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Simultaneous Determination of Strychnine and Brucine In Biological Samples and Herbal Formulations by HPTLC

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ABSTRACT

A simple, precise and accurate high performance thin layer chromatography (HPTLC) method have been developed for the quantification of strychnine and brucine. The strychnine and brucine are isolated from biological samples and herbal formulations using a liquid–liquid extraction procedure. The clean-up procedure is performed using an acid solution. The method has a linear range of 45 – 1000 ng/spot for strychnine and 95 – 1000 ng/spot for brucine. The method precision was found to be 0.86 - 2.61 (% RSD) and 0.77 - 2.46 (% RSD) for strychnine and brucine respectively. Accuracy of the method was checked by recovery studies conducted at three different concentration levels and the average percentage recovery was found to be 101.34 % for strychnine and 101.23 % for brucine.

Keywords : HPTLC, strychnine, brucine, biological samples, herbal formulations.

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INTRODUCTION

Strychnos nux-vomica Linn., commonly known as kuchla belongs to the family Loganiaceae, is a medium-sized tree distributed widely in India in the deciduous forest of the eastern and southern parts of the country[1]. Kuchla fruit is used as appetizer, tonic, astringent to bowels, and antipyretic and useful in the treatment of hiccups, leukoderma, blood disorders, piles, ulcers, pneumonia, hemoptysis, occipital headache, cold and cough, anaemia, jaundice, itching, ear troubles, renal colic and urinary infection[2,3]. Some of the major chemical constituents of S. nux-vomica include alkaloids strychnine, brucine, brucine-n-oxide and also traces of strychnicine, a glucoside-loganin, 7-O-acetyl loganic acid[4], caffeotannic acid, and trace of copper. Its alcoholic seed extract showed good lipid peroxidation effect in rat liver[5]. Crude extract of S. nux-vomica has been reported to exhibit an inhibitory effect on the reverse transcriptase of RNA tumor virus, protein kinase and HIV-I protease[6-8]. Recent research has shown that excitatory effect of strychnine on central nervous system results from its ability to antagonise the effect of synaptic inhibition[9]. Brucine and brucine-N-oxide has been reported for its analgesic and anti-inflammatory properties[10]. The methods so far reported for the analysis of strychnine and brucine include their estimation using circular chromatography[11], nonaqueous capillary electrophoresis[12-16], UV spectrophotometry[17], thin layer chromatography[18], column liquid chromatography[18], capillary zone electrophoresis[19] and voltametry[20] showed low resolution owing to poor reproducibility. Others have been working on the separation of bioactive components of plants using chromatographic methods. In this respect, Petruczynik et al. have developed a method for the separation of plant alkaloids on a silica gel plate[21] Shalaby and Khalil further modified this technique using an RP chromatographic plate with ion separation for the estimation of more alkaloids derived from plant sources[22]. Saqui-Sannes, et al. adapted this method for the estimation of strychnine and crimidine in biological samples as well[23]. They determined the amounts of these alkaloids in dog stomach and serum, which are generally used as dog poisons. All these methods used earlier for the estimation of strychnine and brucine are tedious, lengthy and less sensitive.

With this background, I herein report a novel, very simple, specific, sensitive, and very economic laboratory friendly validated HPTLC method for the simultaneous quantification of these marker compounds in the bulk, biological samples and in herbal formulations. The method has been validated as per ICH guidelines[24] similar to the methods reported by laboratory [25-30].

MATERIAL AND METHODS

Instrument:

A Camag HPTLC system equipped with Linomate IV sample applicator (Camag, Muttenz, Switzerland), TLC scanner 3 (WINCATS version 1.4.4) and UV cabinet and twin trough glass tank were used for the analysis. The plates were developed in 20x10 cm twin trough glass chamber (Camag, Muttenz, Switzerland). Precoated silica gel aluminium plates $60F_{254}$ (E. Merck, Darmstadt, Germany) with thickness 0.2 mm were used for all determinations.

Reagents and samples:

All chemicals and solvents used were of either pharmaceutical or analytical reagent grade. Strychnine and brucine were supplies gratis by Dr. Reddy's Laboratories Ltd., Hyderabad. Dosage forms of strychnine and brucine, manufactured by different firms, were obtained commercially. The biological samples which were received in forensic science laboratory for toxicological analysis were used. These biological samples were said to be collected from a body of a person who suspected to be died due to the consumption of strychnous nux-vomica fruit.

Stock Solution:

A standard solution containing strychnine and brucine was prepared by dissolving 5 mg each in 10 ml of methanol (500 µg/ml). This stock solution was used to make calibration curves of strychnine and brucine.

Assay methodology for pure drug:

Aliquots of standard solution of strychnine and brucine (0.05 – 1.0 µg/ml) were applied to aluminium backed silica gel $60F_{254}$ plates as 7-mm bands by means of a Camag Linomat IV applicator. The plates were developed in a Camag twintrough chamber previously equilibrated with chloroform: methanol: formic acid (8.5:1.5:0.4, v/v/v) as mobile phase for 10 min. The development distance was 8 cm. Plates were then removed from the chamber and dried in a current of air. Densitometric scanning was done at 358 nm by use of Camag TLC Scanner II with CATS3 software. The HPTLC spectrum was shown in Figure 1.

Assay procedure for Pharmaceutical Preparations:

Twenty Tentex Forte tablets were weighed and powdered. An amount of powder equivalent to one tablet was weighed and dissolved in a minimum volume of methanol. This solution was filtered through a Whatman No. 41 paper and the filtrate was collected in a 10 ml volumetric flask and diluted to volume with methanol. This solution (2 ml) was quantitatively transferred to

a 10 ml volumetric flask and diluted to volume with methanol to furnish a solution containing 20 µg/ml strychnine and brucine. An aliquot was analysed using the procedure described earlier.

Assay procedure for Biological samples:

Strychnine and Brucine were extracted using the method[31]. To biological fluids (5 ml each) and tissue samples (5 g each), cut in very small pieces were added a saturated solution of sodium bicarbonate and the tissue samples were homogenized at 48ºC using ultrasound 600 W for 20 min. Strychnine and brucine extraction was performed with 8 ml of toluene–*n*-heptane–isoamyl alcohol (67:20:4) for tissue homogenates and body fluids. After vortex agitation and centrifugation, the organic layer was transferred to 15 ml glass tubes and the extraction process was repeated. The organic layer was transferred into glass tubes and then evaporated to dryness at 45°C. The residues were reconstituted in 75 µl methanol. An aliquot was analysed using the procedure described earlier.

METHOD VALIDATION:

The method was validated for linearity, accuracy, precision, limit of quantitation, and robustness.

Linearity:

Linearity was evaluated by analysis of working standard solutions of strychnine and brucine at seven different concentrations from 45 – 1000 ng/spot, prepared from the stock solution. These solutions (10 µl) were applied to plates, which were then developed and scanned as described above. Peak areas were recorded for strychnine and brucine and drug concentration was subjected to regression analysis to calculate the regression equations and correlation coefficients. Linearity data and other validation data are given in Table 1.

Recovery studies (Accuracy):

Recovery experiments were conducted to check for the presence of positive or negative interferences from excipients present in the formulation and to study the accuracy of the method. Recovery was determined by the standard addition method. Strychnine and brucine standard was added to the formulation at two different concentrations and analysis was performed as described above. Recovery was calculated for each standard at each concentration. The results obtained are listed in Table 2.

Limit of detection (LOD) and limit of quantification (LOQ):

In order to estimate the LOD and LOQ, blank solution (methanol) was spotted 6 times following the same method as explained above. The signal to noise ratio was determined. LOD was considered as 3:1 and LOQ as 10:1. LOD and LOQ were experimentally verified by diluting

known concentrations of reference solution until the average responses were approximately 3 or 10 times the standard deviation of the responses for 6 replicate determinations.

Specificity:

The specificity of the method was ascertained by analysing standard drug and sample. The spots for strychnine and brucine in sample were confirmed by comparing R_f and spectra of spot with that of standard. The peak purity of strychnine and brucine was assessed by comparing the spectra at three different levels that is peak start, peak apex and peak end positions of the spot. Purity of sample spot corresponding to strychnine and brucine was determined by taking the spectra and by comparing it with that of standard.

Robustness of the method:

By introducing small changes in the mobile phase composition, the effects on the results were examined. The volume of mobile phase was varied in the range of ±5%. The plates were pre-washed by methanol and activated at 60 ± 5ºC for 5, 10 and 12 min prior to chromatography. Robustness of the method was done at three different concentration levels 0.4, 0.6 and 0.8 µg/ml. Plates were developed in varied volume of mobile phase 8, 10 and 12 ml. Time from spotting to chromatography and chromatography to scanning were also varied and % RSD was determined and found to be less than 2 %.

RESULTS AND DISCUSSION:

Optimization of the solvent system

For the development of mobile phase, different trials were made using many solvents in different proportions. When mobile phase consisting of chloroform: methanol was used in the ratio of 8:2 v/v, two spots were observed at the Rf value of 0.60 and 0.69 for strychnine and brucine, respectively. But it was found that the resolution between the peaks was poor. In order to improve the resolution between the peaks, a mobile phase with the composition of chloroform: methanol and formic acid was used in the ratio of 8.5:1.5:0.4, v/v/v. This mobile phase helped in achieving very compact spots at the Rf value of 0.63 and 0.69 (Figure 1) for strychnine and brucine, respectively, with good resolution of more than one.

Method validation parameter

Linearity

It was found between concentration ranges of 45 -1000 ng/spot for strychnine and 95 -1000 ng/spot for brucine with r^2 value of 0.9986 and 0.9977, respectively (Table 1).

Precision

Instrument precision and interday and intraday precision were measured to evaluate the precision of the method. The low % RSD indicated that the method was highly precise for the analysis.

Limit of detection and limit of quantification

The limits of detection and quantification were 15.2 and 44.4 ng/spot for strychnine and 33.2 and 94.7 ng/spot for brucine respectively (Table 1).

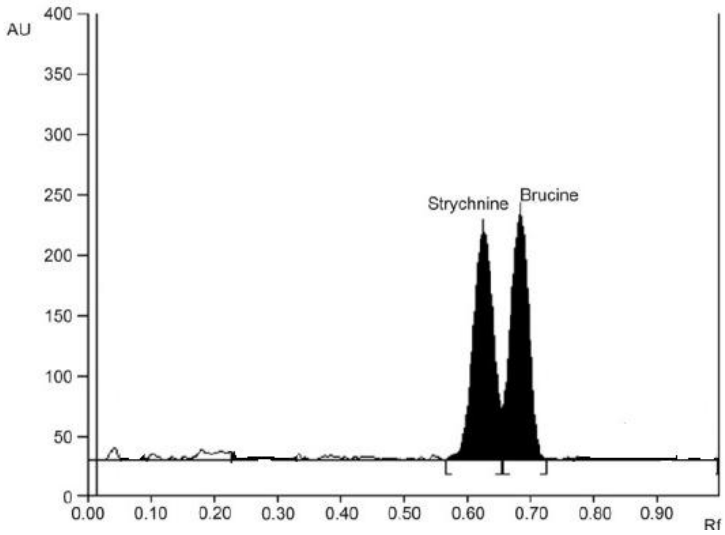

Figure. 1. HPTLC chromatogram of strychnine and brucine

Table 1. Linear regression, LOD and LOQ for strychnine and brucine

Parameter	Results	
	Strychnine	**Brucine**
Linearity range (ng/spot)	45 - 1000	95 – 1000
Correlation coefficient (r^a)	0.9986 ± .0005	0.9977 ± 0.00092
Regression equation	Y = 612.06 + 9.57*X	Y = 638.55 + 8.85*X
LOD (ng/spot)	15.2	33.2
LOQ (ng/spot)	44.4	94.7

a Correlation coefficient

Recovery studies (accuracy)

Average recovery of strychnine and brucine from the formulation was 101.27 % and 101.29 % respectively, which shows the method is accurate and free from interference from excipients present in the formulation (**Table 2)**.

Table 2. Accuracy as recovery data of proposed HPTLC method

Compound	% of standard added	Amount of standard detected (ng/ml)	Recovery* %	RSD %
Strychnine	100	103.35 ± 2.91	103.34	2.61
	250	252.62 ± 6.07	101.04	2.40
	500	497.20 ± 4.28	99.64	0.86
Brucine	100	103.34 ± 2.93	103.18	2.46
	250	252.61 ± 6.07	101.06	2.42

500	497.22 ± 4.29	99.45	0.77

* Recovery value by the proposed method is the mean of five determinations.

Robustness of the method

The effect of deliberate changes in the composition of mobile phase were studied as % RSD and depicted in Table 2. Low % RSD indicates the method is robust. The applicability of the proposed method to the assay of dosage forms was examined by analysing tablets marketed under different trade names. The results obtained were compared statistically by Student t-test and by the variance ratio F-test with those obtained by the reported method[32]. The Student t-values at 95% confidence level did not exceed the theoretical value indicating that there was no significant difference between the proposed and the reported method. It was also observed that the variance ratio F-values calculated for p=0.05 did not exceed the theoretical value indicating that there was no significant difference between the precision of the proposed and the reported method[32]. The results are tabulated in **Table 3**.

Table 3. Analysis of strychnine and brucine in herbal formulation

Formulation	Strychnine		Brucine (mg/tablet)	
	Reported method[32](mg/tablet)	Proposed method (%)	Reported method[32](mg/tablet)	Proposed method(%)
Tentex Forte Tablets[a]	2.54	99.12 ± 1.14, F=1.13; t=1.44	1.97	99.88 ± 1.10, F=1.19; t=1.11

a. Average of six determinations. The calculated F- and t- values refer to 95 % confidence limits.

The results of the analysis of femoral blood, urine, liver and kidney are shown in Table 4. The results obtained are compared with those obtained by the reported method [33,34].

Table 4. Analysis of biological samples

Biological samples	Concentrations (µg/ml or µg/g)[a]			
	Strychnine		Brucine	
	Reported method[33]	Proposed method	Reported method[34]	Proposed method
Blood	21.2	20.6	1.51	1.32
Urine	15.3	14.2	1.685	1.56
Liver	49.8	47.5	16.443	15.8
Kidney	15.6	14.3	-	13.2

[a]n = 3

CONCLUSION:

The proposed HPTLC method for the simultaneous determination of strychnine and brucine was simple, rapid and sensitive compared to the reported methods. The utility of the proposed method for the determination of strychnine and brucine in dosage form and biological samples

have been well demonstrated. The method did not involve any stringent experimental conditions and was also found to be eco-friendly (low consumption of solvents). Hence, the proposed method could be used for screening and quantitative determination of selected drug.

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