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Short communication

Liquid chromatographic separation of derivatives of diospyrin, a bioactive bisnaphthoquinonoid plant-product, and analogous naphthyl compounds

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Abstract

Isocratic reversed-phase liquid chromatography (LC) method was developed using acetonitrile and water for the determination of diospyrin, a pharmacologically important bisnaphthoquinonoid plant-product. The method was validated for precision, accuracy and reproducibility, and was found to be linear over the concentration range of 1–1000 μg/ml; the limits of detection and quantitation were 8 and 20 ng, respectively. The technique was used to determine the amount of diospyrin in plant extracts from four climatic regions in India. It was also applied for differentiation and separation of 27 naphthyl compounds. While a composition of 50:50 was preferable for dimeric compounds, the composition 40:60 was a better choice for the monomers. Also, the isomeric α- and β-naphthols and their dimers could be distinguished by conversion into the respective methyl ethers.

Keywords: Diospyrin; Naphthoquinonoids; Naphthyl compounds

1. Introduction

Quinonoid compounds are mostly present as secondary metabolites in all respiring animal and plant cells and possess a wide range of biological activities [1]. Some of the most frequently used anticancer drugs have been derived from quinononoid natural products [2]. Studies carried out during 1940s on lapachol, a plant-derived naphthoquinonoid, have led to the synthesis of a series of analogues with antiparasitic activities [3]. One such drug, atovaquone, has recently obtained clinical approval as a constituent of a novel antimalarial formulation, viz. Malarone [4]. Diospyrin (**1**), a bisnaphthoquinonoid isolated from the stem-bark of *Diospyros montana* Roxb., exhibited significant antitumour and antileishmanial activities in our laboratory [5,6]. Hence, derivatives of **1**, which were more effective than their naturally occurring precursor, were tested against human cancer cell lines, as well as some parasites and microorganisms [7–12]. Since derivatisation of **1** would influence its polarity, and consequently, its pharmacokinetic profile, detection of such compounds might pose a challenge in

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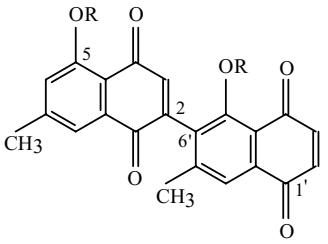
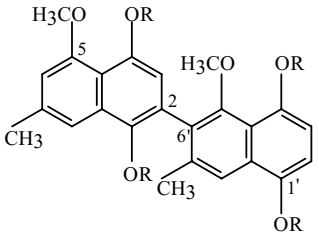
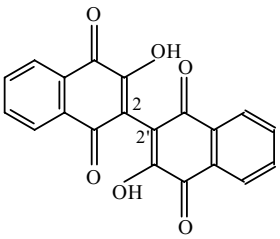


1 R = H, **Diospyrin**
2 R = CH_3
3 R = CH_2CH_3
4 R = $CH_2CH_2CH_3$
5 R = $CH(CH_3)_2$
6 R = $CH_2CH_2CH_2CH_3$
7 R = $COCH_3$



8 R = H
9 R = $COCH_3$



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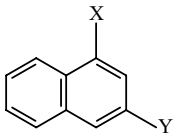
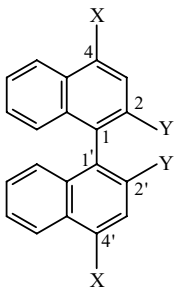
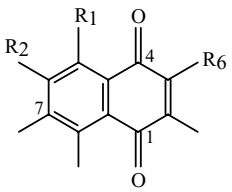


20 : X = OH ; Y = H
21 : X = OCH_3 ; Y = H
22 : X = H ; Y = OH
23 : X = H ; Y = OCH_3



24 : X = OH ; Y = H
25 : X = OCH_3 ; Y = H
26 : X = H ; Y = OH
27 : X = H ; Y = OCH_3



11 - 19

Fig. 1. Structures of diospyrin (**1**), its derivatives (**2**–**9**), monomeric naphthoquinonoids (**11**–**19**) and analogous naphthyl compounds (**10** and **20**–**27**).

Table 1

Compound	R_1	R_2	R_3	R_4	R_5	R_6
11	H	H	H	H	H	CH_3
12	OH	H	H	H	H	H
13	OH	H	CH_3	H	H	H
14	OCH_3	H	H	H	H	H
15	H	H	H	H	H	OH
16	H	H	H	H	H	OCH_3
17	H	H	H	H	H	OC_2H_5
18	OH	H	H	H	CH_3	H
19	OCH_3	H	H	H	CH_3	H

studying their metabolic behaviour [13]. This consideration prompted us to look for a simple and precise analytical method for detection and estimation of **1**, and its analogues.

Earlier workers pursuing different objectives have reported the separation of monomeric naphthoquinonoids [14,15], Vitamin K analogues [16,17], prenylquinones [18], etc. through reversed-phase liquid chromatography (LC) using widely divergent compositions of the eluent; however, no uniform solvent system for separation of naphthyl compounds, in general, and dimeric naphthoquinonoids, in particular, has been established so far. Determination of plumbagin, a plant-derived naphthoquinone, by normal-phase LC was reported by Gupta et al [19]. In the present communication, we are presenting a reversed-phase LC–UV spectrophotometric method, using a binary isocratic solvent system, which has been demonstrated to be sensitive enough for detection of some monomeric and dimeric naphthoquinonoid compounds with minor variations of the substituents (Fig. 1, Table 1). Furthermore, the scope of this study has been extended to include a few naphthyl analogues as well. Finally, the method has been validated and verified for the quantitative estimation of the dimeric naphthoquinonoid, **1**, which was present in semi-purified samples of the stem-bark of *D. montana* Roxb. collected from four different climatic regions in eastern and north-eastern India.

2. Experimental

2.1. Chemicals

Acetonitrile and water (E. Merck, India; Spectrochem, India) were of LC grade. The monomeric naphthoquinonoids, viz. menadione (**11**), juglone (**12**), lawsone (**15**) and plumbagin (**18**), and the naphthols (**20** and **22**) were procured from Sigma, USA. 7-Methyljuglone (**13**), isolated from *Euclea natalensis*, was obtained through the kind courtesy of Dr. N. Lall, Department of Botany, University of Pretoria, South Africa.

2.2. Synthesis of compounds

2.2.1. Diospyrin and its derivatives

Diospyrin (**1**) was isolated from the stem-bark of *D. montana* Roxb. and purified meticulously as described before [5] (Fig. 1). Its structure has been reconfirmed to be 2,6′-bis (5-hydroxy-7-methyl-1,4-naphthoquinone) through total synthesis [20]. The preparation of derivatives (**2**–**9**) of **1** are described below. Diospyrin dimethyl ether (**2**) and its homologues (**3**–**6**) were synthesised from diospyrin (**1**) according to the procedure published before [7]. Compound **8**, the hydroquinonoid derivative of **2**, was obtained through reduction of the latter with sodium dithionite, and **8** was acetylated to get its tetra-acetate derivative, **9** [21]. Acetylation of **1** with acetic anhydride and concentrated sulphuric acid yielded the diacetyl derivative, **7**. Ether derivatives (**14**, **16**, **17** and **19**) of the monomeric hydroxynaphthoquinonoids, as well as those of α- and β-naphthols (**21** and **23**) were prepared as per standard methods [22,23].

2.2.2. Dimeric naphthyl compounds

A novel reagent developed from ammonium metavanadate [24] was used to prepare dimeric derivatives of α- and β-naphthols and their respective methyl ethers (**24**–**27**). This reagent was also applied on lawsone to obtain the dimeric naphthoquinonoid compound **10**, which is a structural analogue of **1**.

All synthetic products were characterised by melting point determination, and through spectroscopy, using Pharmacia Ultrospec 2000 for UV-Vis spectrum, Perkin Elmer RX-1 for FT-IR, Bruker AM 300L Supercon NMR spectrometer for ^{1}H NMR (300.13 MHz) and CMR (75.47 MHz) spectra, and VG Micro mass spectrometer, UK, for EI mass spectral analyses (data available on request).

2.3. LC analysis

The liquid chromatograph consisted of Waters (Milford, MA, USA) modular LC apparatus either

with a M-501 solvent delivery pump, M-481 UV-Vis variable—wavelength detector, U6K injector and M-745B data module with a recorder, or with M-510 solvent delivery pump, 696 Photodiode Array detector and Millennium 32 Chromatography Manager. Acetonitrile and water chosen as eluents were passed through 0.50 and 0.45 μm Millipore filters, respectively, and mixed in the desired proportion, followed by degassing under low-vacuum oil-free pump with stirring. The pH of the isocratic eluent was neutral, except in the cases mentioned where pH of 4 and 9 were obtained by addition of acetic acid (0.5%) and triethylamine, respectively. The compounds were dissolved in acetonitrile (0.5–1.0 mg/2 ml) and injected (2–50 μl) on a μBondapak C_{18} steel column (30 cm × 3.9 mm i.d.; particle size 10 μm) at a flow-rate of 1 ml/min at ambient temperature, followed by UV detection at 255 nm.

2.4. Validation procedure for estimation of diospyrin

A stock solution of diospyrin (**1**) was prepared by dissolving an accurately weighed amount (10 mg) of **1** in chloroform (2 ml) and the volume was made up to 10 ml by adding acetonitrile. This solution was serially diluted with acetonitrile for LC analysis in the range of 1–1000 μg/ml (injection volume = 20 μl) using acetonitrile and water (1:1, v/v). The peak areas were plotted against the amount (μg) of diospyrin injected for each determination. A standard calibration curve was generated over four orders of magnitude containing 0.02–20 μg of **1**. The linearity of the method was checked by linear regression analysis. The limits of quantification (LOQ) and detection (LOD) were established by gradually reducing the injection volume of the solution over at least six successive determinations. The method was validated for precision and accuracy using the aforesaid solutions, stored carefully at 0–4 °C, by carrying out 'intra-day' and 'inter-day' determinations for estimation of 0.2, 2 and 20 μg of diospyrin. Precision of the method was characterised by the coefficient of variation (CV), while the relative error (RE) was calculated as a percentage of accuracy, based on the difference of the true quantity injected and that obtained from the calibration curve by plotting the average of the respective peak areas found from the experiments.

2.5. Application of LC vis-à-vis other methods for estimation of diospyrin in plant bark samples

Samples of *D. montana* stem-bark were collected from the following places in India (State in parenthesis): Bolangir (Orissa; A), Deoghar (Bihar; B), Hooghly (West Bengal; C) and Agartala (Tripura; D). Each sample was processed as per the following scheme given for 'A' to obtain semi-crude material for calculation of its diospyrin-content by LC analysis (Method 1), through crystallisation by established chemical procedure (Method 2), and via quantitative chemical conversion into its dimethyl ether derivative (Method 3), followed by gravimetric estimation.

2.5.1. Method 1

A solution of accurately weighed sample of the semi-crude extract [*] was prepared and subjected to LC analysis as described under Section 2.4; the resultant peak area, X, was noted to calculate the diospyrin content in this sample (Fig. 2). The same experiment was repeated (peak area = Y) by spiking with a known amount of pure diospyrin; the peak area (Z) for the latter was also determined in a separate experiment. Thus, $(Y - Z) \sim X$ would be a measure of accuracy of this method (Table 2).

2.5.2. Method 2

An accurately weighed sample of the semi-crude extract [*] was refluxed for 30 min with ethanol, filtered,

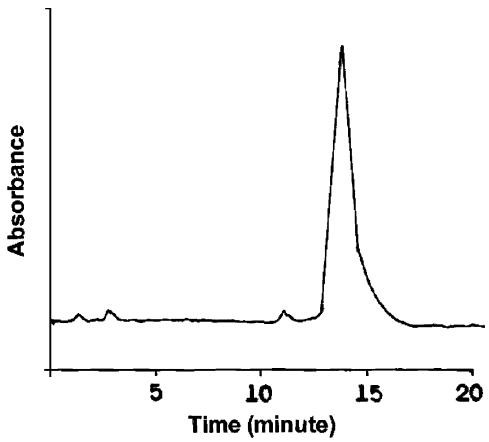
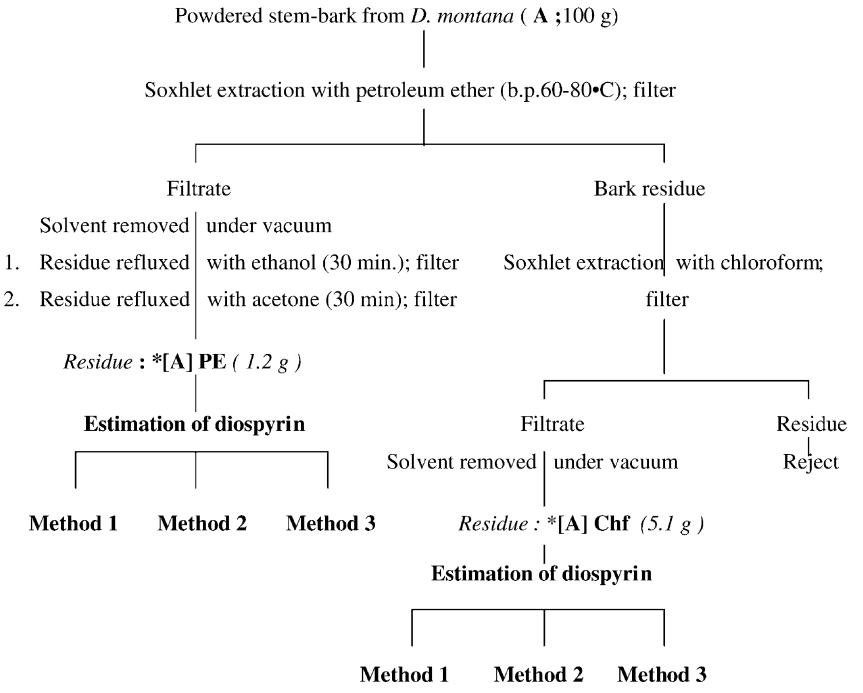


Fig. 2. LC–UV chromatogram of a sample of a semi-crude extract of the stem-bark of *D. montana* showing the peak for diospyrin.



and the residue was similarly treated with acetone. The process was repeated till a bright orange-coloured residue was obtained which was dissolved in chloroform, treated with activated charcoal and filtered. The filtrate was concentrated to obtain crystals of pure diospyrin (mp 258–60 °C), and the percentage of its yield was noted (Table 2).

2.5.3. Method 3

An accurately weighed sample of the semi-crude extract [*] was dissolved in chloroform and treated with methyl iodide and silver oxide [20] to convert its total diospyrin content into the dimethyl ether derivative [2]. The reaction mixture was passed through a column of neutral alumina and the solvent was removed

Table 2
Determination (%) of diospyrin in semi-crude samples of *D. montana* stem-bark collected from different climatic regions in India

Sample no.	Place of collection	Method 1[a] (%)		Method 2[a] (%)	Method 3[a] (%)
		Observed	Calculated		
(A) Chf	Bolangir	86	78	9.1	88
(A) PE	Bolangir	27	25	3.1	33
(B) Chf	Deoghar	52	54	4.7	55
(B) PE	Deoghar	4.1	4.3	0.4	5.1
(C) Chf	Hooghly	61	56	5.6	65
(C) PE	Hooghly	13	12	0.8	15
(D) Chf	Agartala	78	82	6.4	85
(D) PE	Agartala	19	20	1.3	20

[a] Methods 1–3 described in Section 2.5; percentages based on quantity in the semi-crude extracts [*] obtained as intermediate in purification scheme as given therein; $n = 4$.

to get **2** quantitatively, the yield of which was a measure to calculate the total content of diospyrin in the sample (Table 2).

3. Results and discussion

3.1. Retention times (RT) of naphthoquinonoids and naphthyl analogues

An isocratic eluent composed of acetonitrile and water was used for the analysis of 27 naphthoquinonoids. The retention times (Table 3) of the monomeric as well as dimeric naphthyl analogues were clearly dependent on the relative proportions of acetonitrile and water. A 40:60 composition led to satisfactory separation for the monomeric compounds. This composition could also be used for the separation of dimeric compounds, if present in the same mixture. However, the dimers themselves would be more conveniently separated at a 50:50 composition.

The elution pattern was found to follow the expected order in the reversed-phase mode in which the most polar compound was least retained. Thus, **2**, the simplest methyl ether of **1**, showed the lowest RT in this series and **6**, with *n*-butyl substituents, had the highest value. However, by the same argument, the compound **1** with free –OH groups is expected to possess the highest polarity, and hence, to be eluted faster than its ethers, which was, however, not the case (Table 3). A plausible explanation could be the phenomenon of intramolecular H-bonding of the hydroxy groups to the nearest carbonyl functions; this would override, to some extent, the intermolecular H-bonding between the solute and the protic solvent in the eluent which generally determines the rate of elution in reversed-phase chromatography [14]. In this context, the exceptionally low RT (∼1.7 min) for lawsone (**15**), which was again the same for both its mono- (**15**) and dimeric (**10**) forms, could be due to its –OH group being *ortho*- to a carbonyl function, thereby raising its polarity. The anomalously low RT value of lawsone was also reported previously [15,25].

While extending the scope to other naphthyl compounds, it was observed that this method was not sensitive enough to differentiate between the isomeric α- and β-naphthols, nor between their dimers; however, simple conversions into their respective methyl ethers could overcome this difficulty (vide Table 3).

Table 3
Retention times (min) for naphthoquinones and naphthyl analogues using acetonitrile–water (A = 60:40, B = 50:50 and C = 40:60) as isocratic eluent

Compound	A (pH 7)	B (pH 4–9)	C (pH 7)
Dimeric naphthoquinones			
1	7.1	14[a]	31
2	4.8	6.9	12
3	6.0	11	25
4	8.6	20	29
5	7.6	16	18
6	13	24	40
7	5.0	8.5	19
8	5.0	7.7	13
9	6.6	14	30
10	1.8	1.7[b]	1.9
Monomeric naphthoquinones			
11	4.4	5.4	7.4
12	4.1	4.8	6.3
13	4.9	5.8	8.1
14	3.7	3.8	4.5
15	1.8	1.8[b]	1.8
16	3.8	4.2	5.0
17	4.1	4.7	6.2
18	4.6	6.0	8.7
19	3.9	4.4	5.5
Dimeric naphthoquinones			
20	4.0	5.0	6.9
21	6.1	9.5	18
22	3.9	4.6	6.3
23	5.6	8.4	15
Monomeric naphthols and derivatives			
24	4.5	6.9	14
25	19	49	49
26	4.7	7.2	14
27	8.2	17	>49

[a] 12.4 at pH 9.
[b] 3.6 at pH 4.

3.2. Validation of assay procedure

The LC–UV method was validated for solutions containing 0.02–20 μg of diospyrin (**1**). Linear regression analysis showed the correlation coefficient (R) to be 0.9999 over this range (four data points). The limit of quantification of diospyrin was found to be 20 ng, and the minimum detectable quantity 8 ng (peak height more than five times greater than background noise).

Table 4
The intra-day and inter-day determinations of diospyrin

Quantity (μg)		Precision and accuracy	
T	*O*	CV (%); $n = 5$	RE (%)
Intra-day determinations			
0.02	0.017	6.9	15
0.20	0.22	4.2	10
2.0	1.8	5.5	10
20	19.8	1.9	1
Inter-day[a] determinations			
0.02	0.017	12.2	15
0.2	0.17	10.4	15
2.0	1.7	7.6	15
20	19.8	3.2	1

T: 'true' quantity; *O*: 'observed' quantity; RE (relative error) % $= (O - T) \times 100/T$.
[a] The same sample used within a span of 2 weeks.

It may be concluded from Table 4 that reproducibility of the method is satisfactory in terms of its precision, since the CV for all the intra- and inter-day determinations of diospyrin are within 10%. The method is just accurate for estimation of at least 20 ng of diospyrin, while the accuracy improves considerably with the increase in concentration (RE = 1% for 20 μg).

3.3. Determination of diospyrin in bark of *D. montana*

Diospyrin present in a semi-crude extract of the stem-bark of *D. montana* (Table 2) was determined by the present method with verification by spiking with a known quantity of pure diospyrin (Method 1). Obviously, some of the diospyrin would be lost during removal of the undesired materials (before injecting onto the LC column) as described in Section 2.5, particularly during crystallisation. This is evident from the extremely low values obtained by Method 2. To prevent this loss, the semi-crude extract [*] was treated with methyl iodide and silver oxide followed by purification over a neutral alumina column (Method 3). Table 2 shows that the results obtained by the LC method (Method 1) was reasonably accurate (RE ∼ 10%) compared to the calculated value based on the calibration curve, and at par with those obtained by Method 3.

The data can also be compared with a high-performance thin-layer chromatography technique (HPTLC) reported recently by Ravishankar et al. [26] for the determination of diospyrin (RE >50%). Further, the LOD and LOQ of our method were 4–5 times lower than those of the HPTLC technique. LC with electrochemical detection has also been applied for monomeric naphthoquinones [15]; however, the amperometric detection was found to be sensitive to the pH of the eluent (2-propanol –water, 35:65 v/v, pH 6.5). Hence, the proposed LC–UV method may well serve the current global interest for standardisation of herbal medicinal preparations in terms of their naphthoquinonoid constituents.

Acknowledgements

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