Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 46 (2008) 391–394
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

High-performance thin layer chromatography method for estimation of conessine in herbal extract and pharmaceutical dosage formulations

Aman D. Kaur, V. Ravichandran, Prateek K. Jain, Ram K. Agrawal *

Pharmaceutical Chemistry Research Laboratory, Department of Pharmaceutical Sciences, Dr. Hari Singh Gour University, Sagar, MP 470003, India

Received 15 June 2007; received in revised form 25 September 2007; accepted 2 October 2007
Available online 6 October 2007

Abstract

A new, simple, sensitive, precise and robust high-performance thin layer chromatographic (HPTLC) method was developed for the estimation of conessine in herbal extracts and pharmaceutical dosage forms. Analysis of conessine was performed on TLC aluminium plates pre-coated with silica gel 60F-254 as stationary phase. Linear ascending development was carried out in twin trough glass chamber saturated with mobile phase consisting of toluene–ethylacetate–diethyl amine (6.5:2.5:1, v/v/v) at room temperature ($25 \pm 2\,^{\circ}C$). After derivatized the plate with modified Dragendroff's reagent, Camag TLC scanner III was used for spectrodensitometric scanning and analysis of the plate in absorbance mode at 520 nm. The system was found to give compact spots for conessine (R_f value of 0.82). The data for calibration plots showed good linear relationship with $r^2 = 0.9998$ in the concentration range of 1–10 μg with respect to peak area. The present method was validated for precision and recovery. The limits of detection and quantification were determined. Statistical analysis of the data showed that the method is reproducible and selective for estimation of conessine.

Keywords: High-performance thin layer chromatography; Method validation; *Holarrhena antidysentrica*; Conessine; Polyherbal formulations

1. Introduction

The stem bark of *Holarrhena antidysenterica* (Family: Apocynaceae) is used to cure amoebic dysentery and diarrhea [1–3]. The principle called conessine (Fig. 1) isolated from the stem bark has lethal action on *Entamoeba histolytica* [4,5]. The various part of the *H. antidysenterica* has been reported to possess antibacterial activity [6–8]. The bark has been reported to possess anthelmintic appetizing, astringent and antidiarrheal properties [9]. Total alkaloids of *H. antidysenterica* bark vary from 1.1 to 4.72% [10]. The main steroidal alkaloid of *H. antidysenterica* is conessine (0.4%) [11], and the other alkaloids isolated include narconessine, conessimine, holarrhimine, holarrhenine and holarricine. The bark also contains sterol, gum, resin, tannin and lupeol [11–14]. Duez et al. [15] reported comparison of high-performance thin layer chromatography densitometry and gas–liquid chromatography for the determination of conessine in *Holarrhena floribunda* stem bark. Dwivedi et al. [16] reported turbidimetric method for the quantitative estimation of the total alkaloids of Kutaj bark (*H. antidysentrica*) in crude medicinal preparations and in the body fluid of man and rat. Gas chromatography has also been reported as more accurate for the analysis of Conessine [17].

Certain herbal extracts and polyherbal formulations containing active constituents were standardized by high-performance thin layer chromatography (HPTLC) method. HPTLC method was reported to be the most suitable method for the estimation of active constituents of extracts, plant species (raw material) or polyherbal formulations. No HPTLC method has been reported for the estimation of conessine in *H. antidysentrica.* Therefore, an attempt has been made to develop accurate, specific, repeatable and robust HPTLC method for the determination of conessine.

2. Experimental

2.1. Material

H. antidysenterica was procured from local suppliers and was assessed biologically by the Department of Botany, Dr. H.

* Corresponding author. Tel.: +91 7582233934.
E-mail address: dragrawal2001@yahoo.co.in (R.K. Agrawal).

0731-7085/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2007.10.001

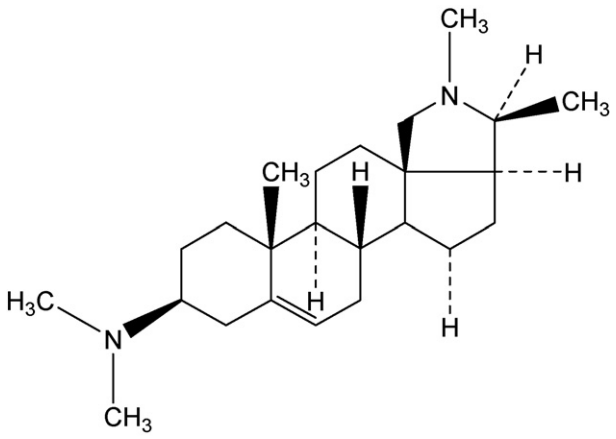

Fig. 1. Chemical structure of conessine.

S. Gour University, Sagar (MP), India. Standard conessine (mp: 340–343 °C; IR: 2970 (C–H str., assym. CH_3), 2944 (C–H str., Sym. CH_3), 2833 (C–H str., assym. CH_2), 2655 (C–H str., 3° carbon in ring), 3190 (C–H str.), 1641(C–C str.), 1477 (assym. CH_3 bending), 1443 (CH_2 scissoring), 1365 (–N $(CH_3)_2$ bending), 1025 (C–N str.), 775 (=C–H str, out of plane bend), 727 (CH_2 rock), 627 (C–C bending, out of plane); ^{1}H NMR: three singlet at δ 0.91, 2.28, 2.32, a doublet at δ 1.05 characteristic of five methyl groups and an olefinic proton at d 5.31, aliphatic protons between d 0.92 and 2.44. These data were consistent with those in Refs. [18,19]). was isolated from *H. antidysenterica*. All chemicals and reagents used were of analytical grade.

2.2. Instrumentation and chromatographic conditions

The samples were spotted in the form of bands of 5 mm with Camag microlitre syringe on pre-coated silica gel aluminium Plate 60F-254 (20 cm × 10 cm) with 200 μm thickness (E. Merck, Germany) using a Camag Linomat V (Switzerland) sample applicator. A constant application rate of 100 nL s^{-1} was employed and space between two bands was 12.5 mm. The slit dimension was kept at 2 mm × 0.45 mm and 20 mm s^{-1} scanning speed was employed. The composition of mobile phase is toluene:ethylacetate:diethylamine (6.5:2.5:1, v/v/v). Linear ascending development was carried out in a twin trough glass chamber saturated with mobile phase. The optimized chamber saturation time for the mobile phase was 30 min at room temperature (25 ± 2 °C). The length of chromatogram run was 175 mm. Then the plate was allowed to dry at room temperature. Derivatized the plate with modified Dragendroff's reagent (Bismuth nitrate + nitric acid + potassium iodide) by dipping for 5 s and dried the plate for 5 min at 45 °C in an oven. The separated bands on the HPTLC plates were scanned over the wavelength of 400–600 nm. The source of radiation utilized was tungsten lamp. The maximum absorbance was found to be at 520 nm.

2.3. Calibration curve of conessine

A stock solution of conessine (1000 μg mL^{-1}) was prepared in methanol. Different volumes of stock solution 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 μL, were spotted on the TLC plate to obtain concentration of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 μg $spot^{-1}$ of conessine, respectively. The calibration graph was plotted by using data of peak areas against corresponding concentrations.

2.4. Method validation

2.4.1. Sensitivity, limit of detection and limit of quantification

These parameters were calculated from the data set obtained from a linear calibration curve in the range 1–10 μg (two replicates for each standard). For this purpose, a 10 μL volume of the corresponding methanolic standard solution was applied in duplicate as bands of 2 mm. Application parameters were the same as above, except for delivery speed, which was changed to 50 nL s^{-1} in order to obtain an appropriate evaporation. The corresponding slope and regression standard deviation ($S_{Y/X}$) values were used to establish sensitivity ($S_{Y/X}/b$). LOD was calculated with the following equation:

$$\mathrm{LOD} = 3\left(\frac{S_{Y/X}}{b}\right)\sqrt{\frac{n-2}{n-1}}$$

LOQ was determined by multiplying by 10 in this equation. A linear calibration curve in the range 1–100 μg (two replicates for each standard) was also calculated.

2.4.2. Accuracy (recovery analysis) and precision

The accuracy of the method was studied by performing experiments by standard addition technique. Three different levels (50, 100 and 150%) of standards were added to a previously analyzed sample, each level being repeated thrice. Precision of an analytical method is expressed as S.D. and R.S.D. of series of measurement. It was ascertained by replicate estimation of the samples by proposed method. The precision of the developed method was also confirmed by different day's analysis. The similar process was repeated under the same set of condition for 3 days. The sample solution and standard solution prepared earlier were used and on HPTLC plate five spots of sample and two spots of standard were applied, the plate was developed and scanned, and densitograms were recorded. Same procedure was followed the next day and the concentration of the sample was calculated by comparing with the standard.

2.4.3. Stability

Sample solutions of the extracts were prepared and stored at room temperature for 10, 30, 60, 120 and 240 min. and then applied on the same chromoplate; after development the chromatogram was evaluated for additional spots. Similarly spot stability was analyzed by keeping the resolved plates and inspecting at intervals of 10, 30, 60, 120 and 240 min.

2.5. Analysis of conessine in herbal extracts

The powdered drug (500 mg) was placed in a conical flask and extracted twice with methanol containing 0.5 mL of the 25% ammonia solution. Filtered and volume was made to 50 mL

with methanol and the solution was analyzed for the drug content. Pipette out 1 mL of this solution to 10 mL volumetric flask and made up to volume with methanol (100 μg mL^{-1}). Five microlitre of the filtered solution (5 μg mL^{-1}) was applied on the TLC plate followed by development and scanning as described in Section 2.2. The analysis was repeated in triplicate. Conessine content in herbal extracts from four different sources (in India) was also studied.

2.6. Analysis of conessine in prepared formulations

Pharmaceutical formulation (solution) equivalent to about 10 mg of conessine, i.e., 10 mL of formulation was made alkaline with 1 mL of 10% ammonia solution and then extracted with chloroform. Removed the solvent from the chloroform extract and the residue was dissolved in 20 mL of 40% alcohol acidified with 2 mL of concentrated HCL. This solution was extracted with chloroform to remove the coloring matter and rejected the chloroform extract. The alcoholic mother liquor was made alkaline and extracted with chloroform. Concentrated the combined chloroform extract and volume was adjusted to 10 mL with methanol. Five microlitre of the filtered solution was applied on the TLC plate followed by development and scanning as described in Section 2.2. The analysis was repeated thrice. Combination of active components was also analyzed separately to study the interference of each active component.

3. Results and discussion

3.1. Selection of mobile phase

The standard solution and the test solution were spotted on HPTLC plates and different individual solvents as well as combination of solvents have been tried to get a good separation and stable peak. The mobile phase toluene–ethylacetate–diethylamine (6.5:2.5:1, v/v/v) was selected for estimation of the drugs by HPTLC method, which gave good resolution with $R_f = 0.82$ for conessine (Fig. 2). Well defined spots were obtained when the chamber was saturated with mobile phase for 30 min at room temperature.

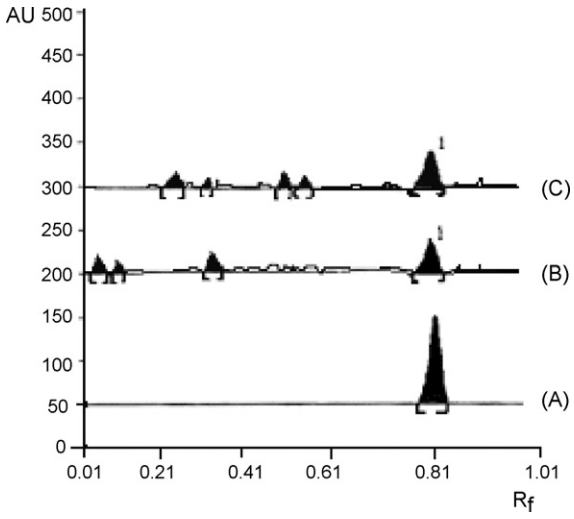

Fig. 2. Chromatogram of conessine (5 μg spot^{-1}). AU: area under curve; MM: distance traveled by solute (conessine) in millimeter; $R_f = 0.82$, mobile phase: toluene–ethylacetate–diethylamine (6.5:2.5:1, v/v/v). A: chromatogram of standard conessine; B: chromatogram of extract of *Holarrhena antidysentrica* and C: chromatogram of conessine containing pharmaceutical formulations.

3.2. Calibration curves

The present HPTLC method for estimation of conessine showed a good correlation coefficient ($r^2 = 0.9998 \pm 0.0441$) in the concentration range of 1–10 μg spot^{-1} with respect to the peak area. The mean value (±S.D.) of slope and intercept were 423.77 ± 4.89 and 638.93 ± 6.76, respectively. No significant difference was observed in the slope of standard curves (ANOVA, $P > 0.001$).

3.3. Method validation

The real goal of validation process is to determine limits of allowed variability for the conditions needed to run the method. The accuracy of the method was determined by recovery experiments. The recovery studies were carried out three times and the percentage recovery were calculated and presented. From the data obtained, recoveries of added standard drugs were found to be accurate (Table 1). Three repeated standard and sample

Table 1
Recovery studies ($n = 3$)

S. no.	Herbal extract/formulation	Amount of conessine present (μg)	Amount of conessine added (μg)	%Recovery[a]	%R.S.D.	S.E.
1	MDK-1	0.060	0.305, 0.610, 0.915	99.720 ± 0.635	0.006	0.364
2	MDK-2	0.070	0.305, 0.610, 0.915	100.005 ± 2.835	0.028	1.635
3	MDK-3	0.077	0.305, 0.610, 0.915	98.208 ± 0.427	0.004	0.242
4	MDK-4	0.079	0.305, 0.610, 0.915	98.825 ± 1.309	0.130	0.751
5	Formulation-I	0.511	0.305, 0.610, 0.915	99.125 ± 0.431	0.004	0.248
6	Formulation-II	0.499	0.305, 0.610, 0.915	99.643 ± 1.336	0.013	0.768
7	Formulation-III	0.499	0.305, 0.610, 0.915	100.782 ± 3.622	0.035	2.092
8	Formulation-IV	0.505	0.305, 0.610, 0.915	98.886 ± 0.418	0.004	0.236

R.S.D.: relative standard deviation; S.E.: standard error.
[a] Average of three different quantities of conessine added (0.3, 0.6, and 0.9 μg).

Table 2
Precision of the HPTLC method ($n = 3$, 5 μg spot^{-1})

Analyst	Conessine from herbal extract/formulations (%)							
	MDK-1	MDK-2	MDK-3	MDK-4	F-1	F-2	F-3	F-4
I	0.302	0.330	0.389	0.390	1.459	1.421	1.420	1.440
II	0.301	0.329	0.389	0.389	1.460	1.421	1.419	1.440
III	0.302	0.330	0.389	0.389	1.460	1.421	1.420	1.439
Days								
Day 1	0.301	0.330	0.389	0.390	1.459	1.421	1.420	1.440
Day 2	0.300	0.330	0.388	0.390	1.456	1.420	1.419	1.440
Mean	0.300	0.330	0.388	0.390	1.457	1.420	1.419	1.440

solutions were made and response factors of drug peaks and %R.S.D. were calculated and presented. From the data obtained, the developed HPTLC method was found to be precise.

The calibration curves were plotted using the response factors versus concentration of standard solution. These data demonstrate that the methods have adequate sensitivity to the concentrations of the analytes. The LOD and LOQ of the developed method were determined by injecting progressively low concentrations of the standard solutions using the developed methods. The LOD is the smallest concentration of the analyte that gives a measurable response. The LOD and LOQ were found to be 4.2 and 42.3, respectively, which indicate adequate sensitivity of the method. The LOD and LOQ values determined are effected by the separation conditions, i.e., HPTLC plates, reagent; instrumentation and detection wavelength and data system solvents other than AR grade solvent can result in large changes in single to noise ratio due to base line noise and drift.

The precision (Table 2) of the methods were studied by carrying out experiments by changing conditions. It was observed that there were no marked changes in the chromatograms. The values obtained demonstrated the suitability of the system for the analysis of the above drug system suitability parameters might fall within ±3% standard deviation range, during routine performance of the method.

3.4. Estimation of conessine in herbal extracts and in pharmaceutical formulations

A single spot at $R_f = 0.82$ was observed in the chromatogram of the conessine isolated from extract along with other components. There was no interference in analysis from the other components present in the extracts. These components appeared in the chromatogram at significantly different R_f values (Fig. 2). The total conessine content in herbal extracts MDK-1, MDK-2, MDK-3 and MDK-4 were found to be 0.301% (w/w), 0.330% (w/w), 0.389% (w/w) and 0.390% (w/w), respectively. A single spot at $R_f = 0.82$ was observed in the chromatogram of the conessine extracted from formulations. There was no interference in analysis from the other active components and excipients present in the formulations. The total conessine content in pharmaceutical formulation-I, formulation-II, formulation-III and formulation-IV were found to be 1.460% (w/w), 1.421% (w/w), 1.420% (w/w) and 1.440% (w/w), respectively. The percentage recovery from the formulations was found to be 98.80–100.7%.

4. Conclusion

From the above studies, it can be concluded that HPTLC technique can be successfully used for estimation of conessine in extracts and polyherbal formulations. The developed HPTLC method for this estimation of conessine is accurate, linear, rugged, simple and rapid. Statistical analysis proves that the method is reproducible and selective for the analysis of conessine.

Acknowledgement

One of the authors Miss. Aman Deep Kaur is grateful to U.G.C for providing fellowship for this work.

References

[1] A.P. Singh, J. Ayur. Med. Res. 10 (1988) 1–3.
[2] P.V. Sharma, Classical Uses of Medicinal Plants, first ed., Oriental Publishers and Distributors, 1996, pp. 101–103.
[3] P.G. Daswani, T.J. Birdi, D.S. Antarkar, N.H. Antia, Indian J. Pharm. Sci. 3 (2002) 164–166.
[4] N.K. Dutta, S.N. Layer, J. Ind. Med. Assoc. 50 (1968) 349.
[5] R.N. Chopra, Indigenous Drugs of India, second ed., CBS Publishers, New Delhi, 1932, pp. 329–332.
[6] M. Ballal, D. Srujan, K.K. Bhatt, A. Shriwadikar, P.C. Shivananda, Indian J. Pharmacol. 33 (2001) 392.
[7] C.I. Jolly, R.M. Nisha, Indian J. Pharm. Sci. 58 (1996) 51–53.
[8] I. Ahmed, Z. Mehmood, F. Mohammad, S. Ahmad, J. Med. Aromatic Plant Sci. 4A (2000) 173–178.
[9] R.N. Chopra, I.C. Chara, K.L. Handa, L.D. Kapur, Chopra's Indigenous Drugs of India, Academic Press, New Delhi, 1982, p. 342.
[10] K.K. Bhutani, S. Raj, D.K. Gupta, S. Kumar, C.K. Atal, M.K. Kaul, Indian Drugs 21 (1984) 212–214.
[11] C.R. Karnick, Pharmacopoeial Standard of Herbal Plants, first ed., Sri Satguru Publications, 1994, pp. 180–182.
[12] C.K. Kokate, A.P. Purohit, S.B. Gokhale, Pharmacology, ninetieth ed., Nirali Prakashan, Pune, 2002, pp. 516–518.
[13] Chemical Abstract, 45 (1951) 1608.
[14] B.N. Siddiqui, M. Pillay, J. Indian Chem. Soc. 9 (1982) 553.
[15] P. Duez, S. Chamart, M. Vanhaelen, M. Hanacq, L. Molle, J. Chromatogr. 351 (1986) 140.
[16] R.K. Dwivedi, R.K. Sharma, J. Ethanopharm. 30 (1990) 75.
[17] P.J. Houghton, M.L.D. Diogo, Pharm. Biol. 34 (1996) 305.
[18] N. Kumar, B. Singh, P. Bhandari, A.P. Gupta, V.K. Kaul, Chem. Pharm. Bull. 55 (2007) 912.
[19] G.N. Zirihi, P. Grellier, F. Guéde, B. Bodo, L. Mambu, Bioorg. Med. Chem. Lett. 15 (2005) 2637.