4.2. Fortified samples

Six identical samples of hairy root dry extract were prepared, each corresponding to 250 mg plant material. The samples (3–3) were dissolved either in 1.25 ml methanol or 1.25 ml stock solution of standards, diluted with 3.75 ml of phosphate buffer (30 mM; pH 8.0). After homogenization they were submitted to SPE procedure described previously. The recovery (R) was calculated as $R = 100(C_{found} - C_{initial})/C_{added}$; ($C_{found}$, concentration in fortified samples; $C_{initial}$, initial concentration in sample; C_{added}, added concentration).

2.5. Extraction efficiency

The completeness of the extraction was determined using six successive extractions of *D. innoxia* hairy roots (500 mg). The first four extracts were obtained following the protocol described in Section 2.2. Further, successive extractions were performed using 20–20 ml solvent for 10–10 min. Only small quantities of the alkaloids were detected in the fifth extract: 2.4% (±0.5%, $n = 3$) 6β-hydroxyhyoscyamine, 0.5% (±0.2%, $n = 3$) scopolamine and 1.2% (±0.2%, $n = 3$) hyoscyamine. Apoatropine was not detected. (Results are expressed as percentages of the alkaloid contents measured in the combined first four extracts). No analytes were detected in the sixth extract.

2.6. Apparatus and HPLC conditions

HPLC analyses were performed on a Surveyor (Thermo Finnigan, San Jose, CA, USA) HPLC system consisting of a quaternary gradient pump with an integrated vacuum degasser, a PDA detector and an autosampler. Thermo Finnigan ChromQuest 4.0 software was used for data acquisition, processing, and reporting. The separation was performed on a Phenomenex Luna 5 μm C_{18} (2) reversed-phase column (250 mm × 4.6 mm I.D., Torrance, CA, USA) coupled with a Phenomenex SecurityGard C_{18} guard column (8 mm × 3 mm I.D., Torrance, CA, USA). The column temperature was 30 °C. The injection volume was 5 μl. The mobile phase consisted of acetonitrile–methanol–30 mM KH_2PO_4–K_2HPO_4 (815:185, g/g) buffer solution, pH 6.00 (12:7.9:80.1, v/v/v). The flow rate was 1.0 ml/min. Peaks were identified by standard addition and diode-array detection.

2.7. Standard solutions

A stock solution containing 20.00 mg amounts of hyoscyamine, scopolamine, and 6β-hydroxyhyoscyamine and 10.00 mg amount of apoatropine in methanol was prepared in a 25 ml volumetric flask. The standard solutions containing apoatropine 4, 8, 20, 40, 60, 100 μg/ml, scopolamine, hyoscyamine, and 6β-hydroxyhyoscyamine 8, 16, 40, 80, 120, 200 μg/ml were obtained by serial dilution of the stock solution with methanol, 0.1% trifluoroacetic acid. Stock solution and working solutions were stored at 4 °C and brought to room temperature before use.

2.8. Plant material

In vitro cultures of *D. innoxia* Mill. were established as earlier described [30]. Among hairy roots cultivated on antibiotic-free MS solid medium [31], clones #410, #411 and #415 were selected for further investigations according to their growth and tropane alkaloid production. These clones were transferred to 40 ml Ms liquid medium and cultivated (100 ml Erlenmeyer flask) at 24 ± 1 °C, in the dark and in a climatised shaker apparatus (Certomat BS 4, B. Braun, Melsungen, Germany) at 100 rpm.

A. belladonna L. hairy roots were established as already described [32]. These cultures were grown in petri dishes on solid B5 medium [33] supplemented with 2% sucrose. The best alkaloid producing hairy root clones (#K4, #K5, #K8) were selected and cultivated in B5 liquid medium and used for further investigations.

3. Results and discussion

3.1. SPE method

As strong interferences from sample matrix are expected at low UV wavelengths (205–210 nm), where sensitive detection of tropane alkaloids is possible, the enhanced purification of hairy root extracts is important. Solid phase extraction is frequently applied for tropane alkaloid analyses in plant material. While Extrelut sorbent is highly popular [34–42], C_{18} sorbent is less frequently used [8,43–46]. Though Extrelut produces fairly clean extracts, C_{18} sorbent can protect the

Table 1
Mean recoveries of apoatropine, 6β-hydroxyhyoscyamine, scopolamine and hyoscyamine obtained on SPE octadecyl columns for pure standards (500 μg)

Compound	Found[a] ± SD (μg)	Recovery (%)
Apoatropine	501 ± 6.6	100.2
6β-Hydroxyhyoscyamine	486 ± 6.1	97.2
Scopolamine	488 ± 5.7	97.6
Hyoscyamine	484 ± 4.7	96.8

SPE conditions, see in Section 2.3.
[a] Mean of six replicate analyses.

analytical column from strongly retained interferences (column killers), because it utilizes the same separation mechanism. Using Extrelut SPE, the HPLC sequence runs happened to stop, because of increased noise level or pressure, and we had to change precolumn more frequently. These difficulties disappeared when SPE was performed on Supelco LC-18 micro column with samples dissolved in methanol: 30 mM phosphate buffer (25:75, v/v; pH 8.0). Under the selected alkaline conditions, buffer concentration and pH, alkaloids were retained on the column during the sample application and washing steps. To ensure the full elution of tropane alkaloids, different solvent combinations were evaluated for pH control. In this way optimal conditions were obtained resulting in high recoveries of hyoscyamine, scopolamine, 6β-hydroxyhyoscyamine and apoatropine with good reproducibility both for pure standards and fortified samples (Tables 1 and 2).

3.2. Optimization of separation

Tropane alkaloid separations were performed on Phenomenex Luna new-generation silica-based columns (C_8, then C_{18}) to find simple operating conditions at optimized pH allowing the use of a mobile phase free of ion-pair reagents and triethanolamine.

3.2.1. Mobile phase

3.2.1.1. Solvent type selectivity. It was investigated on a C_8 column with eluents as mixtures of an organic solvent and 0.1% TFA in water. Acetonitrile provided well resolved peaks, but the time required for the separation of four tropane alkaloids was relatively long (23 min); on the other hand methanol produced partly resolved peaks for scopolamine and apoatropine. The critical peak pairs were different for methanol and for acetonitrile (scopolamine/6β-hydroxyhyoscyamine), therefore, these solvents can be combined to obtain the required resolution. Full separation of apoatropine, scopolamine, 6β-hydroxyhyoscyamine and hyoscyamine, was achieved using a 10.5:7:82.5 (v/v/v) mixture of acetonitrile, methanol and 0.1% TFA as eluent (Fig. 1A and D). Methanol added to the mobile phase helped to fine tune the position of scopolamine and decreased the separation time (18.22 min).

3.2.1.2. pH. The separation was further improved by using ammonium acetate buffered (50 mM) mobile phase at optimized pH (6.00). (Fig. 1B and E). The retention time changed dramatically for scopolamine, but not for the other tropanes, due to their different pK_a values (7.6 for scopolamine, ∼8.9 for 6β-hydroxyhyoscyamine, 9.7 for hyoscyamine and ∼10.0 for apoatropine [4]).

3.2.2. Column-type selectivity

Changing the column type revealed that apoatropine and 6β-hydroxyhyoscyamine were better separated on the C_{18} column, than on the C_8 one. Therefore, the former column was selected for further studies. The fine tuned mobile phase was a 12 + 7.9 + 80.1 (v/v/v) mixture of acetonitrile, methanol, 30 mM phosphate buffer, pH 6.00 (Fig. 1C). The same mobile phase on conventional non-endcapped C_8 column, Hypersil MOS produced very poor peak shape for apoatropine (As 5% = 4.1) and hyoscyamine (As 5% = 2.2). In combination with modern Luna C18 column the selected eluent provided excellent peak shapes and improved separation of apoatropine (R_t = 9.15 min), 6β-hydroxyhyoscyamine (10.60 min), scopolamine (15.07 min) and hyoscyamine (18.56 min). The last three alkaloids can be separated only with retention times, 14.3 min, 15.0 min, and 19.0 min respectively using an improved version of ion-pair chromatographic methods [21]. Therefore, our method can be considered as more selective for these tropane alkaloids. Fig. 1F shows the chromatogram of *D. innoxia* hairy root extract. Peeks corresponding to tropane alkaloids are

Table 2
Mean recoveries of apoatropine, 6β-hydroxyhyoscyamine, scopolamine and hyoscyamine obtained on SPE octadecyl columns for fortified samples of *D. innoxia* hairy roots

Compound	Initial amount[a] ± SD (μg)	Added μg	Found[b] ± SD (μg)	Recovery (%)
Apoatropine	36 ± 0.6	500	504 ± 7.0	93.6
6β-Hydroxyhyoscyamine	784 ± 20.5	500	1272 ± 17.3	97.6
Scopolamine	549 ± 8.2	500	1024 ± 14.2	95.0
Hyoscyamine	1494 ± 13.9	500	1982 ± 18.9	97.6

SPE conditions see in Section 2.3.
[a] Means of three replicate analyses.
[b] Means of three replicate analyses.

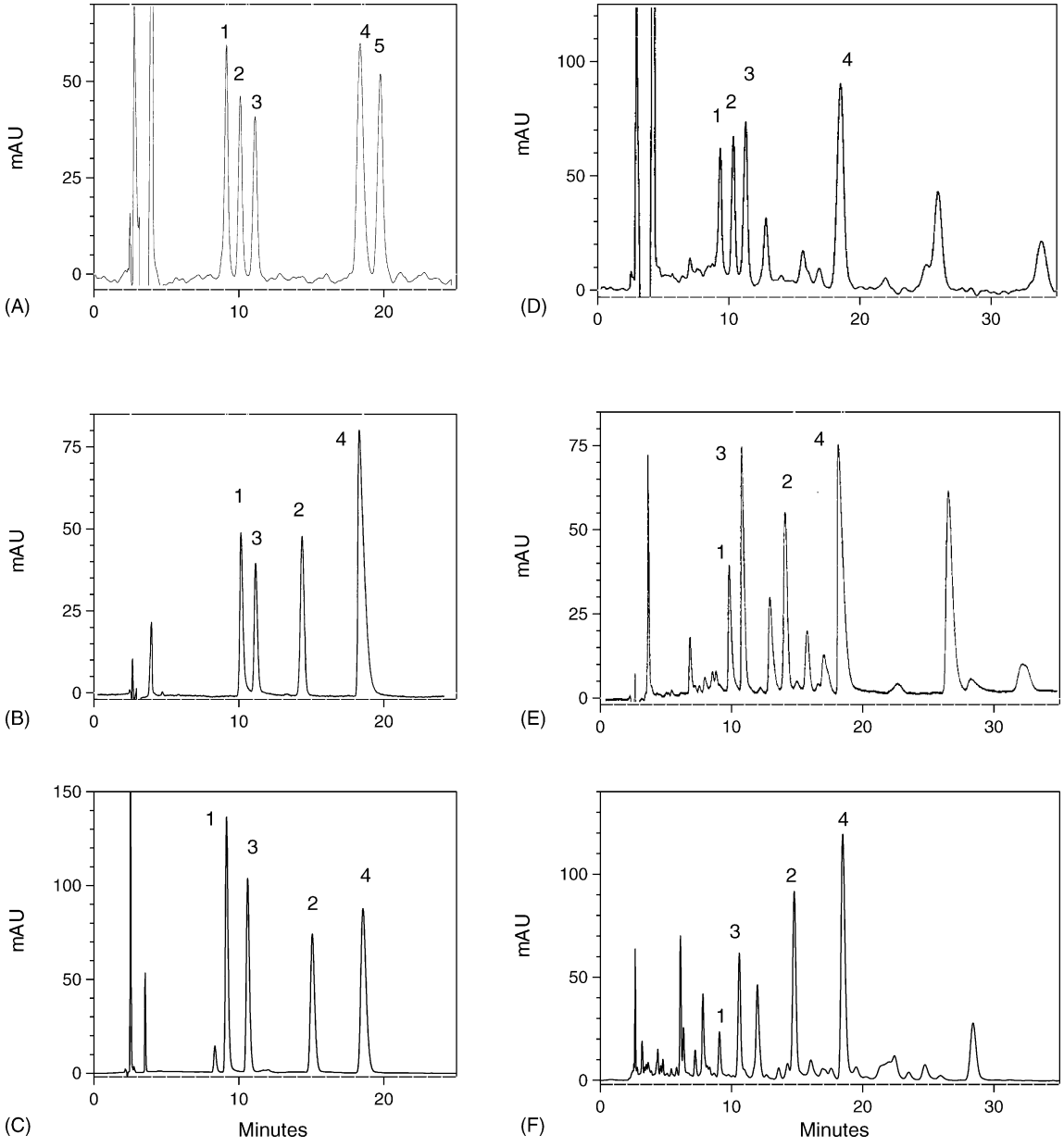

Fig. 1. HPLC-DAD chromatogram of standards (A)–(C) and *D. innoxia* hairy root extracts (D)–(F); detection at 210 nm. Peaks: 1: apoatropine, 2: scopolamine, 3: 6β-hydroxyhyoscyamine, 4: hyoscyamine, 5: tropic acid. (A), (D) Luna C_8 column. Mobile phase: acetonitrile–methanol–0.1% TFA in water (10.5:7:82.5, v/v/v). (B), (E) Luna C_8 column. Mobile phase: acetonitrile–methanol–50 mM ammonium acetate, pH 6.00 (12.7:8.5:78.8, v/v/v). (C), (F) Luna C_{18} column. Mobile phase: acetonitrile–methanol–30 mM K_2HPO_4–KH_2PO_4 buffer solution, pH 6.00 (12:7.9:80.1, v/v/v); flow rate: 1 ml/min.

well resolved from other co-extracted materials. The application of an ion-pair reagent and triethanolamine free mobile phase ensured greater convenience in method development and resulted in excellent column performance for a long period of time (Tables 3 and 4). The pH was controlled within narrow limits (±0.02 units) by accurate measurement of buffer ingredients. As a result, retention times did not change by more than 3% over time. The application of potassium phosphate buffer instead of ammonium acetate resulted in a fourfold increase in detection sensitivity.

3.3. Method validation and application

Quantitative determination of apoatropine, 6β-hydroxyhyoscyamine, scopolamine, and hyoscyamine was performed by an external standard method at 210 nm. A good relationship was obtained between the injected concentration (x) and the uncorrected peak area (y) for each component of calibration solutions within the investigated range. The calculated linear regression parameters for calibration curves are given in Table 5. Absolute limit of detection (LOD) was 0.3 ng for apoatropine, 0.6 ng for 6β-hydroxyhyoscyamine and

Table 3
HPLC repeatability (RSD, $n = 6$) of peak areas for alkaloid standard solutions without SPE clean up

Concentration of standards (μg/ml)	Peak area	
	Mean ± SD × 10^3	RSD (%)
Apoatropine		
20.00	597 ± 4.95	0.8
40.00	1190 ± 7.42	0.6
60.00	1806 ± 3.83	0.2
100.00	2985 ± 4.16	0.1
6β-Hydroxyhyoscyamine		
40.00	592 ± 1.72	0.3
80.00	1150 ± 2.19	0.2
120.00	1727 ± 2.77	0.2
200.00	2835 ± 7.08	0.3
Scopolamine		
40.00	719 ± 3.31	0.5
80.00	1434 ± 2.30	0.2
120.00	2170 ± 2.82	0.1
200.00	3550 ± 8.52	0.2
Hyoscyamine		
40.00	701 ± 1.55	0.2
80.00	1399 ± 2.33	0.2
120.00	2118 ± 4.04	0.2
200.00	3495 ± 8.09	0.2

Chromatographic conditions, see in Section 2.6.

Table 4
HPLC repeatability (RSD, $n = 6$) of peak areas for two SPE purified extracts of *D. innoxia* hairy roots

Concentration of alkaloids (μg/ml)	Peak area	
	Mean ± SD × 10^3	RSD (%)
Apoatropine		
4.98	149 ± 2.40	1.6
9.70	287 ± 9.79	3.4
6β-Hydroxyhyoscyamine		
39.30	578 ± 2.37	0.4
121.93	1752 ± 12.60	0.7
Scopolamine		
34.56	624 ± 11.62	1.9
122.16	2206 ± 32.52	1.5
Hyoscyamine		
39.82	699 ± 15.45	2.2
145.59	2554 ± 16.61	0.7

Chromatographic conditions see in Section 2.6.

Table 6
Precision (RSD, $n = 6$) of the HPLC method using *D. innoxia* hairy roots

Compound	Alkaloid content (μg/g)	
	Mean ± SD	RSD (%)
Apoatropine	190 ± 16	8.4
6β-Hydroxyhyoscyamine	2390 ± 78	3.3
Scopolamine	2330 ± 114	4.9
Hyoscyamine	2820 ± 98	3.5

hyoscyamine, and 0.8 ng for scopolamine on the chromatographic system with a signal-to-noise ratio, $S/N > 3$. Reported ion-pair chromatographic methods provided approximately 10 ng as LOD for the last three alkaloids [18,20]. Intra assay precision was determined by analysing six identical samples of *D. innoxia* hairy roots. The results are given in Table 6 and are corresponding to the intended use of the method. The whole procedure was validated for *D. innoxia* hairy root samples at 1000 μg/g level. Therefore, homogenized hairy root samples were spiked with adequate methanol solution volume before following the described alkaloid extraction procedure. Three replicate analyses were run for both the fortified samples and the matrix tested. Overall average recoveries were 94.2% for hyoscyamine, 95.0% for scopolamine, 85.2 for 6β-hydroxyhyoscyamine and 85.4% for apoatropine (Table 7). Results obtained for hyoscyamine and scopolamine are corresponding to their SPE recovery. On the other hand, the small initial amount of apoatropine and higher water solubility of 6β-hydroxyhyoscyamine can explain their lower recovery in the total procedure.

This method was applied for the chemical analysis of *A. belladonna* and *D. innoxia* hairy root cultures cultivated for 5 weeks in the dark. Results (Table 8) reflect that there are some differences in the biosynthetic capacity of clones of the two species. Still hyoscyamine levels are comparable, about ten times higher scopolamine and three times higher 6β-hydroxyhyoscyamine contents were measured in *D. innoxia* clones. The low scopolamine level in *A. belladonna* hairy roots is a characteristic of this species, intact plants are considered as source of hyoscyamine. The best alkaloid producer, a *D. innoxia* clone (#410-D), selected for further alkaloid production studies, contained 5050 μg/g hyoscyamine, 1890 μg/g scopolamine, 2170 μg/g 6β-hydroxyhyoscyamine, and 306 μg/g apoatropine.

Table 5
Regression parameters of calibration lines (area = $A + BX$)

Compound	X range (μg/ml)	A	B	r	n[a]
Apoatropine	4–100	−2322.29	29962.10	0.99997	6
6β-Hydroxyhyoscyamine	8–200	19559.60	14208.40	0.99977	6
Scopolamine	8–200	−52.16	18058.80	0.99996	6
Hyoscyamine	8–200	−2053.73	17633.40	0.99995	6

[a] n: number of calibration points (each calibration solution measured in duplicate in three separate runs).

Table 7
Mean recoveries of apoatropine, 6β-hydroxyhyoscyamine, scopolamine and hyoscyamine obtained for the global method

Compound	Initial amount[a] ± SD (μg)	Added (μg)	Found[b] ± SD (μg)	Recovery (%)
Apoatropine	163 ± 3.6	500	590 ± 4.0	85.4
6β-Hydroxyhyoscyamine	566 ± 8.2	500	992 ± 5.2	85.2
Scopolamine	745 ± 18.6	500	1220 ± 25.4	95.0
Hyoscyamine	1085 ± 20.8	500	1556 ± 56.5	94.2

[a] Means of three replicate analyses.
[b] Means of three replicate analyses.

Table 8
Alkaloid contents (mean ± SD; $n = 3$) in *D. innoxia* (D) and *A. belladonna* (B) hairy root clones as determined by HPLC

Clone	Hyoscyamine (μg/g)	Scopolamine (μg/g)	6β-Hydroxyhyoscyamine (μg/g)	Apoatropine (μg/g)
410-D	5050 ± 710	1890 ± 175	2170 ± 440	306 ± 20
411-D	2400 ± 190	1440 ± 145	1870 ± 130	186 ± 40
415-D	4670 ± 770	1860 ± 430	2450 ± 50	60 ± 15
4-B	3880 ± 215	230 ± 30	675 ± 110	n.d.
5-B	3270 ± 570	152 ± 20	590 ± 10	n.d.
8-B	2970 ± 590	225 ± 23	645 ± 10	n.d.

n.d.: not detected.

4. Conclusion

A new HPLC method using a mobile phase free of ion-pair reagents and triethanolamine is described for the simultaneous determination of apoatropine, 6β-hydroxyhyoscyamine, scopolamine and hyoscyamine in *D. innoxia* and *A. belladonna* hairy root cultures. This method is simple, reproducible and sensitive. Simplified operating conditions and improved selectivity, in particular for 6β-hydroxyhyoscyamine and scopolamine, make this method more efficient for tropane alkaloid analysis than ion-pair chromatographic methods applied earlier. As a reliable method, it is practicable for the chemical selection of hairy root clones overproducing tropane alkaloids and alkaloid production/medium optimization studies.

References

[1] K.M. Oksman-Caldentey, R. Arroo, in: R. Verpoorte, A.W. Alfermann (Eds.), Metabolic Engineering of Plant Secondary Metabolism, Kluwer Academic Publishers, Dordrecht, The Netherlands, 2000, p. 253.
[2] A. Giri, M.L. Narasu, Biotechnol. Adv. 18 (2000) 1.
[3] A. Lanoue, M. Boitel-Conti, J.-C. Portais, J.-C. Laberche, J.-N. Barbotin, P. Christen, B. Sangwan-Norreel, J. Nat. Prod. 65 (2002) 1131.
[4] B. Dräger, J. Chromatogr. A 978 (2002) 1.
[5] M. Friedman, C.E. Levin, J. Agric. Food Chem. 37 (1989) 998.
[6] M.G.P. Mahagamasekera, P.M. Doran, Phytochemistry 47 (1998) 17.
[7] J. Palazón, E. Moyano, R.M. Cusidó, M. Bonfill, K.M. Oksman-Caldentey, M.T. Piñol, Plant Sci. 165 (2003) 1289.
[8] K.-H. Plank, K.G. Wagner, Z. Naturforsch. Ser. C 41c (1986) 391.
[9] E. Gontier, B.S. Sangwan, J.N. Barbotin, Agro-Food-Ind. Hi-Tech. 5 (1994) 26.
[10] C.R. Celma, J. Palazón, R.M. Cusidó, M.T. Piñol, M. Keil, Planta Med. 67 (2001) 249.
[11] J. Payne, J.D. Hamill, R.J. Robins, M.-J.C. Rhodes, Planta Med. 53 (1987) 474.
[12] M.A. Fliniaux, F. Manceau, A. Jacquin-Dubrenil, J. Chromatogr. 644 (1993) 193.
[13] E. Gontier, M.A. Fliniaux, J.N. Barbotin, B.S. Sangwan-Norreel, Planta Med. 59 (1993) 432.
[14] E. Gontier, B.S. Sangwan, J.N. Barbotin, Plant Cell Rep. 13 (1994) 533.
[15] S.-M. Kang, H.-Y. Jung, Y.-M. Kang, D.-J. Yun, J.-D. Bahk, J.-K. Yang, M.-S. Choi, Plant Sci. 166 (2004) 745.
[16] D.V. McCalley, J. Chromatogr. A 967 (2002) 1.
[17] S.M.C. Buckenmaier, D.V. McCalley, M.R. Euerby, J. Chromatogr. A 1060 (2004) 117.
[18] T. Oshima, K. Sagara, Y.Y. Tong, G. Zhang, Y.H. Chen, Chem. Pharm. Bull. 37 (1989) 2456.
[19] Y. Mano, H. Ohkawa, Y. Yamada, Plant Sci. 59 (1989) 191.
[20] L.Y. He, G.D. Zhang, Y.Y. Tong, K. Sagara, T. Oshima, T. Yoshida, J. Chromatogr. 481 (1989) 428.
[21] F. Nakanishi, K. Sasaki, K. Shimomura, Plant Cell Rep. 18 (1998) 249.
[22] M. Sauerwein, M. Wink, K. Shimomura, J. Plant Physiol. 140 (1992) 147.
[23] L.R. Snyder, J.J. Kirkland, J.L. Glajch, Practical HPLC Method Development, second ed., Wiley-Interscience, New York, NY, 1997, p. 317.
[24] P. Sandra, G. Vanhoenacker, F. Lynen, L. Li, M. Schelfaut, LC-GC European Guide to LC-MS, 2001, p. 8.
[25] J. Nawrocki, Chromatographia 31 (1991) 177.
[26] J. Nawrocki, Chromatographia 31 (1991) 193.
[27] U.W. Neue, C.H. Phoebe, K. Tran, Y. Cheng, Z. Lu, J. Chromatogr. A 925 (2001) 49.
[28] J.M. Herrero-Martinez, A. Méndez, E. Bosch, M. Rosés, J. Chromatogr. A 1060 (2004) 135.
[29] H. Hank, É. Szőke, K. Tóth, I. László, L. Kursinszki, Chromatogr. Suppl. 60 (2004) S55.
[30] I. László, É. Szőke, Zs. Németh, L. Kursinszki, L. Albert, E. Tyihák, in: S.A. Kiss (Ed.), Magnesium and Interaction of Magnesium with Trace Elements., Hungarian Chemical Society, Budapest, 1998, p. 353.
[31] T. Murashige, F. Skoog, Physiol. Plant. 15 (1962) 473.
[32] I. Bálványos, K. Vida, K. Tóth, É. Szőke, in: Y. Rayssiguier, A. Mazur, J. Durlach (Eds.), Advances in Magnesium Research: Nutrition and Health, John Libbey and Co. Ltd., London, 2001, p. 491.

[33] O.K. Gamborg, R.A. Miller, K. Ojima, Exp. Cell Res. 50 (1968) 151.
[34] T. Hartmann, L. Witte, F. Oprach, G. Toppel, Planta Med. 52 (1986) 390.
[35] L. Witte, K. Müller, H.A. Arfmann, Planta Med. 53 (1987) 192.
[36] B. Dräger, A. Schaal, Phytochemistry 35 (1994) 1441.
[37] A. El-Shazly, A. Tei, L. Witte, M. El-Domiaty, M. Wink, Z. Naturforsch. 52c (1997) 729.
[38] L. Mateus, S. Cherkaoui, P. Christen, K.M. Oksman-Caldentey, Phytochemistry 54 (2000) 517.
[39] N. Khanam, C. Khoo, R. Close, A.G. Khan, Phytochemistry 56 (2001) 59.
[40] P. Rocha, O. Stenzel, A. Parr, N. Walton, P. Christou, B. Dräger, M.J. Leech, Plant Sci. 162 (2002) 905.
[41] A. Namera, M. Yashiki, Y. Hirose, S. Yamaji, T. Tani, T. Kojima, Forensic Sci. Int. 130 (2002) 34.
[42] G. Rothe, B. Dräger, Plant Sci. 163 (2002) 979.
[43] M. Ylinen, T. Naaranlahti, S. Lapinjoki, A. Huhtikangas, M.-L. Salonen, L.K. Simola, M. Lounasmaa, Planta Med. 52 (1986) 85.
[44] I.N. Papadoyannis, V.F. Samanidou, G.A. Theodoridis, G.S. Vasilikiotis, G.J.M. Kempen, G.M. Beelen, J. Liq. Chromatogr. 16 (1993) 975.
[45] A.A. Vitale, A. Acher, A.B. Pomilio, J. Ethnopharmacol. 49 (1995) 81.
[46] B.J. Savary, D.K. Dougall, Phytochemistry 29 (1990) 1567.