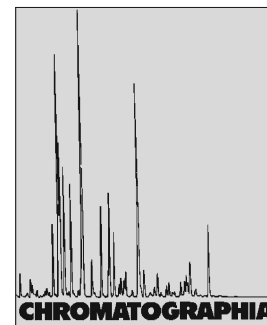


Quantitative Determination of Protoberberine Alkaloids in *Tinospora cordifolia* by RP-LC-DAD



Dada Patil¹, Manish Gautam², Sanjay Mishra¹, Prajakta Kulkarni³, Karupothula Suresh², Sunil Gairola², Suresh Jadhav², Bhushan Patwardhan^{1,4,✉}

¹ Interdisciplinary School of Health Sciences, University of Pune, Pune, Maharashtra 411007, India

² Serum Institute of India Ltd, Hadapsar, Pune, Maharashtra 411028, India

³ Sinhgad College of Pharmacy, Pune, Maharashtra 411041, India

⁴ Manipal Education, HAL Airport Road, Bangalore 560 008, India;

E-Mail: bhushan@unipune.ernet.in; bhushan.patwardhan@manipalu.com

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Abstract

Tinospora cordifolia, known as Guduchi in Ayurveda, is a medicinal plant popular mainly for immunomodulatory activity. Its therapeutic activity may be attributed to protoberberine alkaloids such as jatrorrhizine, palmatine and berberine. A new, simple RP-LC-DAD method has been developed for separation, simultaneous identification and quantitative estimation of these protoberberine alkaloids in *T. cordifolia* extracts and formulations. The developed method was validated based on ICH-Q2B guidelines and was found to be accurate, precise and linear over a relatively wide range of concentrations (0.65–83.33 $\mu\text{g mL}^{-1}$). This method can serve as a useful quality control tool for *T. cordifolia* and its formulations.

Keywords

Column liquid chromatography-diode array detection

Protoberberine alkaloids

Berberine, jatrorrhizine and palmatine

Tinospora cordifolia

Introduction

The global herbal drugs and nutraceutical market is growing and so is the incidence of adulteration. It is essential to detect counterfeits and ensure quality, safety and efficacy of herbal materials and formula-

tions [1]. Instrumental analytical methods play a significant role in assessing the authenticity and quality of herbal drugs and hence are widely used for their quality control. *Tinospora cordifolia* (Willd) Miers (Menispermaceae) is an official herb in Indian Pharmacopoeia and also in

Ayurvedic Pharmacopoeia [2, 3]. The stems of *T. cordifolia* (TC) known as Guduchi in vernacular, are widely used in Ayurveda as Rasayana to enhance general body resistance, promote longevity and as antistress and adaptogen [4, 5]. Three major groups of compounds; protoberberine alkaloids, terpenoids and polysaccharides are considered as putative active constituents of TC [6, 7]. Comprehensive analytical approaches simultaneously targeting three alkaloids are important for quality control of TC containing formulations. Protoberberine alkaloids such as berberine, palmatine are reported to have anti-cancer [8–10], anti-infective [11, 12], anti-diabetic [13] and immunomodulatory [14, 15] activities. Several methods have been reported to estimate these alkaloids in herbal drugs by using TLC [16], CE [17], LC [18] and LC-ESI-MS [19] methods.

Here, we report a comprehensive extended method for simultaneous quantitative analysis of such alkaloids in TC and its formulations. We have used a validated RP-LC-DAD method for the simultaneous quantitative estimation of berberine and palmatine in *T. cordifolia*. LC-ESI-MS was employed for identification of the jatrorrhizine, berberine and

Table 1. Method validation and applicability for markers' identification and quantification

		Linearity		Precision			Accuracy	
		Regression equation	LOD ^b ($\mu\text{g mL}^{-1}$)	LOQ ^b ($\mu\text{g mL}^{-1}$)	Intraday mean $\mu\text{g mL}^{-1}$ ($N = 15$)	Interday mean $\mu\text{g mL}^{-1}$ ($N = 15$)	Repeatability mean $\mu\text{g mL}^{-1}$ ($N = 5$)	Mean % ($N = 3$)
Method validation								
Compound 1 Jatrorrhizine $t_R = 19.28$	$Y = 2.1992X - 0.2295$	0.11	0.32	NA	NA	NA	NA	NA
Compound 2 palmatine $t_R = 23.49$	$Y = 1.9162X + 0.0411$	0.12	0.35	15.77 (1.80) ^a	14.52 (3.09)	14.89 (3.15)	101.59 (2.22)	
Compound 3 berberine $t_R = 24.76$	$Y = 1.9746X - 0.1734$	0.10	0.30	20.96 (1.12)	21.36 (2.45)	20.63 (2.60)	101.91 (2.34)	
Sample code	Batch number	Form	Supplier	Dosage form	Marker content			
					Jatrorrhizine ($\mu\text{g mg}^{-1}$)	Palmatine ($\mu\text{g mg}^{-1}$)	Berberine ($\mu\text{g mg}^{-1}$)	
Method applicability								
TM-1	ACD/418	Crude	Natural Remedies	Powder	+	0.027 ± 0.003	0.0027 ± 0.0007	
TM-2	ACD/02	Crude	Natural Remedies	Powder	+	0.106 ± 0.03	0.010 ± 0.008	
TM-3	TC 006001	Aqueous extract	Natural Remedies	Powder	+	0.023 ± 0.005	0.031 ± 0.009	
TM-4 Formulation	F137001G Single herb	Aqueous extract	Himalaya Drugs	Capsule	ND	$0.0012 \pm .0008$	0.00075 ± 0.0005	
TM-5 Formulation	A106009 Multi herb	Aqueous extract	Himalaya Drugs	Tablet	ND	0.00072 ± 0.0001	0.0023 ± 0.0007	
TM-6 Formulation	WPA 8001 Single herb	Aqueous extract	Wockhardt Limited	Tablet	ND	0.0030 ± 0.0005	0.00048 ± 0.0001	

Accuracy and precision was not established for Jatrorrhizine as it showed poor resolution in TC samples

NA not applicable. t_R retention time in min, ND not detected

^a Values in parenthesis indicate RSD. Values are given as mean \pm SD; +: indicates presence

^b Noise level was determined by repeated analysis of blank injections ($n = 3$) at 345 nm in the t_R range of ± 1 min of respective markers using Chromelon software

palmatine alkaloids in TC extracts and marketed formulations.

Experimental

Chemicals

The chemicals for LC studies were of LC grade, while all others were of analytical grade. Protoberberine alkaloids, jatrorrhizine (compound 1), palmatine chloride (compound 2) and berberine hydrochloride (compound 3) were used as reference standards. Compounds 1 and 2 were procured from Chromadex; CA; USA and compound 3 was procured from Inga Laboratories Mumbai, India. Acetonitrile, methanol, ammonia

solution and dichloromethane were purchased from Merck, India. Purity of all the compounds was found to be >99%.

Test Materials

The study was carried out using six different samples (TM 1–TM 6) procured from local market and their details are described in Table 1. The crude samples were authenticated at the National Institute of Science Communication and Information Resources, New Delhi, India (Voucher: NISCAIR/RHM/F-3/Consult/451/27/1). The samples were dried to constant weight at 50–60 °C prior to analysis.

Preparation of Standard Solution

The standard stock solution mixture of compounds 1, 2 and 3 ($100 \mu\text{g mL}^{-1}$) was prepared in methanol. Aliquots of stock solution were suitably diluted to yield eight linear concentrations in the range of $0.65\text{--}100 \mu\text{g mL}^{-1}$. These dilutions were then used for calibration curve studies.

Sample Preparation

Isolation of alkaloids was done using an earlier reported method [20]. Samples (TM-1–TM-6) were extracted with dilute glacial acetic acid (12% v/v) in the ratio

of 1:40 followed by centrifugation at 400 g for 5 min. The supernatants were basified to pH 8 with ammonia solution (25% v/v) and were partitioned with dichloromethane (three times; 900 mL) and organic phase was dried under vacuum. The working concentrations of TM 3 (4 mg mL⁻¹) and TM 1, 2, 4, 5 and 6 (8 mg mL⁻¹) were prepared using methanol.

LC Conditions

LC analysis was performed using Dionex System (Germering, Germany) with thermostated column oven (TIC100), diode array detector (340U) and a quaternary gradient pump (P680). Compounds were separated at 25 °C on a Hypersil Gold C-18 column (250 × 4.6 mm; 5 μm; Thermo Electron Corporation; Bellefonte, USA) with a C18 Hypersil BDS guard column (50 × 4.6 mm; 5 μm). Mobile phase consisted of water (18 mΩ cm) containing 20 mM ammonium acetate and 0.2% formic acid (pH 4.8) as component A and acetonitrile as component B. The linear gradient program was: 0–5 min with 20% B, 5–40 min with 20–50% of B, 40–45 min with 50% B and followed by equilibration period for 10 min. Separations were monitored at 265 and 345 nm with a flow rate of 0.6 mL min⁻¹. Samples were filtered through a 0.2 μm filter prior to injections and 20 μL were injected to the LC system. Data was processed using Chromeleon 6.70 software (Germering, Germany).

LC-ESI-MS Conditions

LC-MS system consisted of a liquid chromatograph (Shimadzu Prominence, Japan), binary gradient pump (LC-20AD), auto-sampler (SIL HTC) and column oven (CTO-10ASVP). For LC-ESI-MS analysis, a triple quadrupole mass spectrometer (API 4000; Applied Biosystems/MDS SCIEX, CA, USA) with SIM acquisition in the positive ion mode was used. The operating parameters were: curtain gas (25 psi); gas 1-nebulizer gas (25 psi); gas 2—heater gas (15 psi); turbo ion spray voltage (IS) 5,500 V; source tempera-

ture at 300 °C; declustering and entrance potential were set at 80 and 10 V respectively.

Method Validation

Linearity, accuracy and precision (inter, intra and repeatability) studies were carried out as per ICH-Q2B guidelines [21]. LOD and LOQ were reported as 3 and 10 times the noise level obtained from three replicate injections of blank samples respectively. Precision of the method was based on % RSD of compounds 2 and 3 on replicate injections of TM-3 (4 mg mL⁻¹). Method repeatability was assessed by analysis of five different working solutions prepared from the same sample of TM-3. Accuracy assay was carried out by spiking (100% of detected amount) compounds 2 and 3 (15.77 and 20.96 μg mL⁻¹) into TM-3. Percent recoveries obtained have been reported in Table 1.

Results and Discussion

Optimization of the Separation Conditions

During method development, different mobile phase compositions were tried. The compositions (water:acetonitrile) with modifiers such as ammonium acetate and ammonium chloride showed good resolution of alkaloid peaks. However, ammonium acetate was preferred to ammonium chloride due to better compatibility with MS analysis which is consistent with previous observations [22]. Hypersil Gold C18 column was chosen as stationary phase as it enabled better separation compared to Luna C18 and Hypersil BDS C18 columns. Suitable changes were made to gradient conditions and flow rate (0.6 mL min⁻¹) to improve the resolution of alkaloid peaks. Typical chromatograms of TC extract (TM-3) are shown in Fig. 1a, in which baseline separation of compound 2 and 3 was achieved. These LC conditions could not resolve compound 1 from the samples. Detailed analysis of DAD spectrum indicated that compounds 1, 2 and 3 showed wavelength

maxima at 265 and 345 nm which is consistent with their previous reports [23]. Wavelength at 345 nm was used for quantification as it exhibited better resolution and minimal spectral interferences arising due to coexisting components in samples (TM-1–TM-6).

LC-DAD and LC-MS Analysis

The LC chromatograms of TM-3 were compared to the chromatograms of marker compounds: jatrorrhizine, palmatine and berberine (Fig. 1a). These alkaloids were identified in the samples by comparing their retention times, UV spectra at 345 nm and MS data with that of the marker compounds. In MS analysis, selective ion monitoring (SIM) was used because of higher sensitivity [24]. To reduce interference of other components, higher dilutions of samples and marker compounds were selected for analysis [24]. Figure 1b shows the SIM chromatograms of *m/z* 338, 352 and 336 as identified in TM-3 for compounds 1, 2 and 3 respectively in positive ion mode. The SIM chromatogram of compound 1 showed two peaks at *m/z* 338.1. Based on these observations, it was concluded that the method is more suitable for quantification of compounds 2 and 3 in TC samples.

Method Validation

The method showed excellent linearity ($r^2 > 0.9999$) in the range of 0.65–83.33 μg mL⁻¹ for marker compounds. The regression equations, LOD and LOQ of respective marker are given in Table 1. The developed method was found to be reproducible and precise as the % RSD values obtained for compounds 2 and 3 were less than 5%. Mean percent recoveries for compounds 2 and 3 were found to be 101.59 and 101.91% respectively. Hence the developed method was found to be accurate (Table 1).

Method Applicability

The developed method is able to identify protoberberine alkaloids including

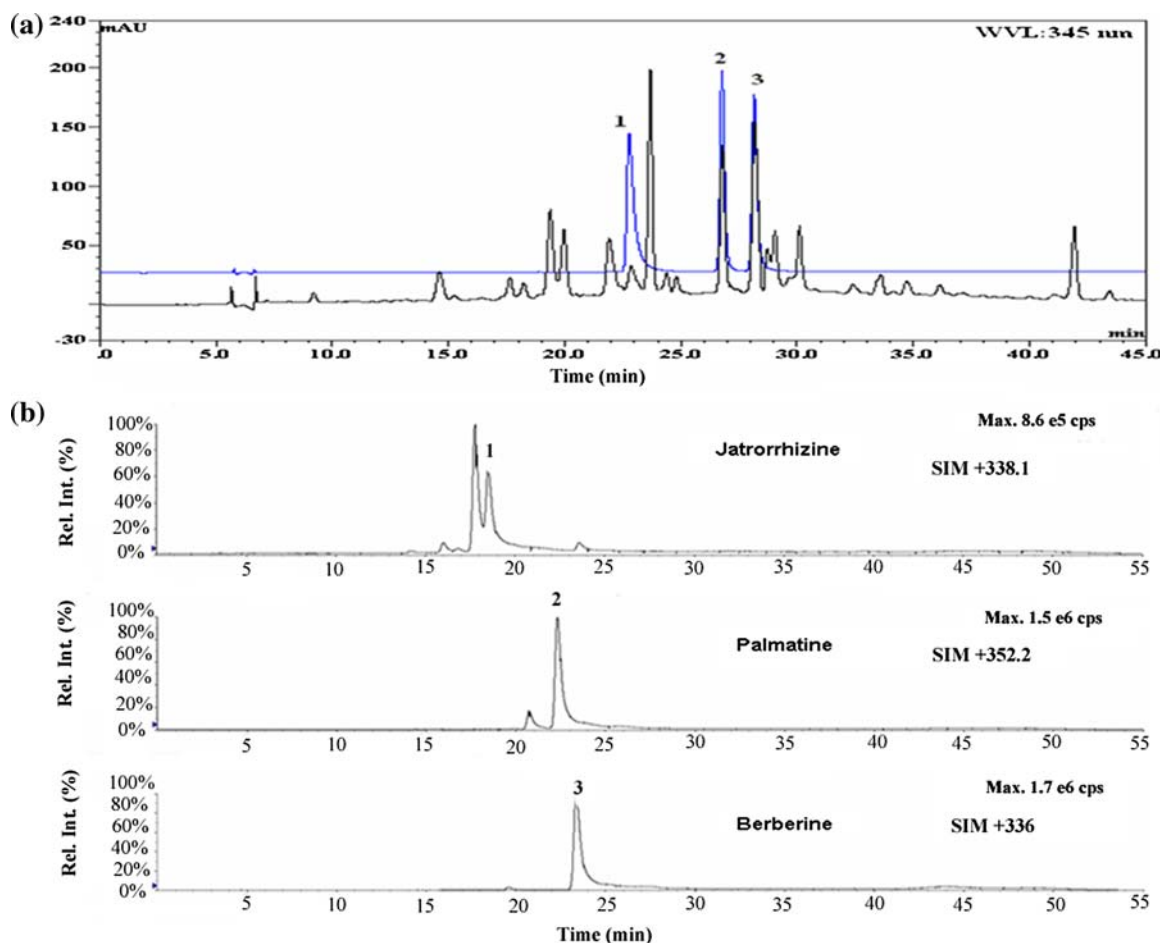


Fig. 1. a LC-DAD chromatograms at 345 nm showing overlay of markers (standard mix) and TM-3. TM-3 was chosen as it commonly used in formulations. Peaks: **1** = jatorrhizine; **2** = palmatine; **3** = berberine. Peak **1** in TM-3 did not show good resolution. b SIM chromatograms of marker compounds as identified in TM-3 at m/z 338, 352 and 336. Jatorrhizine showed merged peaks in SIM which was consistent with LC-DAD observations

jatorrhizine, palmatine and berberine in TC and its formulations (Table 1). It can also be used for quantitative estimation of compounds **2** and **3** in the range of 0.0007–0.10 and 0.0007–0.03 $\mu\text{g mL}^{-1}$ respectively.

Conclusion

T. cordifolia is marketed globally in various dosage forms such as powders, tablets and capsules either as a single herb or in poly herbal formulations. This extended and validated method allows simultaneous estimation of protoberberine alkaloids and can be used for routine quality control of crude drugs and polyherbal formulations.

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References

1. Warude D, Patwardhan B (2005) *J Sci Ind Res* 64:83–92

2. Indian Pharmacopoeia (2007) Indian Pharmacopoeia Commission, Ghaziabad, pp 2037–2038
3. The Ayurvedic Pharmacopoeia of India (2001) The Controller of Publications, Delhi, 1st edn, pp 41–42
4. Patwardhan B, Gautam M (2005) *Drug Discov Today* 10:495–502
5. Patil M, Patki P, Kamath HV, Patwardhan B (1997) *Ind Drugs* 34:211–215
6. Chintalwar G, Jain A, Sipahimalani A, Banerji A, Sumariwalla P, Ramakrishnan R, Sainis K (1999) *Phytochemistry* 52:1089–1093
7. Bisset N, Nwaiwu J (1983) *Planta Med* 48:275–279
8. Diwanay S, Gautam M, Patwardhan B (2004) *Curr Med Chem ACA* 4:479–490
9. Hur JM, Hyun MS, Lim SY, Lee WY, Kim D (2009) *J Cell Biochem* 107:955–964
10. Diwanay S, Chitre D, Patwardhan B (2004) *J Ethnopharmacol* 90:49–55
11. Iwasa K, Kim C (1997) *Phytochemistry* 46:1359–1363

12. Iwasa K, Nishiyama Y, Ichimaru M, Moriyasu M, Kim H, Wataya Y, Yamori T, Takashi T, Lee D (1999) *Eur J Med Chem* 34:1077–1083
13. Leng S, Lu F, Xu L (2004) *Acta Pharmacol Sin* 25:496–502
14. Rockova L, Majekova M, Kost D, Stefek M (2004) *Bioorg Med Chem* 12:4709–4715
15. Patwardhan B, Kalbag D, Patki PS, Nagsampagi BA (1990) *Indian Drugs* 28:56–63
16. Rout KK, Pradhan S, Mishra SK (2008) *J AOAC Int* 91:1149–1153
17. Unger M, Stockigt D, Belder D, Stockigt J (1997) *J Chromatogr A* 767:263–276
18. Shi P, Zhang Y, Shi Q, Zhang W, Cheng Y (2006) *Chromatographia* 64:163–168
19. Luo X, Chen B, Yao S (2005) *Talanta* 66:103–110
20. Sarkozi A, Janicsak G, Kursinszki L, Kery A (2006) *Chromatographia Suppl* 63:S81–S86
21. ICH-Q2B, validation of analytical procedure: methodology. International conference on harmonization, Geneva 1996 March
22. Shi Q, Yan S, Liang M, Yang Y, Wang Y, Zhang W (2007) *J Pharm Biomed Anal* 43:994–999
23. Grycova L et al (2007) *Phytochemistry* 68:150–175
24. Villagrasa M, Guillamón M, Eljarrat D, Barceló E (2007) *J Chromatogr A* 1157:108–114