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Separation of forskolin derivatives by automated multiple development high-performance thin-layer chromatography

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Abstract

An optimized gradient enabling the separation and quantification of eleven forskolin derivatives by automated multiple development on high-performance thin-layer chromatography (HPTLC) plates is presented. The HPTLC development uses a 25-step gradient with a polarity range of methylene chloride–methanol to hexane and detection by chlorosulfonic acid reagent. Multiple correspondence analysis has allowed correlation of the molecular structures of forskolin derivatives (with hydroxyl substitutions) to the migration distance. © 1997 Elsevier Science B.V.

Keywords: Forskolin; Labdanes

1. Introduction

Forskolin is a labdane, 7β-acetoxy-1α,6β,9α-trihydroxy-8,13-epoxy-labd-14-en-11-one, primarily extracted from *Coleus forskohlii* Briq. (*Lamiaceae*) roots [1]. This diterpenoid has been investigated as a promising new drug: it shows positive inotropic, hypotensive activity, inhibits thrombocyte aggregation and has been proposed in the treatment of ocular hypertension and stimulation of skin melanogenesis [2–4]. It acts by directly stimulating adenylate cyclase, resulting in an increase in the ''second messenger'' c-AMP [5,6].

Forskolin (FSK) derivatives have been proposed as a treatment against hair loss and grey hair and their pharmacological properties have also been widely studied [7–10].

In this paper, we present an optimized automated multiple development (AMD) technique applied to high-performance thin-layer chromatography (HPTLC) of forskolin and derivatives. The main advantages of this recent technique are that it is fully automated and it avoids band broadening during migration due to a band reconcentration effect [11]. Optimization of the AMD–HPTLC experimental conditions for FSK derivatives led to their ''one experiment'' separation with high resolution. This method has been used for separation and quantification of a complex mixture of eleven structurally related labdanes.

2. Experimental

2.1. Standards

Forskolin (F1), deacetylforskolin (F2), 1-deoxyforskolin (F3), 1,9-dideoxyforskolin (F4), 7-

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deacetyl-1,9-dideoxyforskolin (F5), 7-deacetyl-1-deoxyforskolin (F6), 6-acetyl-7-deacetylforskolin (F7), 9α-hydroxy-8,13-epoxy-labd-14-en-11-one (F8), 6β-hydroxy-8,13-epoxy-labd-14-en-11-one (F9), 7-deacetylforskolin 7-hemisuccinate (F10) and 9-deoxyforskolin (F11) (all structures are described in Table 1) were purchased from Sigma (Saint-Quentin-Fallavier, France). All standards were from *Coleus forskohlii* and were at least 98% pure, except for F6 and F11 which were at least 95% pure. A standards mixture was prepared in methanol at 1 mg ml^{-1} of each standard and diluted 1:2, 1:4, 1:5 and 1:10 with methanol. Unless otherwise stated, all other chemicals were from Merck (Nogent, France) and were of the highest purity available.

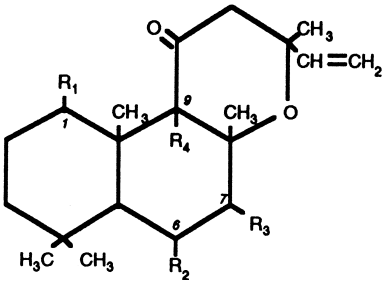
2.2. Chromatographic conditions

Chromatography was performed on silica gel HPTLC plates (60F254 Ref. 5642, Merck), 10×20 cm. A 1 μl volume of each standard solution was applied in duplicate to the plates as 8 mm wide bands with an Automated TLC Sampler III, ATS3 (Camag, Muttenz, Switzerland), 8 mm from the bottom of the plate, band velocity 10 mm s^{-1}, dosing speed 200 nl s^{-1}. These application parameters were identical for all analyses performed.

Prior to development, plates were activated at 110°C for 24 h, then prewashed twice with chloroform–methanol (2:1, v/v). Development was performed using a Camag AMD instrument with the solvent system described in Table 2.

The experimental AMD parameters used in this study were as follows: number of steps, 25; drying times, 3 min; preconditioning with nitrogen for 15 s for each step. Feeding bottles were filled with high precision using only one precision flask for all solvent mixtures. The development times are reported in Fig. 1.

Table 1
Structures of forskolin derivatives: forskolin (F1), deacetylforskolin (F2), 1-deoxyforskolin (F3), 1,9-dideoxyforskolin (F4), 7-deacetyl-1,9-dideoxyforskolin (F5), 7-deacetyl-1-deoxyforskolin (F6), 6-acetyl-7-deacetylforskolin (F7), 9α-hydroxy-8,13-epoxy-labd-14-en-11-one (F8), 6β-hydroxy-8,13-epoxy-labd-14-en-11-one (F9), 7-deacetylforskolin 7-hemisuccinate (F10) and 9-deoxyforskolin (F11)



Forskolin derivative	R1	R2	R3	R4
F1	–OH	–OH	–O-CO-CH_3	–OH
F2	–OH	–OH	–OH	–OH
F3	/	–OH	–O-CO-CH_3	–OH
F4	/	–OH	–O-CO-CH_3	/
F5	/	–OH	–OH	/
F6	/	–OH	–OH	–OH
F7	–OH	–O-CO-CH_3	–OH	–OH
F8	/	/	/	–OH
F9	/	–OH	/	/
F10	–OH	–OH	–O-CO-$(CH_2)_2$-COOH	–OH
F11	–OH	–OH	–O-CO-CH_3	/

Table 2
Chromatographic conditions for AMD separation describing the composition of each bottle for each step

Starting with step No.	1	2	5	10	15	20
Use bottle No.	1	2	3	4	5	6
Methanol	5	3	2	1		
Methylene chloride	95	97	98	99	100	
n-Hexane						100
Drying time (min)	3	3	3	3	3	3
Wash bottle			Nitrogen			

2.3. Detection of forskolin and derivatives

After chromatography, the plates were fully sprayed until wet with the chlorosulfonic reagent prepared as follows: glacial acetic acid (10 ml) added to chlorosulfonic acid (5 ml) then water (35 ml). Plates were then heated for 30 min at 110°C and examined in daylight and UV light (365 nm). Spots appeared brown in daylight and yellow to orange in UV light.

2.4. Quantification

After spraying, the FSK derivatives were quantified with a TLC2 scanner and Camag TLC-Software 3 (Camag) under the following conditions: slit width 8×0.4 mm, wavelength 365 nm, photomode reflection, scan mode, sensitivity 182 and span 22.

2.5. Statistical analysis

Multiple correspondence analysis (MCA) was performed on the migration distance (MD) values in relation to the planar structures of the FSK hydroxyl and acetyl derivatives.

F10 was excluded due to its atypical succinate substitution.

The basic data presented in Table 3 order the FSK derivatives in ten rows and eight columns corresponding to eight qualitative variables (number and position of hydroxyl group, presence or not of acetyl substitutions). Furthermore, an index was defined corresponding to a molecular migration each 10 to 10 mm.

MCA aims to generate quantitative scores which maximize the mean correlation ratio among the previously described qualitative variables. MCA was performed using the multivariate descriptive statistical ADE-4 Program Library software (CNRS URA 2055, Lyon I, Villeurbanne, France).

3. Results and discussion

A mixture of forskolin and derivatives was chromatographed by HPTLC–AMD with the solvent gradient described in Table 2. Gradient optimization was performed according to Lodi et al. [12]. A good separation resolution and narrow bands were ob-

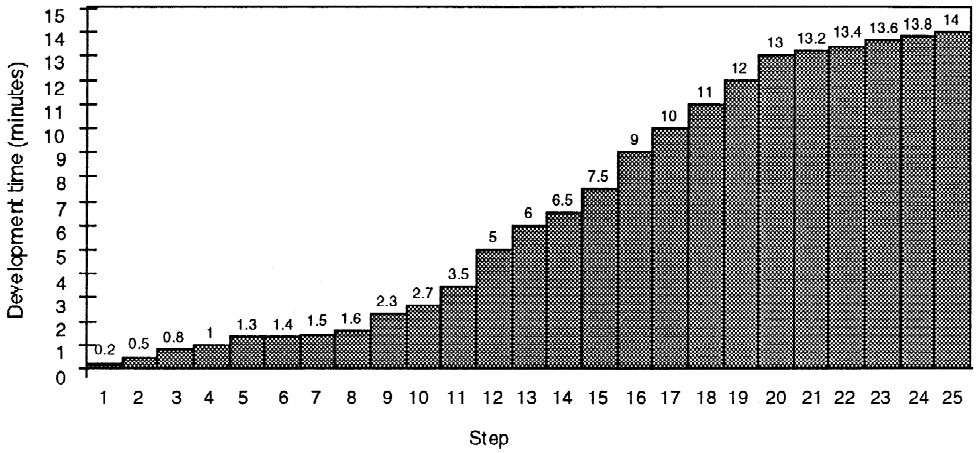


Fig. 1. Development time determining the running distance for each step.

Table 3
Multiple correspondence analysis data

Name	MD (mm)	MD index[a]	NB OH[b]	OH in 9[c]	OH in 1[c]	OH in 6[c]	OH in 7[c]	$COCH_3$ in 7[c]	$COCH_3$ in 6[c]
F7	20.0	1	3	2	2	1	2	1	2
F2	24.5	1	4	2	2	2	2	1	1
F11	27.9	1	2	1	2	2	1	2	1
F1	30.8	2	3	2	2	2	1	2	1
F5	39.8	3	2	1	1	2	2	1	1
F9	42.3	3	1	1	1	2	1	1	1
F6	45.9	3	3	2	1	2	2	1	1
F4	47.6	3	1	1	1	2	1	2	1
F3	55.1	4	2	2	1	2	1	2	1
F8	58.3	4	1	2	1	1	1	1	1

[a] Code: 1=from 20 to 29; 2=from 30 to 39; 3=from 40 to 49 and 4=from 50 to 59.
[b] Code: 1=1 OH; 2=2 OH; 3=3 OH and 4=4 OH.
[c] Code: 1=no and 2=yes.

tained except for F6 and F4 which have to be considered jointly if one is in excess. The labdane separation spanned from 14.2 mm to 58.3 mm. All standards were separated, beginning with F10, the most polar diterpenoid, and ending with F8, the least polar. Scanning of the standards plate at 365 nm is reported in Fig. 2 which illustrates the high resolution of the separation. A linearity study was performed on all the standards to allow quantification of the labdanes. For all these compounds, a linear relationship was observed between the integrated area and the quantity of compound applied over the range 0.1–1 μg. The regression equations of the calibration curves are shown in Table 4. The equation that gave the closest fit under the conditions employed for the standards is of the type $y=ax+b$. The standard deviation is less than 8% (on 9 independent applied samples). The limit of detection of each compound is 40 ng for 8 mm bands with a ratio response baseline signal of 2.

MCA was carried out to correlate the migration distance on the plates to the planar molecular

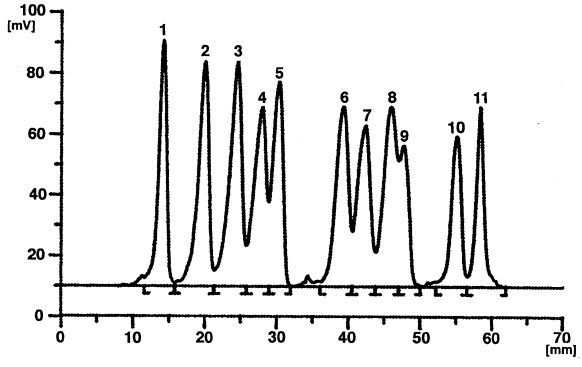


Fig. 2. Scanning profile of the forskolin and derivatives standards mixtures. 1=7-Deacetylforskolin 7-hemisuccinate (F10), 2=6-acetyl-7-deacetylforskolin (F7), 3=deacetylforskolin (F2), 4=9-deoxyforskolin (F11), 5=forskolin (F1), 6=7-deacetyl-1,9-dideoxyforskolin (F5), 7=6β-hydroxy-7,13-epoxy-labd-14-en-11-one (F9), 8=7-deacetyl-1-deoxyforskolin (F6), 9=1,9-deoxyforskolin (F4), 10=1-deoxyforskolin (F3), 11=9α-hydroxy-8,13-epoxy-labd-14-en-11-one (F8).

Table 4
Migration distances and linear regressions for all forskolin derivative standards

Forskolin derivative	Migration distance (mm)	Equation	r
F10	14.2	$y=870.0x+32.8$	0.998
F7	20.0	$y=1044.8x+49.0$	0.998
F2	24.5	$y=1128.9x+47.3$	0.995
F11	27.9	$y=1152.9x+90.8$	0.992
F1	30.8	$y=917.9x+46.1$	0.997
F5	39.8	$y=1144.6x+118.8$	0.995
F9	42.3	$y=1081.6x+75.5$	0.993
F6	45.9	$y=994.7x+85.4$	0.994
F4	47.6	$y=899.6x+106.8$	0.998
F3	55.1	$y=1340.7x+84.4$	0.994
F8	58.3	$y=1040.9x+172.2$	0.984

Forskolin (F1), deacetylforskolin (F2), 1-deoxyforskolin (F3), 1,9-dideoxyforskolin (F4), 7-deacetyl-1,9-dideoxyforskolin (F5), 7-deacetyl-1-deoxyforskolin (F6), 6-acetyl-7-deacetylforskolin (F7), 9α-hydroxy-8,13-epoxy-labd-14-en-11-one (F8), 6β-hydroxy-8,13-epoxy-labd-14-en-11-one (F9), 7-deacetylforskolin 7-hemisuccinate (F10) and 9-deoxyforskolin (F11).
y: Area (mV mm^{-1}); x: quantity of forskolin derivative applied on the plate (μg); r: correlation coefficient.

structures of the labdanes. MCA (44.3% of the total inertia) indicates that the relationship between migration order (polarity) and structure can be explained by the planar structures when the molecules contain only hydroxyl substitution in positions 1, 6 and/or 7. The number of hydroxyl groups associated with their position in 1 and 7 gives the largest contribution to the order of migration, the hydroxyl group in position 6 having a smaller effect (F9). F8 which contains no hydroxyl substitutions (except in position 9) is effectively the most apolar FSK derivative and has an MD higher than F6, F9, F5 and F2. Surprisingly, F6 which contains an additional hydroxyl group in position 9 migrates after F5 and F9. The 9-hydroxyl position seems to have no influence on MD (the MDs of F1 and F11 are very close). Acetyl groups do not seem to affect the order of migration.

The statistical analysis, based on the planar structure, has clarified the relationship between the hydroxyl structures of FSK derivatives and the migration distances. Stereochemical analysis could then be used to explain the polarity of molecules with substitutions other than hydroxyl groups and is currently under investigation in our laboratory.

The AMD–HPTLC analysis presented here appears to be suitable for the separation and quantification of forskolin and its derivatives in spite of their very similar structures. This method can also be transposed to a crude *Coleus forskohlii* extract in development for pharmacological use.

References

[1] S.V. Bhat, B.S. Bajwa, H. Dornauer, N.J. De Souza, Tetrahedron Lett. 19 (1977) 1669.
[2] E. Lindner, A.N. Dohadwalla, B.K. Bhattacharya, Arzneim.-Forsch. Drug Res. 28 (1978) 284.
[3] N.J. De Souza, A.N. Dohadwalla, J. Reden, Med. Res. Rev. 3 (1983) 201.
[4] A. Meybeck, F. Bonté, M. Dumas, P. André and G. Redziniak, Eur. Pat. 0486595, 1995.
[5] H. Metzger, E. Lindner, Arzneim.-Forsch. Drug Res. 31 (1981) 1248.
[6] K.B. Seamon, J.W. Daly, J. Cyclic. Nucl. Res. 7 (1981) 201.
[7] F. Bonté, A. Meybeck and C. Maréchal, US Pat. 5 510 113, 1996.
[8] S.V. Bhat, A.N. Dohadwalla, B.S. Bajwa, N.K. Dadkar, H. Dornauer, N.J. De Souza, J. Med. Chem. 26 (1983) 486.
[9] H.G. Joost, A.D. Habberfield, I.A. Simpson, A. Laurenza, K.B. Seamon, Mol. Pharmacol. 33 (1988) 449.
[10] K. Schmidt, W.R. Kukovetz, J. Cardiovasc. Pharmacol. 13 (1989) 353.
[11] C.F. Poole, M.T. Belay, J. Planar Chromatogr. 4 (1991) 345.
[12] G. Lodi, A. Betti, E. Menziani, V. Brandolini, B. Tosi, J. Planar Chromatogr. 4 (1991) 106.