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Standardisation and quality control of herbal drugs-1 TLC fluorodensitometric determination of marker compounds of *Plumbago indica* L.

Ajita M. Abeysekera[a]*, T. M. S. G. Tennakoon[b], Tuley De Silva[a]

a. Department of Chemistry, University of Sri Jayewardenepura,Nugegoda, Sri Lanka
b. Research Laboratory, Link Natural Products Ltd., Malinda, Kapugoda, Sri Lanka

Abstract

Objective: Develop a method to quantify a mixture of isoshinanolone and *epi*- isoshinanolone (ISMIX) as a specific marker for *Plumbago indica* L. in a commercial herbal drug, "Dasamoolarishtaya" (DMA). Materials and Methods: The ISMIX content in different DMA samples were analyzed by TLC fluorodensitometry. Isoshinanolone isolated from the root of *P.indica* was used as the standard. The precision and accuracy of the method were determined. Results: The concentrations of ISMIX in different samples of DMA varied widely from < 0.0012 mg/ml (detection limit of method) to 0.045 mg/ml. The precision was 1.52 % CV (n = 4) and recoveries of 99.2 – 100.8 % were obtained in standard addition recovery experiments. Conclusions: The method described is inexpensive, rapid and is of acceptable precision and accuracy for routine quality control work.

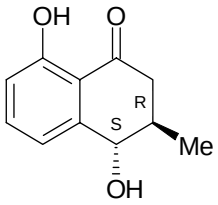
Keywords: Fluorodensitometry, isoshinanolone, *epi*- isoshinanolone, quality control, standardization, herbal drugs.

1. Introduction

Developing methods for the identification and quantification of plant ingredients in herbal drugs through specific marker compounds is important for the quality control and standardization of these drugs. "Dasamoolarishtaya" (DMA), is a complex, fermented herbal drug widely used in the ayurvedic system of medicine in Sri Lanka. The root of *Plumbago indica* L. (Plumbaginaceae) is used in the manufacture of DMA, along with the parts of over 60 other plant species [1].

A number of naphthoquinones and flavonoids have been reported from the plant [2-6]. The major secondary metabolite of *P.indica* is the naphthoquinone, plumbagin. Due to the method of manufacture of DMA, plumbagin is not incorporated (< 0.0002 %) in the final product, and cannot be detected visually by TLC. However, two related compounds found in the plant, isoshinanolone (I) and *epi*-isoshinanolone (II) can be detected by fluorescence (excitation at 366 nm) in the drug

* Corresponding author
e-mail : ajitabey@sjp.ac.lk



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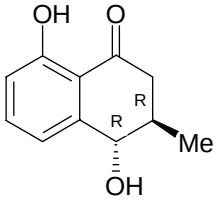

II

and serve as specific marker compounds for *P.indica* in DMA. Isoshinanolone has not been reported previously from *P.indica* but has been reported from the related species *P.zeylanica* and *P.scandens* [7,8].

As (I) and (II) had the same rf value in a variety of TLC systems, a method to quantify the total amount of (I) and (II) (ISMIX) in DMA by TLC densitometry was developed. Although ISMIX could be separated and quantified by HPLC, the method described in this paper is more suitable for routine quality assurance work than HPLC, as it is cheaper and faster and is of acceptable precision and accuracy.

2. Materials and methods

2.1 Drug samples

DMA manufactured by Link Natural Products was used as the reference drug. DMA produced by four other manufacturers were purchased as market samples. The preparation of DMA is described in detail in reference 1.

2.2. Isolation of Isoshinanolone

The dried root of *P.indica* (1 kg) was extracted in a soxhlet apparatus with 5 l of n-hexane for 72 h, and the solvent was evaporated to obtain 35 g of a sticky solid. Thirty grams of the solid was chromatographed on a silica column using a n-hexane/ethyl acetate gradient elution. Plumbagin eluted with n-hexane (1.5 g). Isoshinanolone eluted with the n-hexane:ethyl acetate (1:1) fractions. The dark red semi-solid obtained (15 g) by evaporation of the solvent from these fractions was dissolved in 250 ml of hexane and partitioned with 1 M aqueous potassium hydroxide (250 ml x 4).

The aqueous layer was acidified with concentrated hydrochloric acid and extracted with diethyl ether (250 ml x 4). The impure isoshinanolone obtained by evaporation of the solvent was purified by subjecting it twice to preparative TLC. Isoshinanolone was identified as a yellow fluorescent band at R_f = 0.5 in the first solvent system (n-hexane:ethyl acetate, 7:3), and R_f = 0.35 in the second solvent system (toluene: chloroform, 1:1). The product (200 mg) was obtained as a pale yellow semi solid). NMR and HPLC analysis (see results and discussion) indicated that the product obtained from the above procedure was generally a mixture of isoshinanolone and its 1-epimer, epi-isoshinanolone (ISMIX).

One particular commercial sample of *P.indica* root bark however yielded only isoshinanolone, which was used in drawing the standard curve for the densitometric studies.

Isoshinanolone: UV λ_{max} (MeOH) 215,259,332 nm. IR ν_{max} (film) 3441,2930,1631,1524,1452 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.17 (3H, *d*, *J* = 6 Hz, 2-CH_3), 2.10-2.98 (4H, m, H-2,H_2-3,1-OH), 4.72 (1H, *d*, *J* = 2.5 Hz, H-1), 6.92 (2H, m, and 7.49, 1H, m, H-6,H-7,H-8), 12.35 (1H, s, 5-OH). ^{13}C NMR ($CDCl_3$) δ 16.14(2-CH_3), 34.38(C-2), 40.67(C-3), 71.00(C-1), 114.88(C-10), 118.11(C-8), 118.62(C-6), 136.91(C-7), 144.97(C-9), 162.62(C-5), 204.75(C-4). GCMS *m/z* 192 [M+] (100), 177(17), 150 (50), 122(42), 121(86).

2.3 Densitometry

Densitometry was carried out on a Shimadzu CS 9000 densitometer with excitation at 384 nm and a 450 nm interference filter.

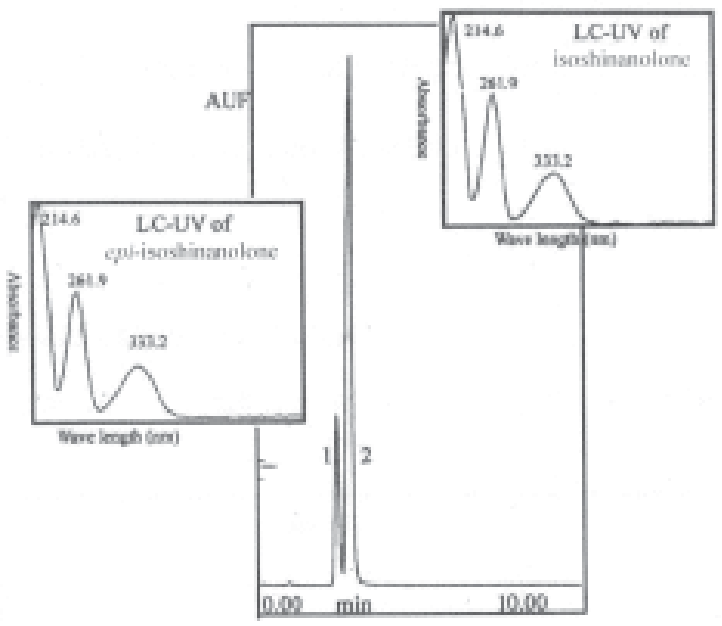

Figure 1. HPLC chromatogram of ISMIX and HPLC –UV spectra of isoshinanolone and epi- isoshinanonlone.

2.4 Standard curve

The absorption spectra of isoshinanolone and *epi*- isoshinanolone were shown to be identical by HPLC-UV (figure 1). It was assumed that their fluorescence emission characteristics would be the same, and the standard curve was drawn with isoshinanolone, which was isolated from a sample of *P.indica* that contained only isoshinanolone. The purity and identity of the standard was established by TLC, HPLC, UV, IR, ^{1}HNMR, ^{13}CNMR and GC-MS [8,9].

Standard solutions of isoshinanolone in methanol containing 5,10,15,20 and 40 µg/ml were prepared by dilution of a stock solution containing 1000 µg/ml. One microlitre of each solution (6 replicates) was applied on the TLC plate (pre coated silica, Merck) manually, using a Hamilton syringe. The chromatogram was developed in a pre-saturated chamber with toluene:ethyl acetate (8:2) as solvent.

The plate was then dried in a stream of hot air and dipped in 1% ethanolic aluminium chloride hexahydrate reagent and heated at 100°C for 2 min and immediately immersed in hexane reagent. Isoshinanolone was visualized at rf 0.62 as a blue fluorescent spot when illuminated at 366 nm. Densitometric scanning was carried out after keeping the plate in the dark for 30 min to stabilize the fluorescence. The standard curve was a straight line passing through the origin (R^2 = 0.9936).

2.5 Sample preparation

DMA (50.00 ml) was dealcoholized by reducing its volume (to ca. 25 ml) by evaporation under vacuum in a rotary evaporator, and extracted with methylene chloride (50 ml x 4). The combined methylene chloride extract was dried over anhydrous sodium sulphate and the solvent was evaporated under reduced pressure. The resultant solid was dissolved in 1.00 ml of methanol. One microlitre of the solution was applied on the TLC plate manually, using a Hamilton syringe.

2.6 Recovery studies

A standard solution of isoshinanolone (2 mg/ ml) was prepared by dissolving 200 mg of isoshinanolone in 100 ml of chloroform. Aliquots of 50 µl, 75 µl, and 100 µl, from the solution corresponding to 100 µg, 150 µg, and 200 µg, respectively of isoshinanolone were added separately to samples of DMA

Table 1.
Recovery of isoshinanolone added to DMA

ISMIX in Sample (µg)	Added isoshinanolone (µg)	Total (µg)	Found (µg)	Recovery %
755	100	855	854.2	99.20
755	200	955	955.55	100.27
1320	200	1520	1521.6	100.80
2230	150	2380	2383.39	102.26

(50.00 ml each) drawn from DMA stocks where the initial concentration of ISMIX had been determined previously. The spiked DMA samples were analyzed as described above.

2.7 HPLC of ISMIX

HPLC of ISMIX was carried out on a Waters instrument equipped with a model 616 quaternary gradient system, 55920 manual injector and a 57002 model 996 photodiode array. A Chromospher C 18 column (200 mm, ID = 3mm) was used with methanol:water (50:50 v/v) as the mobile phase at a flow rate of 0.5 ml/min. The sample volume injected was 5 µl, and the monitoring wave length was 254 nm.

3. Results and discussion

The accuracy of the method was determined by the standard addition recovery method, and recoveries of 99.2 – 100.8 % were obtained (table 1). The precision of the method was determined to be 1.52 % CV (n = 4). The limit of detection as determined by serial dilution, was 60 ng per spot (S/N = 3).

Both the intensity and the stability of fluorescence of a compound on a TLC plate can be enhanced by spraying with the appropriate reagents and solvents [10].

It was found that post-chromatographic treatment of the TLC plate with aluminium chloride enhances the fluorescence while causing a bathochromic shift of the absorption maximum to 384 nm, and that treatment with hexane stabilizes the fluorescence.

The results of the analysis of five different batches of five different brands of DMA purchased from the market are given in table 2. The related densitometric chromatograms are given in figure 2. There is a wide variation in the ISMIX content amongst the different brands as well as within the different batches of the same brand.

These variations reflect the differences in the quality of the crude drug used in the manufacture of DMA as well as inadequate process control during manufacture. In the manufacturing process of DMA, the root of *Plumbago indica* is washed with a dilute

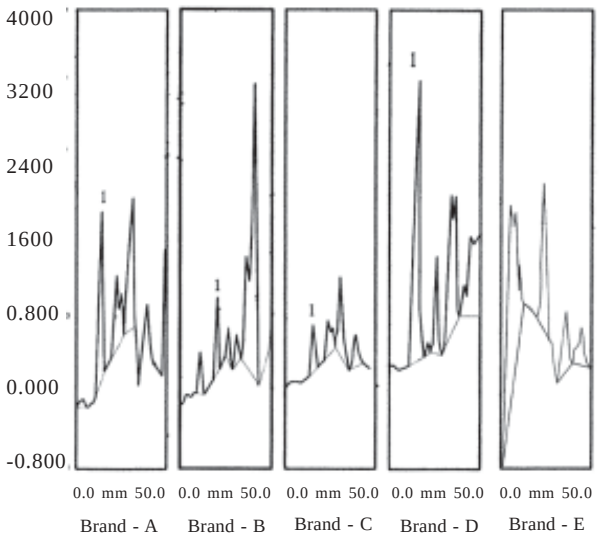

Figure 2. Densitograms of different brands of DMA. (Peak 1- ISMIX).

Table 2
ISMIX contents for five commercially available brands of DMA

Brand	ISMIX content (mg/ml)				
	Batch 1	2	3	4	5
A	0.0151	0.0092	0.0446	0.0264	0.0053
B	ND	ND	0.0071	ND	ND
C	0.0068	0.0079	0.0050	0.0049	0.0054
D	ND	0.0044	0.0080	0.0063	0.0058
E	0.0060	ND	ND	ND	0.0096

ND = not detected, A = Reference sample (Link Natural Products)
B,C,D,E = labels assigned to commercial samples.

Table 3.
^{13}C NMR data of ISMIX

	δ	
C	Isoshinanolone	epi-isoshinanolone
1	71.01	73.52
2	34.34	37.36
3	40.62	43.31
4	204.83	203.75
5	162.52	162.33
6	118.01	118.67
7	136.89	136.97
8	117.12	117.12
9	145.00	145.90
10	114.86	115.00
2-CH_3	16.11	17.74

Table 4.
Ratio of isoshinanolone to *epi* - isoshinanolone in *Plumbago indica* from different locations in Sri Lanka

Location	Ratio of isoshinanolone and *epi* - isoshinanolone
Matale	4 : 1
Anuradhapure	4 : 1
Gampha	only isoshinanolone detected
Kurunagala	only isoshinanolone detected
Colombo	only isoshinanolone detected

solution of calcium hydroxide and then extracted with boiling water. In both these processes low molecular weight phenolics are removed by solubilization and steam volatilization, and clearly the two processes need to be standardized.

The ^{13}C NMR spectrum of ISMIX showed clearly the signals for the two epimers as eleven closely lying pairs (table 3). In the ^{1}H NMR spectrum of ISMIX, the H-1 signal is clearly differentiated for the two epimers, occurring at δ 4.72 and δ 4.47 for isoshinanolone and e*pi*-isoshinanolone respectively. The HPLC of ISMIX (figure 1) confirmed the presence of two compounds having similar UV spectra. The NMR and UV spectral data agreed well with published values [8,9].

The ratio of (I) to (II) in different samples of *Plumbago indica* collected from different parts of Sri Lanka was determined by HPLC and 1H NMR (table 4).These studies showed that there were two distinct varieties of *Plumbago indica* growing in Sri Lanka. One variety has (I):(II) in a ratio of 4:1 while the other contains only isoshinanolone. However, no correlation of the morphology of the plant or total ISMIX content with the variety could be observed.

It is to be noted that DMA contains other plants containing flavonoids and furanocoumarins as fluorescent compounds. The method described achieves selectivity firstly by the choice of extraction solvent (methylene chloride) in which most flavonoids are not very soluble. Secondly, although furanocoumarins are soluble in this solvent, they appear at a higher rf (~ 0.8) in the chromatographic system used. Finally, the fluorescence enhancement method used enables very dilute solutions to be chromatographed, reducing interference from other substances.

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