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Automated multiple development thin layer chromatography for calystegines and their biosynthetic precursors

Yvonne Scholl[a], Naoki Asano[b], Birgit Dräger[a,*]

[a]*Martin-Luther-University Halle-Wittenberg, Institute of Pharmaceutical Biology, Hoher Weg 8, D-06120 Halle/Saale, Germany*
[b]*Faculty of Pharmaceutical Sciences, Hokuriku University, Ho-3 Kanagawa-machi, Kanazawa 926-1181, Japan*

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Abstract

Automated multiple development thin layer chromatography (AMD-TLC) was used for separation of calystegines, a class of nortropane alkaloids, and of precursors of their biosynthesis. The calystegines differ in the number of hydroxyl groups at the nortropane ring system and in the substitution pattern. A combination of TLC methods allows the separation of the individual calystegines and separation of possible precursors of the biosynthesis. Solvent combination, development time, the number of development steps, the drying time between each run and the preconditioning parameters of the silica gel TLC plates were optimised. Limits of detection were evaluated for several detection reagents. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Calystegines

1. Introduction

Calystegines, hydroxy nortropane derivatives, were first isolated from transformed root cultures of *Calystegia sepium* [1]. They occur in several plants of the Solanaceae together with medicinally important tropane alkaloids, such as hyoscyamine and scopolamine [2]. Calystegines have a nortropane ring system with several hydroxyl groups substituted in various positions. They have been subdivided into three groups, namely calystegines A (three hydroxyl groups), calystegines B (four hydroxyl groups) and calystegines C (five hydroxyl groups). N-methylated tropane derivatives like *N*-methyl-calystegine B2 were also identified (Fig. 1) [3].

It is proposed that calystegines are biosynthesised by the tropane alkaloid pathway which is expressed in some, but not all Solanaceae [4]. An important branch point in the biosynthetic pathway of tropane alkaloids is the stereospecific reduction of tropinone (Fig. 2). Tropinone is reduced by two different enzymes which catalyse the formation of either tropine or pseudotropine [5,6]. The two isomeric 3-hydroxytropanes are incorporated into hyoscyamine/scopolamine or, presumably, into calystegines; the latter needs experimental evidence.

For the elucidation of biosynthetic steps, radiolabelled precursor feeding is the method of choice. The monitoring of incorporation and turnover of radio-labelled substrates requires selective separation of all putative metabolites preferably from crude

*Corresponding author. Tel.: +49-345-552-5765; fax: +49-345-552-7021.

E-mail address: draeger@pharmazie.uni-halle.de (B. Dräger).

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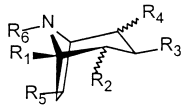

Calystegines	R1	R2	R3	R4	R5	R6
A_3	✖	✖	✖			H
A_5	✖		✖	✖		H
B_1	✖	✖	✖		●	H
B_2	✖	✖	✖	✖		H
B_3	✖	■	✖	✖		H
B_4	✖	✖	✖	■		H
C_1	✖	✖	✖	✖	●	H
C_2	✖	■	✖	✖	●	H
Alkaloid 1				✖	●	H
Alkaloid 2			✖	✖		H
N-Me-B_2	✖	✖	✖	✖		CH_3

Fig. 1. Structures of calystegines. ✖-equatorial hydroxyl group, ■-axial hydroxyl group, ●-exo hydroxyl group.

plant extracts. Calystegines and hydroxyl tropanes can be separated by thin layer chromatography [7,8], the groups of calystegines according to their number of hydroxyl groups are separated by simple or twofold TLC with manual development. Separation of individual calystegines, however, and of putative biosynthetic precursors such as nortropane alcohols, is insufficient because of the structural similarity. Highly selective TLC in combination with a radio-scanner would enable identification and quantification of labelled metabolites.

Automated multiple development (AMD) is an instrumental technique for separation of compounds of similar chemical properties, even stereoisomers, by using a solvent gradient on high-performance thin layer plates (HPTLC). The introduction of AMD has provided the separation of complex mixtures like biogenic amines [9], and pesticide residues in soil and water [10,11]. Gradient development leads to a band re-concentration improving the separation [12]. A successful separation depends mainly on choice of the solvent components, optimisation of the shape of the gradient and the stepwise movement of the elution front [13]. This paper shows a method for separation of the calystegines and putative biosynthetic precursors. Separation of individual calystegines was achieved by the same method and was enhanced by a variation of this method. Calystegine analysis by AMD-TLC is applied for screening plant extracts for the accumulation of calystegines.

2. Experimental

2.1. Apparatus and materials

The AMD 2 System was used for separation

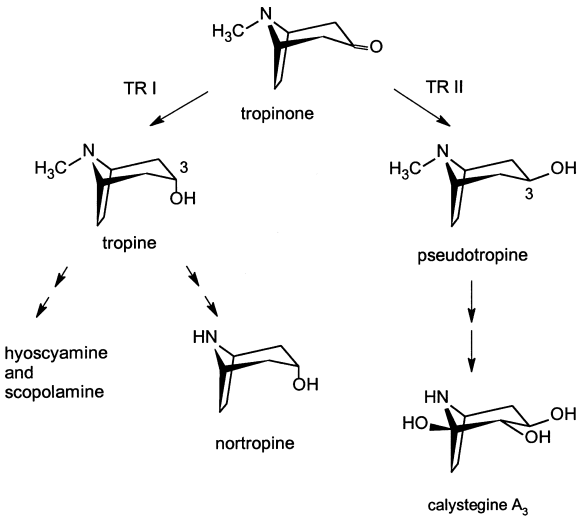

Fig. 2. Biosynthesis of calystegines: TR I, tropine forming tropinone reductase; TR II, pseudotropine forming tropinone reductase.

(Camag, Muttenz, Switzerland). Standard tropinone and tropine were obtained from Sigma (Deisenhofen, Germany), nortropine was a gift from Boehringer Ingelheim (Ingelheim, Germany). Pseudotropine was synthesised according to Ref. [14]. All calystegines were gifts from Dr. Naoki Asano, Kanasawa, Japan. All other chemicals were of the highest grade available.

Silica gel 60 HPTLC-plates (F_{254}, 200 μm layer, 10×20 cm, on glass, Merck, Darmstadt, Germany) and silica gel 60 plates (250 μm layer, 10×20 cm, on glass, Merck) were used. Samples were applied as 5 mm bands, 6 mm distance between the bands, with a 100-μl sample syringe (Hamilton, Bonaduz, Switzerland) using a Linomat IV (Camag).

2.2. Detection

For calystegines: silver nitrate reagent as dip, (1) 0.2% silver nitrate in acetone for 60 s, dry by cold fan, (2) 2% sodium hydroxide in ethanol until spots become visible.

For secondary amines: chlorine-tolidine as two-step spray reagent, 0.8% sodium hypochlorite, dried by cold fan, 320 mg *o*-tolidine dissolved in 30 ml concentrated acetic acid, filled up to 500 ml with water, mixed with 0.4% potassium iodide solution (1:1), freshly prepared.

For tropanes: Dragendorff var. Munier as spray (10% KI and 1.2% bismuth oxynitrate in 20% tartaric acid, stock solution, diluted 1:10 in 20% tartaric acid before use) [15].

2.3. Sample preparation

Plant material: root cultures of *Atropa belladonna* (gift from M. Rhodes, Norwich, UK), root cultures of *Hyoscyamus muticus* (gift from M. Leech, Norwich, UK), and root cultures of *Calystegia sepium* (gift from P. Bachmann, Braunschweig, Germany) were cultivated in Gamborg's B5 medium under standard conditions. Sprouts of *Solanum tuberosum* were harvested as described [8]. Root cultures were harvested, blotted dry, fresh mass was determined for all tissues. The plant tissues were homogenised in methanol–water (1:1). For calystegine measurements, the crude extract was neutralised and applied to a cation exchange column as described [16]. For analysis of tropinone, tropane and nortropane alcohols, the crude extract was evaporated to a volume of 1 ml and made alkaline with 50 μl ammonia. The alkaline residue was applied to 1 g of an Extrelut column (Merck), the column was soaked at room temperature for 20 min. Alkaloids were eluted from the column by washing with 8 ml chloroform and 4 ml chloroform–methanol (4:1). The eluate was concentrated by vacuum evaporation. Dry residues were taken up in methanol.

2.4. Feeding experiments

Stems of *Calystegia sepium* were collected at the banks of the river Saale in Halle, Germany. They were dipped into a solution of tropinone (neutralised to pH 7.0 with HCl) and in water as control. After 48 h the stems were extracted with methanol–water (1:1). The crude extract was evaporated and the aqueous residue was applied to an Extrelut column (Merck) as described under Section 2.3.

2.5. Quantitative analysis of calystegines by gas chromatography

Derivatization: 0.5 ml of the extract after ion-

exchange purification were lyophilised and derivatized with 40 μl HMDS and 10 μl TMCS. The mixture was kept at 50°C for 15 min, then 50 μg azobenzene (1 mg/ml stock solution in hexane) were added as internal standard, and the mixture was diluted with hexane to a final volume of 0.5 ml.

Instrumentation: gas chromatograph HP 6890, detection simultaneous FID and PND, column HP5 (30 m×0.25 mm I.D., 0.25 μm film thickness), injection split ratio 1:20, carrier gas helium (1 ml/min constant flow), injection temperature 250°C, detector temperature 310°C, temperature programme 160°C, 5°/min up to 240°C.

Analysis: 1 μl injection. Analyses were done in triplicate, RSD among GC samples of the same extract is less than 4%, RSD with repeated injections of the same GC sample is less than 1%.

3. Results and discussion

3.1. Separation of calystegines and biosynthetic metabolites

The type and the mixture of solvents, the development time, the number of development steps, the drying time between each run and the preconditioning parameters of the silica gel TLC plates are the parameters that were optimised for AMD separation. Firstly, putative metabolites of the biosynthesis of calystegines, i.e. tropinone, tropine, pseudotropine and nortropine were separated with a gradient given in Fig. 3A (method 1). Table 1 shows R_f values.

Further solvents, e.g. *n*-hexane, ethyl acetate and acetonitrile, were tested instead of chloroform, but they did not improve the separation. A drying time of 2 min after each run was found sufficient when TLC plates with 250 μm layer were used. The preconditioning of the TLC plate between each development run is an important parameter for separation. Ammonia in the gas phase is applied for 6 s by leading compressed air through a stock bottle in the apparatus. Ammonia concentration in this bottle has to be 9 *M*. With lower ammonia concentration or without fumigation at all, band broadening was observed and separation was impaired. With higher concentrations of ammonia higher R_f values resulted with less separation.

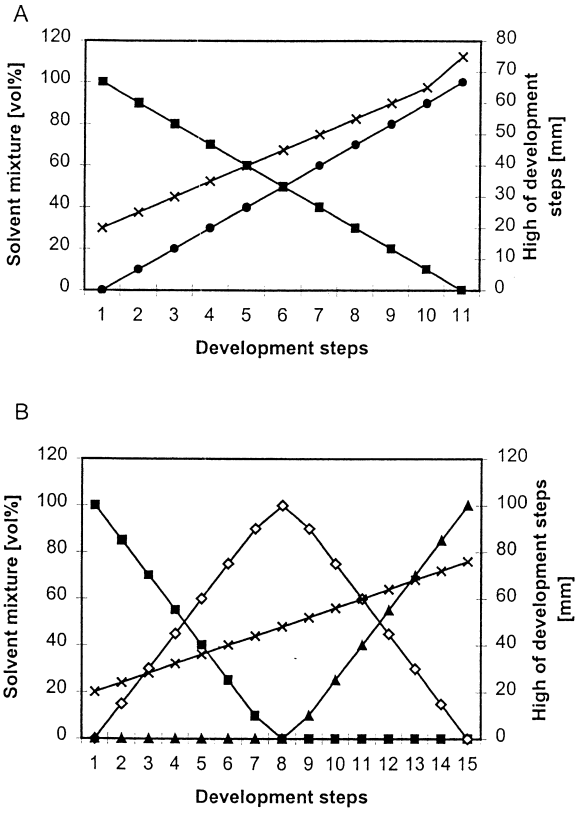

Fig. 3. Elution gradient profile. ■, methanol; ●, chloroform; ▲, propanol; ◇, ethanol; ×, eluent front. (A) Method 1 for separation of tropinone, tropane alcohols, and calystegines; (B) method 2 for separation of calystegines.

Table 1
R_f values for calystegines and their putative precursors; $n=6$; standard deviations in brackets

Analyte	R_f values	
	Method 1 (Fig. 3A)	Method 2 (Fig. 3B)
Tropinone	0.84 (0.027)	
Pseudotropine	0.63 (0.029)	
Tropine	0.53 (0.034)	
Nortropine	0.39 (0.059)	
Alkaloid 1	0.62 (0.033)	0.79 (0.041)
Alkaloid 2	0.57 (0.043)	0.69 (0.058)
Cal. A_3	0.52 (0.03)	0.57 (0.044)
Cal. A_5	0.55 (0.029)	0.63 (0.033)
Cal. B_1	0.40 (0.026)	0.46 (0.032)
Cal. B_2	0.35 (0.025)	0.39 (0.031)
Cal. B_3	0.36 (0.008)	0.40 (0.037)
Cal. B_4	0.38 (0.026)	0.41 (0.04)
Cal. C_1	0.24 (0.022)	0.27 (0.018)

The separation of the calystegines A_3, A_5, B_1, B_2, B_3, B_4, C_1, and two dihydroxylated calystegine-like compounds (described as alkaloid 1 and 2 by Asano et al. [17]) by the same method 1 was attempted. A separation between the calystegines and the tropanes in one gradient is possible. The R_f values of the calystegines in general are lower than of tropinone, tropine and pseudotropine (Table 1). For further distinction of the two groups of analytes, tropinone and the tropane alcohols are detected with Dragendorff's reagent — calystegines are not detected. For routine screening of plant material for calystegines, the silver nitrate reagent is the most feasible, but it is less sensitive for dihydroxylated compounds. The chlorine-tolidine reagent is very sensitive for all secondary amines. In crude plant extracts, however, chlorine-tolidine reagent also reacts with many amino acids giving similar blue spots, this may lead to false-positive results.

Detection limits with Dragendorff's reagent are 1 μg for tropinone, and 0.5 μg for tropine, pseudotropine and nortropine. With chlorine-tolidine reagent, the detection limit for nortropine is 50 ng. The detection limit with silver nitrate for di- and trihydroxylated calystegines is 400 ng and for calystegines of the B- and C-group 200 ng. The detection limits for chlorine-tolidine reagent are 100 ng for all calystegines.

Further experiments for an improved separation of individual calystegines included ethanol and propanol in the solvent system. Two dimensional development with propanol–ethanol–water (4:1:1) in the first dimension methanol–water–chloroform–ammonia (66:22:11:1) in the second dimension leads to broadening of substance zones and does not largely improve the resolution. The stepwise change of the solvent system and the intermediate preconditioning with ammonia obviously are decisive for narrow bands and separation of calystegines. Propanol–ethanol–water (4:1:1, single development) leads to some separation of individual calystegines, but the R_f values in general are low (R_f 0.05–0.3) [18].

For this reason these solvents were combined in different mixtures and tested. A separation for individual calystegines was obtained with a gradient of 15 steps shown in Fig. 3B (method 2). In general, an increased number of development steps results in higher R_f values but with the disadvantage of a long development time. A reasonable separation is obtained by the 15 steps of method 2 in 280 min. Drying time depends on the volatility of the solvents used and the thickness of the silica gel layer. For methanol–ethanol mixtures 2 min drying time is sufficient (steps 1–7). Increasing propanol requires drying times of 3 min (steps 8–15). TLC plates with 250 μm layer are used here. Ammonia (14 *M*) is used for preconditioning, with similar effects as described for tropinone and tropane alcohols. When diluted ammonia concentrations were tested, lower R_f values for dihydroxy and trihydroxy calystegines were observed.

R_f values obtained with method 2 for calystegines are shown in Table 1. Individual B-calystegines are partly separated. Calystegine B_1 with one hydroxyl group in position 6 shows a higher R_f value compared to calystegines B_2, B_3 and B_4. Attempts for further separation of B-calystegines included e.g. methanol and dichloromethane as solvents. With a gradient of methanol–dichloromethane over 15 steps and 14 *M* ammonia for preconditioning the separation of the B-calystegines can be further improved (R_f values: B_1 0.67; B_2 0.59; B_3 0.61; B_4 0.64). Calystegine B_1 and B_2 were the major B-calystegines in all plant extracts examined so far. They can be clearly separated here. If, however, an exact identification of all B-calystegines is required, GC–MS must be used in addition [7].

3.2. Applications

Method 1 is applicable for different purposes: (i) identification of turnover products after precursor feeding to plant tissue, in particular with radioactive labelled precursors. (ii) Identification of products of enzyme assays. (iii) Screening of different plant species for calystegines.

Method 1 was used to investigate tropinone turnover in plant tissues of *Calystegia sepium* (Fig. 4).

In gas chromatography the differences in retention times between tropinone, tropine and pseudotropine are low [8]. High amounts of tropinone may mask the tropine peak. The same problem arises in enzyme assays of tropinone reductases, where tropinone as substrate has to be added in high concentrations. The AMD-TLC method allows an unambiguous detection

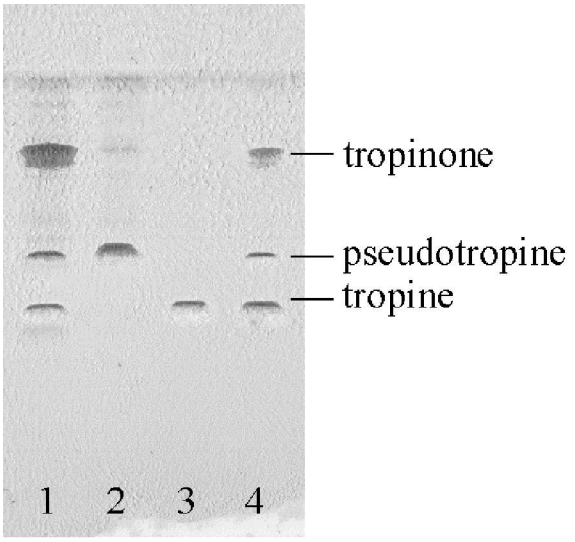

Fig. 4. Separation of tropinone, tropine and pseudotropine by method 1 for analysis of feeding experiments of stem tissue of *Calystegia sepium* with tropinone and pseudotropine, detection reagent: Dragendorff var. Munier. (1) Stem extract, fed with tropinone; (2) stem extract, fed with pseudotropine; (3) standard tropine 3 μg; (4) mix of standards (each 3 μg).

of tropine and pseudotropine in the presence of 50-fold higher amounts of tropinone.

The postulated biosynthesis of the calystegines proceeds via the reduction of tropinone to pseudotropine and unknown further steps, which are supposed to be a demethylation at the ring-nitrogen as well as the implementation of hydroxyl groups to the ring. Nortropine and alkaloids 1 and 2 were included as model substances of these possible intermediates and separated with method 1 in comparison to known precursors tropinone, pseudotropine, and tropine. Differential detection is required here as well, because Dragendorff's reagent works best with tertiary amines and does not react with hydroxylated nortropane derivatives. For this reason the chlorine-tolidine reagent was used for alkaloids 1 and 2. The separation of possible precursors will now allow feeding experiments with radioactive labelled substrates, their turnover and analysis by planar chromatography in combination with a radioscanner.

Fig. 5 shows the identification of calystegines in four different plant extracts by method 1. The crude plant extracts were purified before by cation exchange columns (see Section 2). For calystegines A_3, A_5, B_1, and B_2 a semi-quantitative evaluation is reliably obtained by comparison with defined concentrations of reference compounds.

If the first screening of a plant extract with method 1 results in a strong band of calystegines that may contain several different compounds (e.g. Fig. 5, lane 8), method 2 is applied for additional distinction. The intensive zone of calystegine B_2 of *H. muticus* extract did not separate into different zones with method 2, because it contains no further calystegines.

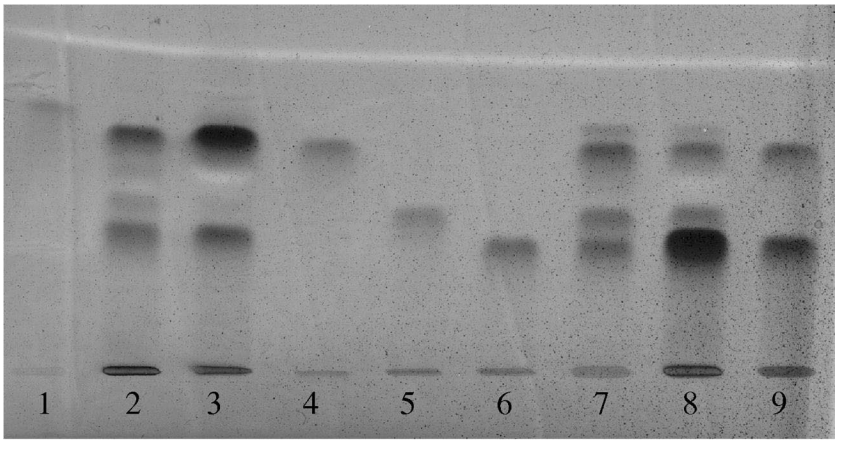

Fig. 5. Separation of calystegines of different plant extracts by method 1, detection reagent $AgNO_3$/NaOH. (1) Cal. A_5 1 μg; (2) *Calystegia sepium*; (3) *Atropa belladonna*; (4) Cal. A_3 1 μg; (5) Cal. B_1 1 μg; (6) Cal. B_2 1 μg; (7) mix of 1, 4–6; (8) *Hyoscyamus muticus*; (9) *Solanum tuberosum*; corresponding quantification of plant extracts is given in Table 2.

With method 2, tropinone, tropine, pseudotropine and nortropine move together to R_f values >0.8. Calystegines are routinely quantified by gas chromatography with internal standard [7]. Results of the same extracts by GC are shown in Table 2. The detection limits for calystegines with the silver nitrate reagent are higher than by GC with a detection limit of ≥1 μg/ml. An advantage of the AMD method is a reduction of time. One TLC plate of 10×20 cm may carry up to 20 samples. One run with method 1 takes about 120 min, that is 7 min per sample compared to 30 min for one run by GC, and the tedious sample derivatisation for GC is not considered. Different detection methods can be applied on one TLC plate by scratching a vertical line in to the silica gel layer to prevent solvent diffusion when dipping into silver nitrate reagent. The other half of the plate is sprayed by Dragendorff's reagent or chlorine-tolidine reagent. This again prevents the time consumption of several separations.

AMD-TLC of calystegines is a fast method for screening plant extracts alternatively or in combination with gas chromatography and nitrogen sensitive detection [8].

TLC plates with thin layers of 250 μm were used for all investigations shown here. The method can be transferred to HPTLC plates. These plates require less ammonia fumigation, probably due to a thinner chromatographic layer. R_f values for calystegines with the same chromatographic methods are ca. 20% higher and closer together compared with TLC plates, thus methods must be adopted. Omission of some steps and less ammonia fumigation result in somewhat shorter separation times, but the improvement of resolution is insignificant. It is recommended to use HPTLC plates for AMD, but we show here that reasonable results can be obtained with simple TLC plates of lower cost.

Table 2
Quantification (μg/spot) of calystegines by GC in different plant extracts, corresponding to Fig. 5

	Cal. A_3	Cal. A_5	Cal. B_1	Cal. B_2
Calystegia sepium Fig. 5, lane 2	1.08	–	0.39	0.51
Atropa belladonna Fig. 5, lane 3	2.44	0.48	0.06	0.7
Hyoscyamus muticus Fig. 5, lane 8	0.56	0.28	0.88	4.88
Solanum tuberosum Fig. 5, lane 9	0.94	–	–	1.62

4. Conclusion

Automated multiple development has proved to be a powerful tool that provides increased separation in particular for compounds with similar structures. Calystegines and their precursors are separated by using different elution gradients. In combination with a TLC scanner a quantitative evaluation will be elaborated. AMD-TLC for labelled metabolites and evaluation by radioactivity scanning is a useful tool for investigation of biosynthesis.

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