Vol. 1/1 (2001) 55 - 59

JOURNAL OF NATURAL REMEDIES

An improved HPLC method for estimation of phyllanthin and hypophyllanthin in *Phyllanthus amarus*.

B. Murali*, A. Amit, M.S. Anand, T.K. Dinesh, D.S. Samiulla.

Quality Control Department, Natural Remedies Pvt. Ltd. Veerasandra Indl Area, Bangalore - 561229

Received 28 June 2000; Revised and accepted 2 September 2000

Abstract:

Objective: To develop an improved HPLC method for estimation of phyllanthin and hypophyllanthin in *Phyllanthus amarus*. Materials and methods: An Isocratic, reversed phase (RP) HPLC procedure has been adopted using a mixture of pH 2.8 Phosphate buffer and acetonitrile as mobile phase, CN column as stationary phase and UV detector. Results: The developed method shows high resolution (R = 1.9), accuracy and reproducibility. Conclusion: The method developed is relatively better in terms of separation than the previously reported methods.

Key Words: *Phyllanthus amarus,* Phyllanthin, Hypophyllanthin, HPLC.

1. Introduction

Liver diseases have become common due to a variety of factors like alcohol abuse, usage of CNS active drugs, antibiotics, toxins like aflatoxins in food, use of chlorinated hydrocarbon as pesticides and hepatitis viruses [1]. Currently, there is no suitable drug to treat hepatotoxicity in orthodox medicine. On the contrary, phytomedicines offer a great potential to treat liver disorders, *Phyllanthus amarus* is one such plant [2-4].

Phyllanthin and hypophyllanthin are reported as active principles of *Phyllanthus amarus* [5], hence this plant could be standardised with reference to these markers. A HPLC method is already reported for the same [6]. However, the method lacks desired level of resolution between phyllanthin and hypophyllanthin, hence we have attempted to present a HPLC profile with increased resolution.

2. Materials and methods

2.1 Plant material

Whole plants of *Phyllanthus amarus* Schum. and Thonn. (Family: Euphorbiaceae) were collected from Ponnamalli outskirts, Chennai, India in February 1999 and taxonomically identified by Dr. S.P. Thyagarajan (Head of the Microbiology Department, Dr. A.L.M. PG Institute of Basic Medical Sciences, Taramani, Madras). The plants were sun dried for 3- 4 days, herbaria were prepared and voucher specimens(PA/E/01) were deposited at the pharmacognosy department of Natural Remedies Pvt. Ltd.

* Corresponding Author
Email: balmurali@hotmail.com

Table 1
Phyllanthin and hypophyllanthin content in *Phyllanthus amarus*

Sl. No.	Sample	Phyllanthin (% w/w)	Hypophyllanthin (% w/w)
1.	Sample 1	0.75	0.28
2.	Sample 2	0.34	0.12
3.	Sample 3	0.33	0.1

2.2 Solvents and reagents

Solvents used were acetonitrile and methanol (from Ranbaxy, HPLC grade), orthophosphoric acid, potassium hydroxide and potassium di-hydrogen phosphate (from Ranbaxy, AR grade).

2.3 Extraction of plant material [6]

7 g of plant material was pulverised and mixed with 2.1g of lime and 30ml of water and macerated for 1 day and marc was extracted with 20ml of 3% methanolic potassium hydroxide (KOH) for 1 hour, process was repeated 3 times. The methanolic KOH extract was combined and concentrated to 50 ml.

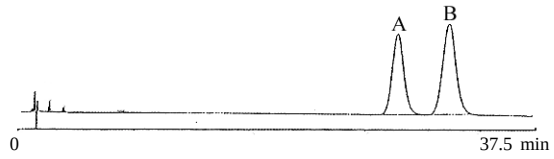

Fig. 1: HPLC Chromatogram of (A) Phyllanthin; (B) Hypophyllanthin

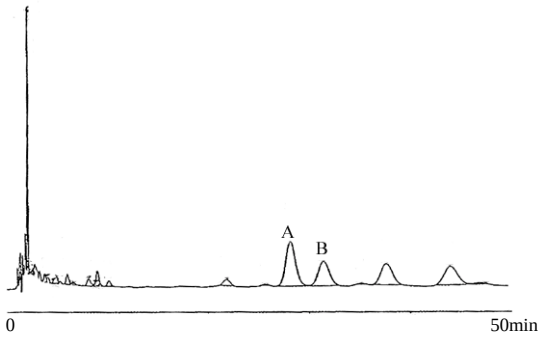

Fig 2: HPLC Chromatogram of whole plant of *Phyllanthus amarus* (A) Phyllanthin; (B) Hypophyllanthin

2.4 Equipments

The Shimadzu chromatographic system comprising of LC 8A model dual pump, photo diode array detector (SPD-M10AVP) and Class LC-10 software and E-Merck nitrile column (250mm x 4mm) Lichrocart 250-4, Lichrosphere 5µ was used for analysis.

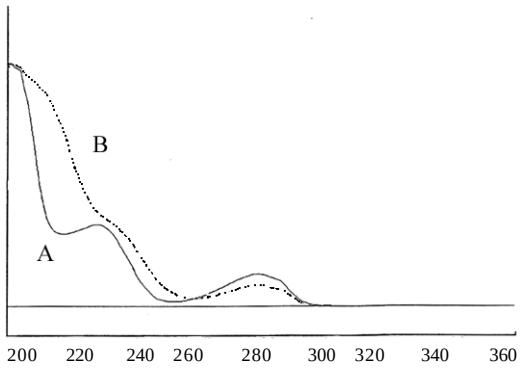

Fig 3: Photodiode array spectra of *Phyllanthus amarus* (A) Phyllanthin; (B) Hypophyllanthin

2.5 Experimental conditions

The analysis was performed at the flow rate of 1.9ml/min using phosphate buffer pH 2.8 (1.36 g potassium dihydrogen phosphate in 1000 ml water, pH adjusted to 2.8 with orthophosphoric acid) and acetonitrile (83:17) as mobile phase. The photo diode array detector was set at a wavelength of 230 nm (for quantification) and the spectral scan performed between 200-360 nm.

The two lignans were isolated from *Phyllanthus amarus* as per the method of Sripathi [7] and their identities were confirmed by comparing the UV, IR, ^{1}HNMR and ^{13}CNMR of the isolated with those reported in the literature [5, 8].

Table - 2
Method of validation

Sl. No	Test Characteristics	Observed results
1.	Specificity	Very specific & no interference (Confirmed by PDA spectrum) Peak purity ratio >0.99
2.	Linearity	Linear up to 1000mcg/ml, Co-relation coefficient $r^2 > 0.99$
3.	Range of quantification	50 -1000mcg/ml.
4.	Accuracy	± 2%
5.	Precision	Related standard deviation < 2%
6.	Repeatability & Reproducibility	Related standard deviation < 2%
7.	System suitability	The resolution between compounds of interest is1.9 (>1.5). Relative retention time of phyllanthin is 1 and hypophyllanthin is 1.14. The assymetric factor is < 1.2

Table 3
Recovery of phyllanthin and hypophyllanthin from *Phyllanthus amarus.*

Sl. No.	Sample 1 (mg)	P content	HP content	Amount of P added	Amount of HP added	Total P content	Total HP content	Total P found	Total HP found	% recovery P	% recovery HP
1.	7000	52.5	19.6	5	5	57.5	24.6	56.9	24.9	99.0	101.2
2.	7000	23.8	8.4	10	10	33.8	18.4	34.1	18.6	100.9	98.8

P- Phyllanthin; HP- Hypophyllanthin; Values are in % w/w

2.6 Identification of phyllanthin and hypophyllanthin peaks

Separately 500 mcg/ml of methanolic solution of Phyllanthin and Hypophyllanthin was prepared and 10µl of each solution was injected to identify the retention time.

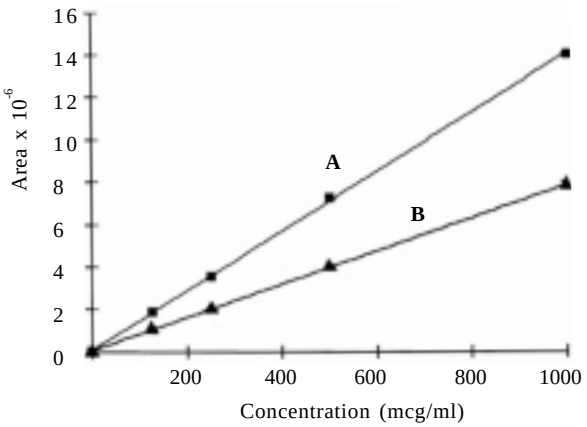

Fig. 4: Regression plot of - (A) Phyllanthin and (B) Hypophyllanthin

2.7 Calibration curves:

25 mg of each of phyllanthin and hypophyllanthin were accurately weighed and added to a 25 ml volumetric flask, dissolved in HPLC grade methanol and the volume was made upto 25ml with HPLC grade methanol to obtain 1000mcg/ml.

This solution was appropriately diluted further to get a concentration of 500, 250, and 125 mcg/ml of phyllanthin and hypophyllanthin. 10 µl of each of these solutions was injected in triplicate and the average area was calculated for phyllanthin and hypophyllanthin. Calibration graphs were plotted for phyllanthin and hypophyllanthin and the regression co-efficient was calculated.

2.8 Estimation of phyllanthin and hypophyllanthin in samples and recovery studies

The HPLC estimation was carried out by injecting 10 µl of the sample solution (refer 2.3). Percentage of phyllanthin and hypophyllanthin were estimated using the area under the curve obtained from the sample by comparing the same with standard. The accuracy of estimation is validated using spike recovery studies.

3. Results and Discussion

An improved HPLC method for standardization of *P.amarus* has been attempted using the two of its bioactive lignans phyllanthin and hypophyllanthin as markers.

The calibration curves for phyllanthin and hypophyllanthin were found to be linear over the range of 0 to 1000 mcg/ml (Table 2). The regression co-efficient for both phyllanthin and hypophyllanthin was 1.0 (Fig. 4).

Mean relative retention time in the identification of phyllanthin and hypophyllanthin under the conditions described above were 1 and 1.14 (asymmetric factor was less than 1.2) and the resolution was 1.9.

The phyllanthin and hypophyllanthin content and recovery studies using the improved HPLC method under the above-mentioned condition were reported in the Table-1 and Table-3 respectively. Recovery studies show that more than 98% of phyllanthin and hypophyllanthin could be extracted using the extraction procedure as described above.

In conclusion, the assay method described herein is simple, precise and reproducible for quantifying phyllanthin and hypophyllanthin, the two bioactive lignans in *P. amarus.*

References

1. Kiso Y, Hikino H. (1991), In: Hostettmann K. (Ed.) *Methods in Plant Biochemistry,* Academic Press: London; 219-233.

2. Thyagarajan SP, Subramaniam S, Thirunalasundari T, Venkateshwaran PS, Blumberg BS. (1988) *Lancet* 764-766.

3. Sane RT, Kuber VV, Chalissery MS, Menons S. (1995) *Current Science* 68(12): 1243-1246.

4. Mehrotra R, Rawat S, Kulshreshtha DK, Goyal P, Patnaik GK, Dhawan BN. (1991) *Indian J. Med. Res.* 93: 71-73

5. Row LR, Srinivasulu C, Smith M, Rao GSR, Rao S. (1966) *Tetrahedron* 22: 2899-2908.

6. Sharma A, Singh RT, Handa SS (1993) *Phytochem. Anal.* 4: 226-229.

7. Sripathi (1999) Isolation and standardisation of markers from *Phyllanthus amarus* (M. Pharm degree dissertation) Rajiv Gandhi University of Health Science: Bangalore; 26-54.

8. Anjaneyulu ASR, Rao KJ, Row LR, Subramanyam C. (1973) *Tetrahedron* 29: 1291-1298.