Development and Validation of a TLC-Densitometric Method for the Simultaneous Quantitation of Strychnine and Brucine from *Strychnos spp.* and its Formulations

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Abstract

A simple, sensitive, and specific thin-layer chromatography densitometric method has been developed for the simultaneous quantitation of strychnine and brucine. These two marker compounds are quantitated in the seeds of *Strychnos nux-vomica, Strychnos ignatii,* and its formulations. The method involves densitometric evaluation of strychnine and brucine after resolving it by high-performance TLC on silica gel plate with toluene–ethyl acetate–diethyl amine–methanol (7:2:1:0.3 v/v) as the mobile phase. The method is validated for precision (interday and intraday), repeatability, and accuracy. The relationship between the concentration of standard solutions and the peak response is linear within the concentration range of 160 to 480 ng/spot for strychnine and 80 to 480 ng/spot for brucine. Instrumental precision is found to be 0.54 and 0.78 (% CV), and repeatability of the method is 1.01 and 1.06 (% CV) for strychnine and brucine, respectively. Accuracy of the method is checked by recovery study conducted at three different levels and the average percentage recovery is found to be 99.13% for strychnine and 100.16% for brucine. The proposed HPTLC method for the simultaneous quantitation of strychnine and brucine is found to be simple, precise, specific, sensitive, and accurate, and it can be used for routine quality control of raw material of *Strychnos spp.* It also can be applied in quantitating any of these marker compounds in other formulations.

Introduction

Strychnos nux-vomica Linn. (Fam. Loganiaceae) is a native plant of India, growing in Bengal, Malabar, Konkan, on the Coromandel Coast, in Ceylon, in many islands of the Indian Archipelago, China, and in northern Australia. It is commonly known as Kuchila, poison nut, and semen strychnos. In herbal medicine, it is traditionally recommended for upset stomach, vomiting, and bitter stomachic. It stimulates the muscular coat of the intestine, increases peristalsis, and hence is given for constipation in an atonic condition of the intestine, problems related to menopause, and migraine headaches (1,2). Some of the major chemical constituents of Strychnos nux-vomica include the alkaloids–strychnine, brucine, brucine-n-oxide (3), and also traces of strychnicine, a glucoside-loganin, 7-O-acetyl loganic acid (4), caffeotannic acid, and trace of copper. The pulp of the fruit contains about 5% of loganin together with the alkaloid strychnicine. A study done on processing of nux-vomica suggested that the crude alkaloid fractions of nux-vomica have distinct antinociceptive potency, even after treatment with licorice-, oil-, vinegar-, and sand-processing (5). *S. nux-vomica* alcoholic seeds extract showed good lipid peroxidation effect in rat liver (6). *S. nux-vomica* crude extract has been reported to exhibit an inhibitory effect on reverse transcriptase of RNA tumour virus (I), protein kinase, and HIV-1 protease (7,8,9). Brucine and brucine *n*-oxide as a major alkaloid of Strychnos nux-vomica has been reported for its analgesic and anti-inflammatory properties (10). The method so far reported for the analysis of strychnine and brucine include separation of alkaloids by high-performance liquid chromatography (HPLC) (11), by high-speed counter current chromatography (12), and by capillary zone electrophoresis (13). In the last two decades, high-performance thin-layer chromatography (HPTLC) has emerged as an efficient tool for the phytochemical evaluation of herbal drugs (14,15,16). The work presented in this paper is an attempt to develop and validate a simple, specific, and sensitive TLC densitometric method using HPTLC for the simultaneous quantitation of these marker compounds in the seeds of Strychnos nux-vomica and Strychnos ignatii. Strychnine and brucine contents were also estimated from the marketed herbal formulations containing Strychnos nux-vomica as one of the ingredients.

Experimental

Materials and reagents

Seeds of *Strychnos nux-vomica* and *Strychnos ignatii* were obtained from the Konkan region of Maharashtra, India. The samples were authenticated and voucher specimens were deposited in our Pharmacognosy and Phytochemistry depart-

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ment. The *Strychnos nux-vomica* formulations selected for TLC analysis were procured from a local market and designated as MS-1 and MS-2. Seeds were ground in a grinding machine to form powder, as the grinding of seeds hastens extraction process. The samples were stored at 25°C in an air-tight container.

All of the chemicals used in the experiments were of analytical grade. Reference standard strychnine (purity 98%, w/w) and brucine (purity 98%, w/w) were purchased from Natural Remedies Pvt. Ltd (Karnataka, India).

Apparatus

The spotting device was a Linomat V Automatic Sample Spotter (Camag, Muttenz, Switzerland); the syringe was 100 µL (Hamilton Bonaduz, Switzerland); The TLC chamber was a glass twin trough chamber (20 × 10 × 4 cm) (Camag); the densitometer was a TLC Scanner 3 linked to WINCATS software (Camag); the HPTLC plates were 20 × 10 cm, 0.2-mm thickness, precoated with silica gel 60 F_{254} (E. Merck KgaA, Cat. no. 1.05548, Darmstadt, Germany).

Preparation of standard solutions

Standard solution of strychnine

A stock solution of strychnine was prepared by dissolving 2 mg of accurately weighed strychnine in methanol and making up the volume to 25 mL with methanol. From this stock solution, standard solutions of 16 µg/mL to 48 µg/mL were prepared by transferring aliquots (2 to 6 mL) of stock solution to 10-mL volumetric flasks and adjusting the volume with methanol.

Standard solution of brucine

A stock solution of brucine was prepared by dissolving 2 mg of accurately weighed brucine in methanol and making up the volume to 25 mL with methanol. From this stock solution, standard solutions of 8 µg/mL to 48 µg/mL were prepared by transferring aliquots (1 to 6 mL) of stock solution to 10 mL volumetric flasks and adjusting the volume with methanol.

Preparation of sample solutions

Dried powder of seeds (2.5 g) of *Strychnos nux-vomica* and *Strychnos ignatii* were exhaustively extracted with methanol (3 × 50 mL) separately under reflux for 1 h on a water bath. The extracts were filtered and concentrated. The volume was made up to 50 mL in a volumetric flask with methanol.

Strychnos nux-vomica formulation

The content of 10 powdered tablets was mixed together, and the amount of sample equivalent to the average weight of tablet content was taken. The samples were extracted with methanol (3 × 50 mL) separately under reflux for 1 h on a water bath. The pooled extracts were transferred separately to 25 mL of volumetric flask, and the volumes were made up to the mark with methanol.

Calibration curve for strychnine and brucine

Ten microliters of each of the standard solutions of strychnine and brucine were applied in triplicate on a TLC plate. The plates were developed in a solvent system [toluene–ethyl acetate–diethyl amine–methanol (7:2:1:0.3 v/v) at 25 ± 2°C temperature and 40% relative humidity up to a distance of 8 cm]. After development, the plate was dried in air and scanned at 257 nm for strychnine and at 304 nm for brucine. The peak areas were recorded. Calibration curves of strychnine and brucine were prepared by plotting peak areas *vs* concentration.

Simultaneous quantitation of strychnine and brucine in *strychnos spp.* and its formulations

Ten microliters of sample solution were applied in triplicate on a precoated silica gel 60 F_{254} TLC plate (E. Merck) (0.2 mm thickness) with the Camag Linomat V Automatic Sample Spotter. The plates were developed and scanned at 257 nm for strychnine and at 304 nm for brucine. The peak areas and absorption spectra were recorded. To check the identity of the bands, UV absorption spectrum of each standard was overlayed with its corresponding band in the sample track. The purity of the bands in the sample extract was checked by overlaying the absorption spectra at the start, middle, and end positions of the bands. The amount of strychnine and brucine in the samples and its formulation were calculated using the linear regression equation derived from the calibration curves.

Validation of the Method

ICH guidelines (CPMP/ICH/381/95; CPMP/ICH/281/95) were followed for the validation of the analytical procedure. The method was validated for precision, repeatability, and accuracy. Instrumental precision was checked by repeated scanning (n = 7)

Table I. Method Validation Parameters for the Quantitation of Strychnine and Brucine by Proposed HPTLC Method

Parameters	Strychnine	Brucine
Instrumental precision (% CV, n = 7)	0.54	0.78
Repeatability of standards (% CV, n = 6)	1.01	1.06
Repeatability of sample (% CV, n = 6)	1.08	0.97
Limit of detection	80 ng	40 ng
Limit of quantitation	160 ng	80 ng
Specificity	Specific	Specific
Linearity (Correlation coefficient)	0.991	0.996
Range (ng/spot)	160–480	80–480
Robustness	Robust	Robust

Table II. Intraday and Interday Precision Study

Marker compound	Concentration (ng/spot)	Intraday precision*	Interday precision*
Strychnine	240	1.34	1.03
	320	1.16	1.51
	400	1.70	1.20
Brucine	240	0.98	1.11
	320	1.61	1.24
	400	1.12	1.13

*Relative standard deviation (% CV, n = 3)

of the same spot of strychnine (240 ng/spot) and brucine (320 ng/spot), and was expressed as coefficient of variance (% CV) of the peak areas. The repeatability of the method was affirmed by analyzing 240 ng/spot of standard solution of strychnine and 320 ng/spot of standard solution of brucine after application on the HPTLC plate (n = 6) and analyzing them as described in the preparation of calibration plot, which was expressed as % CV. Variability of the method was studied by analyzing aliquots of standard solution of strychnine (240, 320, 400 ng/spot) and brucine (240, 320, 400 ng/spot) on the same day (intraday precision) and on different days (interday precision) and the results were expressed as % CV.

Robustness of the method was studied at three different concentration levels: 240, 320, 400 ng/spot for strychnine and 240, 320, 400 ng/spot for brucine. Accuracy of the method was tested by performing recovery studies at three levels (50%, 100% and 125% addition). The percent recovery as well as average percent recovery was calculated. To determine the limit of detection and limit of quantitation, different dilutions of the standard solutions of strychnine and brucine were applied along with methanol as the blank, and determined on the basis of signal-to-noise ratio.

Results and Discussion

For the analysis of raw herbal materials and herbal preparations, TLC is superior to other instrumental analytical techniques because it is simple, economical, and requires minimum sample clean up. The time required for sample analysis in HPTLC is much less when compares with HPLC and liquid chromatography–electrospray mass spectrometry. In HPLC, one sample is injected at a time, and after every injection there is a washing period. On the other hand, in HPTLC more than one sample is applied on a plate and quantitated in a single run. The HPTLC densitometric technique is, therefore, suggested for the determination of strychnine and brucine in *Strychnos spp*. and its formulations. Of the various solvent systems tried, the one containing toluene–ethyl acetate–diethyl amine–methanol (7:2:1:0.3 v/v) gave the best resolution of strychnine [retention factor (R_f)= 0.55] and brucine (R_f = 0.43) from the other components of the sample extract for simultaneous quantitation. The identity of the bands of strychnine and brucine in the sample extract were confirmed by overlaying their UV absorption spectra with those of the standard strychnine and brucine using a Camag TLC Scanner 3. (Figures 1 and 2). The purity of the bands due to strychnine and brucine bands in the sample extract was confirmed by overlaying the absorption spectra recorded at start, middle, and end position of the bands.

The method was validated in terms of precision, repeatability, and accuracy. (Tables I and II). The relationship between the concentration of standard solutions and the peak response was linear within the concentration range of 160 to 480 ng/spot with a correlation coefficient of 0.991 for strychnine and 80 to 480 ng/spot with a correlation coefficient of 0.996 for brucine. The precision and the repeatability at three different concentration levels (Table II) reflect the robustness of the method. The limit of

Table III. Recovery Study of Strychnine and Brucine by the Proposed HPTLC Method

Marker compound	Amount in sample (µg)	Amount added (µg)	Amount found* (µg)	Recovery* (%)	Average Recovery (%)
Strychnine	1395	697.5	2078.33 ± 7.63	99.32 ± 0.36	
	1395	1395.0	2760.00 ± 5.04	98.92 ± 0.18	99.13
	1395	1743.8	3107.10 ± 6.80	99.17 ± 0.21	
Brucine	446	223.00	680.33 ± 4.04	101.49 ± 0.65	
	446	446.00	891.06 ± 3.60	99.88 ± 0.40	100.16
	446	557.50	994.66 ± 4.50	99.11 ± 0.45	

* Mean ± standard deviation (SD, n = 3).

Table IV. Marker Compounds Quantitated by TLC Densitometric Method from Samples of *S. nux-vomica Seeds, S. ignatii Seeds* and its Formulations

Test Material	Content of marker compounds* (% w/w) Strychnine	Brucine
S. nux-vomica	2.790 ± 0.016	0.892 ± 0.021
S. ignatii	2.811 ± 0.019	0.699 ± 0.016
MS-1	0.098 ± 0.009	0.029 ± 0.008
MS-2	0.121 ± 0.018	0.017 ± 0.011

* Mean ± standard deviation (SD, n = 3).

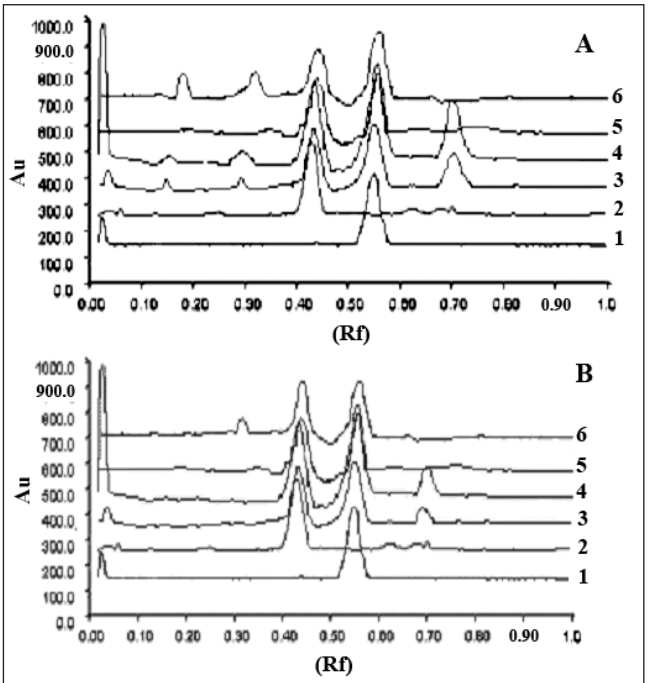

Figure 1. TLC densitograms of sample solutions along with standards. Strychnine standard (Peak 1), brucine standard (Peak 2), *S. nux-vomica* seeds (Peak 3), *S. ignatii* seeds (Peak 4), MS-1 (Peak 5), and MS-2 (Peak 6). Scanned at 257 for strychnine (1A) and scanned at 304 for brucine (1B).

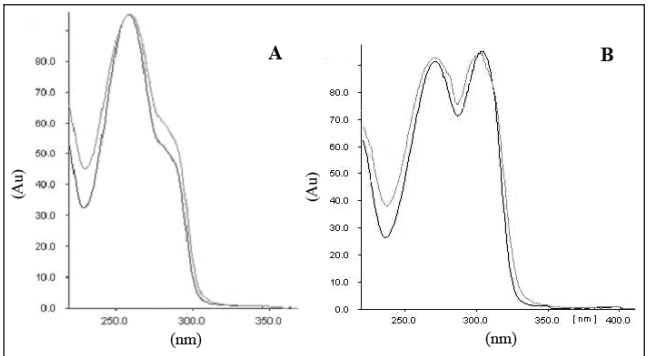

Figure 2. Overlay of UV absorption spectra of the marker compounds in the sample track with respective standards. Strychnine (A) and brucine (B).

detection (LOD) and limit of quantitation (LOQ) were obtained with the signal-to-noise ratio of 3 and 10. LOD represents the lowest concentrations of strychnine and brucine that can be detected, whereas the LOQ represents the lowest concentrations of strychnine and brucine that can be determined with acceptable precision and accuracy. The LOD and LOQ were found to be 80 and 160 ng/spot for strychnine and 40 and 80 ng/spot for brucine, respectively. This indicated that the new method exhibited a good sensitivity for the quantitation of strychnine and brucine. After the addition of standard strychnine and brucine to same amount of the sample solution, at three different concentration levels, the percentage recovery of strychnine was found to be 99.32%, 98.92% and 99.17% with an average of 99.13%, and that of brucine was found to be 101.49%, 99.88% and 99.11% with an average of 100.16%. The results are presented in Table III.

Strychnine and brucine content in the seeds of *S. nux-vomica, S. ignatii* and its formulations were quantitated by the proposed method (Table IV). The densitograms of standard strychnine and brucine along with *S. nux-vomica, S. ignatii*, and its formulation is shown in Figures 1A and 1B. The method developed was found to be suitable for quantitation of these marker compounds in the herbal raw materials.

Conclusion

We established a TLC densitometric method for the simultaneous quantitation of two bioactive compounds (strychnine and brucine) from *S. nux-vomica, S. ignatii* seeds and its formulation, using HPTLC. The method was found to be simple, precise, specific, sensitive, and accurate and can be used for their quantitation in the plant materials, in routine quality control of the raw materials, and in formulations containing these compounds.

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