International Journal of Applied Science and Engineering
2013. 11, 2: 149-158

A Validated Quantitiatve High Performance Thin - Layer Chromatographic Method for Estimation of Strychnine in *Strychnos Nux Vomica* Seed Extract and Marketed Unani Formulation

Mohd Amir [a], M. Mujeeb [a,*], S. Usmani [a], A. Ahmad [b], S. Ahmad [a], W. A. Siddique [c], and Mohammad Aqil[d]

[a] *Bioactive Natural Product Laboratory, Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy Jamia Hamdard, New Delhi, India*
[b] *Pharmakokinetic Research Lab, Department of clinical Pharmacy College of Pharmacy, King Saud University Riyadh*
[c] *Department of Biochemistry, Faculty of Science, Jamia Hamdard, New Delhi, India*
[d] *Department of Pharmaceutics, Faculty of Pharmacy, Jamia Hamdard, New Delhi, India*

Abstract: A new, simple, sensitive, selective, precise and stability-indicating high-performance thin-layer chromatographic method for analysis of strychnine in *Strychnos nux vomica* seed extract, and in marketed formulation was developed and validated. The method was developed on TLC aluminum plates precoated with silica gel $60F_{254}$ as the stationary phase. The solvent system consisted of toluene: ethyl acetate: diethyl amine (7: 2: 1, *v/v/v*). Densitometric analysis of strychnine was carried out in the absorbance mode at 254 nm. This system was found to give compact spots for strychnine (R_f value of 0.42 ± 0.01, for six replicates). Strychnine was subjected to acid and alkali and hydrolysis, hydrogen peroxide-induced degradation and photo degradation. The drug undergoes degradation under all stress conditions. Also, the degraded products were well resolved from the pure drug with significantly different R_f values. The method was validated for linearity, precision, robustness, LOD, LOQ, specificity and accuracy. Linearity was found to be in the range of 100–1000 ng/spot with significantly high value of correlation coefficient $r^2 = 0.9928 \pm 1.02$. The limits of detection and quantification were 12.00 and 36.37 ng/spot, respectively. Statistical analysis proves that the method is repeatable, selective and accurate for the estimation of strychnine in *S. nux vomica* seed extract, and in market formulation. The developed method effectively resolved the strychnine in *S. nux vomica* seed extract, and in marketed formulation hence; it can be employed for routine analysis as a stability indicating method.

Keywords: HPTLC; stability indicating; validation; strychnine; *strychnos nux vomica*; majoon azraqi.

1. Introduction

Strychnine (Figure 1), an indole alkaloid, extracted from the seeds of *Strychnos nux vomica*

* Corresponding author; e-mail: drmmujeeb@rediffmail.com

Received 20 February 2012
Revised 27 November 2012
Accepted 12 January 2013

Linn. acts as stimulant for the central nervous system, causes the sense organ to be more sensitive, stimulate the visceral organs and urogenital tract [1]., laxative and as treatment for other stomach ailments, [2, 3]. Strychnine is rapidly absorbed from the gastrointestinal tract and acts upon the central nervous system. It blocks the uptake of the inhibitory neurotransmitter glycine at the postsynaptic receptor site in the motor neurons of the neural horn in the spinal cord, leading to very powerful tonic contractions [4].

S. nux vomica belongs to family Loganiaceae. It is a medium sized tree distributed widely in deciduous forests of the eastern and southern parts of the India [5]. Almost every part of the plant is used as drug [6] especially the seeds which contain 1.8–5.3% of indole alkaloids, strychnine and brucine. Alkaloids are the main bioactive chemicals in *S. nux vomica* [7], responsible for the pharmacological and toxic effects. Strychnine is physiologically more active than the minor alkaloids which include a-colubrine, b-colubrine, icajine, 3-methoxyicajine, proto-strychnine, vomicine, novacine, N-oxystrychnine, pseudo- strychnine and iso-strychnine [8]. *S. nux vomica* seeds have been used in oriental medicine as activating the channels, alleviating pain, reducing swelling, and enhance blood circulation [9]. The pharmacological effects of this plant have also been known to increase spinal reflexes and stimulate respiratory and sensory centers of the cerebral cortex [10]. In oriental medicine, S. nux-vomica seeds have been used in combination with aconite roots to treat spasms, numbness, or weaknesses associated with wind damp painful obstructions, and with myrrh to treat trauma-induced pain, swelling, fractures and sprains topically, and with sophora roots to treat severe and painful swelling of the throat [9]. In China, *S. nux vomica* is used as an anti-inflammatory and analgesic drug to relieve arthritic and traumatic pains [11].

Methods have been reported for analysis of strychnine employing TLC [12], HPLC [13], and gas chromatography [14, 15]. These methods were developed mainly for pharmacological and toxicological studies. . All of these methods suffer from one or more drawbacks and are not properly validated. Hence, the aim of this work was to develop an accurate, specific, repeatable and stability-indicating method, which was validated as per the ICH guidelines and several other methods reported by laboratory [16, 17]. The proposed method was applied for analysis of strychnine in *S. nux vomica* seed extract and in marketed unani formulation, which proved that method can be applied for quality control of this medicinal plant as well as traditional Unani and Ayurvadic formulations containing it as an ingredient.

Figure 1. Structure of strychnine

2. Experimental

2.1. Drugs and chemicals

S. nux vomica seeds were collected from herbal garden of Hamdard University, New Delhi, identified by Department of Botany and the voucher specimen (No. JH/FP/57/MA) was deposited in the herbarium of the University. A marketed unani formulation (Majoon Azraqi) containing powder of *S. nux vomica* seed procured from a local unani medical shop. Reference standard strychnine (98% purity) was purchased from Sigma-Aldrich Pvt. Ltd. USA. All chemicals and reagents used were of analytical grade and were purchased from Merck Chemicals, India.

2.2. Preparation of standard solution

Standard stock solution was prepared by dissolving 5 mg strychnine standard (98%) in 5 ml methanol in volumetric flask.

2.3. Preparation of sample solutions

The *S. nux vomica* seeds were excised and separately ground to a coarse powder. Coarse powder (10 g) and marketed unani formulation (10g) extracted exhaustively in a Soxhlet apparatus with methanol, (100ml) for 6 h. The methanolic extracts were filtered through Whatmann No. 1 filter paper, concentrated under reduced pressure at a temperature of 45ºC and dried. This residue was again dissolved in methanol (1 ml each) and applied on TLC plate.

2.4. TLC instrumentation and chromatographic conditions

The samples were spotted in the form of bands of 6 mm width with a Camag microlitre syringe on precoated silica gel aluminium Plate $60F_{254}$, (20 × 10 cm with 250 µm thickness; E. Merck, Darmstadt, Germany,) using a Camag Linomat V (Muttenz, Switzerland). A constant application rate of 150 nl/s was employed and space between two bands was 11.6 mm. The slit dimension was kept at 4 × 0.45 mm and 20 mm/s scanning speed were employed. The monochromatic bandwidth was set at 20 nm, each track was scanned thrice and baseline correction was used. The mobile phase consisted of toluene: ethyl acetate: diethyl amine (7.0: 2.0: 1.0, *v/v/v*) and 15 ml of mobile phase was used per chromatographic run. Linear ascending development was carried out in 20 × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) and the top of chamber was covered tightly with the lid. It was saturated (lined on the two bigger sides with saturation pads that had been soaked thoroughly with the mobile phase). The optimized chamber saturation time for mobile phase was 30 min at room temperature (25 ± 2°C) at relative humidity of 60 ± 5%. The length of chromatogram run was 9 cm. The run length of 9 cm results in better apparent resolution with more convenient capability of the detecting device to perform integration of peak area/ height. Subsequent to the development, TLC plates were dried in a current of air with the help of an air dryer in wooden chamber with adequate ventilation. The flow of air in the laboratory was maintained unidirectional (laminar flow, towards exhaust). Densitometric scanning was performed on Camag TLC scanner III in the reflectance– absorbance mode at 254 nm and operated by win CATS software (V 1.4.4.6337, Camag). The source of radiation utilized was tungsten lamp [18, 19].

2.5. Calibration curves of strychnine

Stock standard solution of *strychnine* was prepared in methanol at 1 mg/ml. Standard solutions were prepared by dilution of the stock solution with methanol to give solutions containing *strychnine* in concentration range of 100– 1000 μg/ml. One microlitre from each standard solution was spotted on the TLC plate to obtain final concentration range of 100–1000 ng/spot. Each concentration was spotted six times on the TLC plate.

2.6. Method validation

2.6.1. Precision

Precision of the method was determined with the product. An amount of the product powder equivalent to 100% of the label claim of *strychnine* was accurately weighed and assayed. System intra-day repeatability was determined by six replicate applications and six times measurement of a sample solution at the analytical concentrations of 500 and 1000 ng/spot, respectively. The repeatability of sample application and measurement of peak area for active compound were expressed in terms of % RSD (relative standard deviation) and SE (standard error). Intermediate precision was assessed by the assay of two sets of six samples on different days (inter-day precision). The intra- and inter-day variation for determination of strychnine was carried out at two different concentration levels 500 and 1000 ng/spot, respectively [20].

2.6.2. Robustness of the method

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different composition like toluene: ethyl acetate: diethyl amine (7: 2: 1, *v/v/v*), (6.5: 2.25: 1.25, *v/v/v*), (7.25: 1.75: 1.15, *v/v/v*), were tried and chromatograms were run. The plates were prewashed by methanol and activated at 60 °C ± 5 for 2, 5, 7 min prior to chromatography. Robustness of the method was done at three different concentration levels 500, and 1000 ng/spot [21].

2.6.3. Limit of detection and limit of quantification

In order to estimate the limit of detection (LOD) and limit of quantification (LOQ), blank methanol was spotted six times following the same method as explained above and the signal-to-noise ratio was determined. Limit of detection was considered as 3:1 and LOQ as 10:1. LOD and LOQ were experimentally verified by diluting the known concentrations of strychnine until the average responses were approximately three or ten times the standard deviation of the responses for six replicate determinations.

2.6.4. Recovery studies

The pre-analyzed sample was spiked with extra 50, 100 and 150% of the standard strychnine was re-analyzed by the proposed method. The experiment was conducted six times. This was done to check for the recovery of strychnine at different levels in the seed extract of *S. nux vomica* and Unani formulation (Majoon Azraqi).

2.6.5. Specificity

The specificity of the method was ascertained by analyzing standard drug and sample. The spot for strychnine in sample was confirmed by comparing the R_f values and spectra of the sample with that of standard. The peak purity of strychnine was assessed by comparing the spectra at three different levels i.e., peak start (S), peak apex (M) and peak end (E) positions of the spot [22].

2.7. Determination of strychnine in *S. nux vomica* and marketed formulation

Two micro liter of each sample as prepared above was applied in triplicates on TLC plates. It was developed and scanned as per the method described. The results of peak area obtained corresponding to strychnine were used for quantification in samples using regression equation. The results of triplicate analysis were expressed as average amount of strychnine in % *w/w*.

2.8. Stress degradation studies

2.8.1. Acid and base induced degradation

An amount equivalent to 50 mg of strychnine was dissolved in 50 ml of methanolic solution of 1M HCl and 1M NaOH. The solution was refluxed for 6 h at 90 ºC in the dark in order to exclude the possible degradative effect of light. The resultant solution was diluted 10 times, and applied on the TLC plate in triplicate (2µl each, i.e. 200 ng per spot) [23].

2.8.2. Hydrogen peroxide - induced degradation

To 25 ml of methanolic solution of strychnine, 25 ml of hydrogen peroxide (30.0%, *v/v*) was added. The solution was heated in boiling water bath for 6 h to remove completely the excess of hydrogen peroxide. The resultant solution was diluted appropriately and applied (2 µl each) on TLC plate in triplicate (200 ng per spot).

2.8.3. Photochemical degradation product

Fifty mg of strychnine was dissolved in 50 ml of methanol and exposed to direct sunlight for 24 h. The resultant solution was diluted appropriately and applied on TLC plate (200 ng per spot).

In all degradation studies, the average peak area of strychnine after application (200 ng per spot) of three replicates was obtained.

3. Results and discussion

3.1 Selection and optimization of mobile phase

The HPTLC procedure was developed and optimized. Several easily available solvents in different combinations and concentrations were tried to obtain good resolution, compact spot and better separation. Initially, toluene: ethyl acetate: diethyl amine combitation in varying ratios was tried. Finally The mobile phase toluene: ethyl acetate: diethyl amine (7: 2: 1, *v/v/v*) was

selected which gives a sharp and well-defined peak of strychnine at R_f value of 0.42 (Figure 2). It was found that the solvent system has very good resolution for the separation of strychnine.

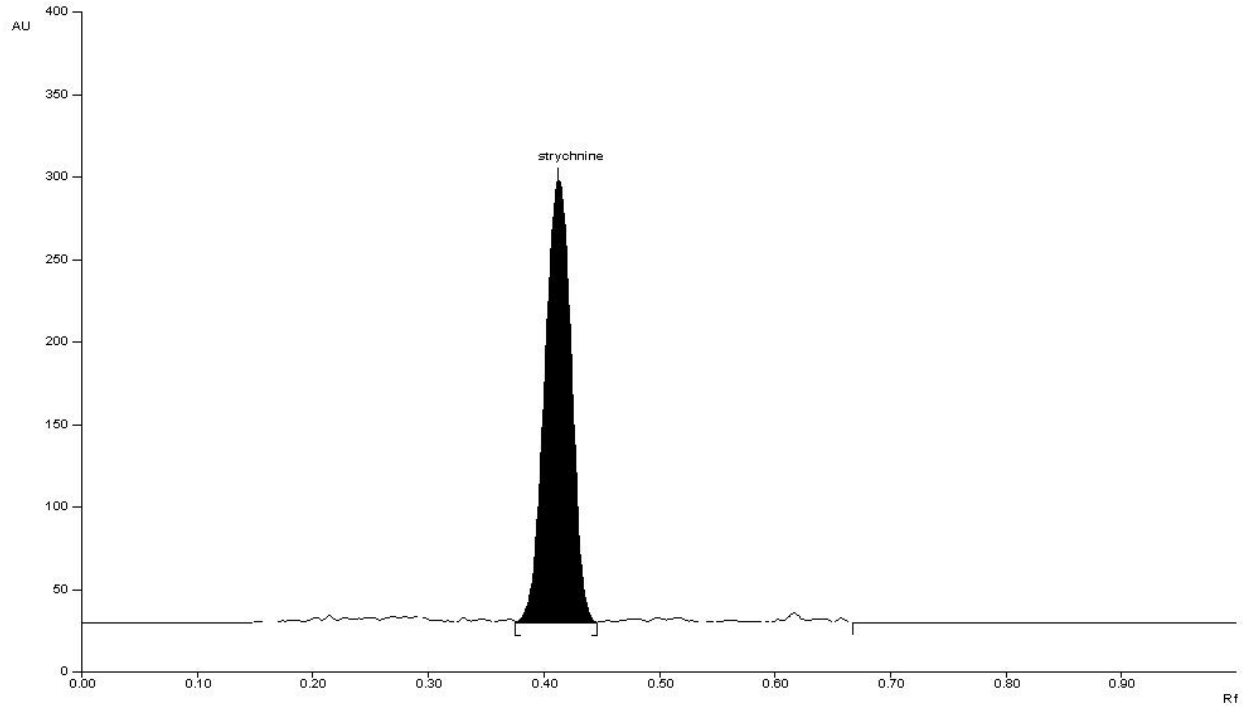

Figure 2. A typical TLC chromatogram of strychnine (R_f =0.42)

3.2. Calibration curves

The linear regression data for the calibration curves (n=3) as shown in Table 1 showed a good linear relationship over the concentration range 100–1000 ng/spot with respect to peak area.

No significant difference was observed in the slopes of standard curves (ANOVA, $P > 0.05$).

Table 1. Recovery studies (n=6), for strychnine

Strychnine		Recovery (%)		% RSD		SEM	
Excess drug added to the analyte (%)	Theoretical content (ng)	Height	Area	Height	Area	Height	Area
0	500	99.12	99.69	0.463	0.330	0.266	0.190
50	750	99.36	100.12	0.441	0.419	0.254	0.243
100	1000	99.25	101.42	0.376	0.378	0.216	0.222
150	1250	102.81	99.85	0.245	0.242	0.186	0.140

3.3. Validation of the method

3.3.1. Precision

The repeatability of sample application and measurement of peak area were expressed in terms of %RSD and results are depicted in Table 2, which revealed intra- and inter-day variation of strychnine at two different concentration levels of 500 and 1000 ng/spot.

Table 2. Intra and inter day precision of HPTLC method (n=6) for strychnine

Strychnine	Mean		SD		% RSD		SEM	
Amount (ng per spot)	Height	Area	Height	Area	Height	Area	Height	Area
a. Intra-day								
500	130.58	1230.89	1.122	1.843	0.859	0.149	0.647	0.583
1000	281.42	2308.83	0.925	1.188	0.328	0.057	0.534	0.518
b. Inter-day								
500	136.88	1256.83	1.496	2.045	1.092	0.162	0.863	1.181
1000	255.82	2389.70	1.660	2.457	0.648	0.102	0.958	1.418

3.3.2. Robustness of the method

The low values of %RSD obtained after introducing small changes in mobile phase composition indicated robustness of the method as indicated in Table 3. There was no significant variation in the slope values (ANOVA, $P > 0.05$).

Table 3. Robustness of the method (n=3) for strychnine

Strychnine	Mobile phase composition			
Amount (ng/spot)	toluene: ethyl acetate: diethyl amine (6.5: 2.25: 1.25, *v/v/v*) % RSD		toluene: ethyl acetate: diethyl amine (7.25: 1.75: 1.15, *v/v/v*) % RSD	
	Height	Area	Height	Area
500	1.959	0.193	1.378	0.179
1000	1.213	0.139	0.685	0.084

3.3.3. LOD and LOQ

The LOD with S/N ratio of 3:1 was found to be 12.00 ng/spot and LOQ with S/N ratio of 10:1 was found to be 36.37 ng/spot.

3.3.4. Recovery studies

The method when used for extraction and subsequent estimation of strychnine from seed extract of *S. nux vomica* and unani formulation after spiking with 50, 100 and 150% of additional standard strychnine, yielded recovery of 99.12 ± 3.69%.

3.3.5. Specificity

The specificity of the method was ascertained by analyzing the standard drug and extract. The spot for strychnine in the sample was confirmed by comparing the R_f values and spectra of the spot with that of the standard. The peak purity of strychnine was assessed by comparing the spectra at three different levels, viz. peak start (S), peak apex (M) and peak end (E) positions of the spot.

3.4. Determination of strychnine in *S. nux vomica* seed and unani formulation

A well resolved single spot of strychnine was observed at R_f 0.42 in the chromatograms of the samples extracted from seeds of *S. nux vomica* and in marketed unani formulation. It was also observed from the super imposed UV spectra that there was no interference from the other components present in the extracts. The strychnine content in the seeds of *S. nux vomica* and in marketed unani formulation were found to be 0.391% *w/w* and 0.126% *w/w*, respectively, with low % RSD value indicated the suitability of this method for routine analysis of strychnine in *S. nux vomica* during the formulation development.

3.5. Stability - indicating property

The sample was subjected to treatment with acid, base, hydrogen peroxide, daylight, well-separated bands of pure strychnine and additional peaks at different R_f. Acid degradation was faster than alkaline. When the drug solution was heated with 1 M HCl at 80ºC for 8 h degradation products were observed at R_f 0.24, 0.39, and 0.52 (Figure 3(a)). The sample of strychnine was found to undergo alkaline degradation very readily in 1 M NaOH at 80ºC – approximately 80% of the drug was degraded in 5 min. Peaks of degradation products were observed at R_f 0.49 (Figure 3(b)). The drug was found to be degraded by 30% hydrogen peroxide when treated at room temperature for 24 h; degradation products were observed at R_f 0.25, 0.28, 0.57, and 0.60 (Figure 3(c)). In daylight the strychnine was almost stable, with only one additional peak at R_f 0.53 (Figure 3(d)).

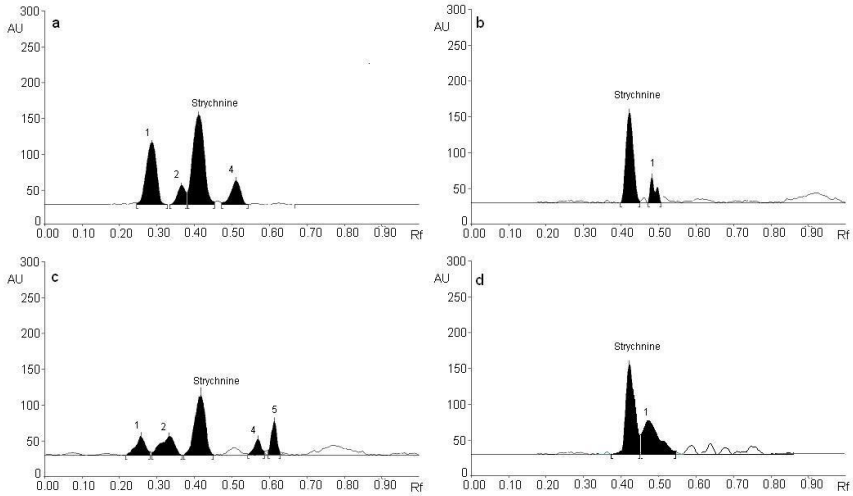

Figure 3. TLC chromatograms of degradation products of Strychnine on exposure to different stress conditions: (a) acid; (b) base; (c) oxidation; (d) day light

4. Conclusion

A validated stability indicating HPTLC analytical method has been developed for determination of strychnine in Strychnos nux vomica seed extract and Marketed Unani Formulation. The results of stress testing undertaken as per the ICH guidelines reveal that the method is selective and stability indicating. This proposed HPTLC method is simple, precise, specific, accurate, less time consuming, cost effective as compared to HPLC, and GC methods and has the ability to separate the drug from its degradation products. The statistical analysis of data obtained proves that the method is reproducible and selective and can be used for routine qualitative and quantitative analysis of strychnine in S. nux vomica seed extract and its Marketed Unani Formulation. . As the method separates the drug from its degradation products, it can be employed as a stability indicating one.

Acknowledgement

Authors are thankful to the Jamia Hamdard, New Delhi (India) and CCRUM, New Delhi (India) for providing us the financial assistance to carry out this research work.

References

[1] Pharmacopoeia of the People's Republic of China, English ed., 1995. *"The Pharmacopeia Commission of PRC"*. Beijing, 38-39, (Part 1).
[2] Gosselin, R. E., Smith, R. P., and Hodge, H. C. 1984. "Clinical toxicology of commercial product". 5th edn. Williams and Wilkins, Baltimore, II- 249, III- 375-379.
[3] Goodman, L. S., Gilman, A. G., and Gilman, A. 1985. *"The pharmacological basis of therapeutics"*. Macmillan Publishing Co., Inc., New York, 582-584.
[4] Charlotte, D., Geoffroy, L. G., Philippe, M., and Jean-Claude, A. 2004. Liquid chromatography / photodiode array detection for determination of strychnine in blood: a fatal case report. *Forensic Science International*, 141: 17-21.
[5] Chopra, R. N., Nayar, S. L., and Chopra, I. C. 1956. *"Glossary of Indian Medicinal Plants"*. National Institute of Science Communication and Information Resources, Council of Scientific and Industrial Research, New Delhi, India, 236, 122.
[6] Sharma, P. V. 1978. *"Dravya Guna Vigyan"*. vol II. Chaukambha Sanskrit Sansthan, Varanasi.
[7] Bisset, N. G. and Phillipson, J. D. 1971. The tertiary alkaloids of some Asian species of strychnos. *Journal of Pharmaceutical and Pharmacology*, 23: 244S.
[8] Trease, C. E. and Evans, W. C. 1989. *"Trease and Evans Pharmacognosy"*. 13th edn. Bailliere Tindal, London, 17-39.
[9] Bensky, D. and Gamble, A. 1986. *"Chinese Herbal Medicine"*. Eastland Press, Seattle, 646.
[10] Chung, B. and Shin, M.K. 1989. *"Dictionary of Folk Medicine"*. Young Lim Press, Seoul, 972.
[11] New Medical College of Jiangsu. 1977. *"Dictionary of Chinese Traditional Medicines"*. Shanghai Scientific and Technological Press, Shanghai, China, 291-293.
[12] Cai, B.C., Hattori, M., and Namba, T. 1990. Processing of nux vomica. II. Changes in the alkaloid composition of the seeds of *Strychno nux-vomica* on traditional drug-processing. Chemical and Pharmaceutical Bulletin. 38: 1295-1298.
[13] Lacassie, E., Marquet, P., Gaulier, J. M., Dreyfuss, M. F., and Lachatre, G. 2001. Sensitive

and specific multi residue methods for the determination of pesticides of various classes in clinical and forensic toxicology. *Forensic Science International*, 121: 116-125.

[14] Marques, E. P., Gil, F., Proenca, P., Monanto, P., Oliveira, M. F., Castanheira, A., and Vieira, D. N. 2000. Analytical method for the determination of strychnine in tissues by gas chromatography/mass spectrometry: two case reports. *Forensic Science International*, 110: 145-152.

[15] Rosano, T. G., Hubbard, J. D., Meola, J. M., and Swift, T. A. 2000. Fatal strychnine poisoning: application of gas chromatography and tandem mass spectrometry. *Journal of Analytical Toxicology*, 24: 642-647.

[16] Ahmad, A., Mujeeb, M., and Panda, B. P. 2010. An HPTLC Method for the Simultaneous Analysis of Compactin and Citrinin in Penicillium citrinum Fermentation Broth. *Journal of Planar Chromatography*, 23: 282-285.

[17] Alam, P., Ali, M., Singh, R., Madhurima, Ahmad, S., and Shakeel, F. 2009. A validated HPLC method for estimation of cordifolioside A in Tinospora cordifolia Miers and marketed formulations. *Journal of Chromatographic Science*, 47: 910-913.

[18] Wagner, H. and Bladt, S. 1996. Plant drug Analysis, A thin layer chromatography atlas. Springer, New York, 359.

[19] Ahmad, S., Rizwan, M., Parveen, R., Mujeeb, M., and Aquil, M. 2008. A Validated Stability-Indicating TLC Method for Determination of Forskolin in Crude Drug and Pharmaceutical Dosage Form. *Chromatographia*, 67: 441-447.

[20] ICH, 1994. Text on Validation of Analytical Procedures, Harmonised Tripartite Guideline prepared within the International Conference on Harmonisation of Technical Requirements for the Registration of Pharmaceuticals for Human Use, ICH Q2A, Geneva.

[21] ICH, 1996. Validation of Analytical Procedures: Methodology, Harmonised Tripartite Guideline prepared within the International Conference on Harmonisation of Technical Requirements for the Registration of Pharmaceuticals for Human Use, ICH-Q2B, Geneva.

[22] ICH, 1995. Draft guidelines on validation of analytical procedures: definition and terminology, federal register, 60. IFPMA, Switzerland.

[23] Ansari, M. J., Ahmad, S., Kohli, K., Ali, J., and Khar, R. K. 2005. Stability-indicating HPTLC determination of curcumin in bulk drug and pharmaceutical formulations. *Journal of pharmaceutical and biomedical analysis,* 39: 132-138.