

Research Article

Development and Validation of Selective High-Performance Liquid Chromatographic Method Using Photodiode Array Detection for Estimation of Aconitine in Polyherbal Ayurvedic Taila Preparations

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Received 9 December 2011; Revised 29 January 2012; Accepted 29 January 2012

Academic Editor: Sibel A. Ozkan

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A simple, sensitive, and selective high-performance liquid chromatographic (HPLC) method has been developed and validated for the analysis of aconitine in marketed ayurvedic *taila* (oil) formulations containing roots of *Aconitum chasmanthum*. Chromatography of methanolic extracts of these formulations was performed on C₁₈ (5 μm × 25 cm × 4.6 mm i.d.) column using isocratic mobile phase consisting of (65 : 35% v/v) acetonitrile and buffer solution (aqueous 0.01 M ammonium bicarbonate buffer, adjusted to pH 9.6 using 30% ammonia solution) at a flow rate of 1 mL/min and SPD-10 A_{VP} photodiode array (PDA) UV-Visible detector. The analytical reference, aconitine, was quantified at 238 nm. The retention time of aconitine was about 42.54 min. The linear regression analysis data for the calibration plot showed a good linear relationship with correlation coefficient of 0.9989 in the concentration range of 15 to 90 μg/mL for aconitine with respect to peak area. The limit of detection and limit of quantitation values were found to be 0.03 μg/mL and 0.1 μg/mL respectively. Repeatability of the method was found to be 0.551–1.689 RSD. Recovery values from 97.75 to 99.91% indicate excellent accuracy of the method. The developed HPLC method is accurate and precise and it can be successfully applied for the determination of aconitine in marketed ayurvedic oil formulations containing *Aconitum chasmanthum*.

1. Introduction

Aconitum chasmanthum (Family, Ranunculaceae) is a most valuable medicinal plant, widely used in the traditional and folk medicines of a number of countries of south east Asia. The chief chemical constituents of *Aconitum chasmanthum* root are aconitine, mesaconitine, and hyaconitine, and their respective hydrolyzed analogs are called monoester alkaloids, that is, benzoylaconine, benzoylmesaconine, and benzoylhyaconine [1–3]. Aconitine being the major diterpenoid responsible for the biopotency of *Aconitum chasmanthum* is recognized as the reference compound [3, 4]. Many polyherbal oil formulations in Indian and Chinese traditional systems of medicine used for control of skin diseases contain aconitum root as major active ingredient [5, 6]. Polyherbal

oil formulations are made with the main objective of incorporating the fat-soluble fraction of the component of herbal drugs to a suitable oil base. Standardization of these formulations in terms of composition is important to ensure quality and safety. Aconitine can be used as analytical reference in the quality control of polyherbal oil formulations containing *Aconitum chasmanthum* [7–10].

There are reports on the application of various analytical methods for isolation and quantitation of aconitine present in *Aconitum chasmanthum*, biological fluids and other botanical sources [10–14]. Its estimation in polyherbal traditional medicines especially oil formulation is challenging. But no reported method deals with estimation of aconitine in complex matrix of ayurvedic *taila* formulations. High-performance liquid chromatography has emerged as an efficient

tool for the phytochemical evaluation of herbal drugs because of its simplicity, sensitivity, accuracy, suitability for high-throughput screening, and so forth [15–21]. Hence it was thought worthwhile to develop a simple chromatographic method for determination of aconitine in ayurvedic *taila* formulations. The method was validated and found to be sensitive and reproducible [22, 23].

2. Material and Methods

2.1. Reference Compound and Reagents. Reference aconitine (95% w/v purity) was purchased from Sigma-Aldrich (Germany). All the chemicals used in analysis were of AR grade except those used for HPLC analyses which were of HPLC grade. All A.R. grade chemicals were procured from S.D. fine chemicals, Mumbai and all HPLC grade solvents were procured from Merck, Mumbai.

2.2. Polyherbal Oil Formulations. Commercial marketed ayurvedic polyherbal oil formulations “*Varnaraksasa taila*” and “*Vipritmalla taila*” which contain *Aconitum chasmanthum* as one of the components were selected for studies. *Varnaraksasa taila* is a polyherbomineral oil containing mercury, sulphur, cinnabar (HgS), realgar (As₄S₄), fine copper powder, orpiment (As₂S₃), *Allium sativum* bulb (family, Alliaceae), and *Aconitum chasmanthum* root (family, Ranunculaceae) digested in mustard seed oil (*Brassica nigra*, Family, Brassicaceae) as per classical Ayurvedic text [5, 6]. *Vipritmalla taila* contains cinnabar (HgO), *Saussurea lappa* root (family, Asteraceae), *Aconitum chasmanthum* root (family, Ranunculaceae), *Ferula asafetida* resin (family, Apiaceae), *Allium sativum* bulb (family, Alliaceae), *Plumbago zylenticum* root (family, Plumbaginaceae), *Valeriana wallichii* root (family, Valerianaceae), and *Gloriosa superba* root (family, Liliaceae) digested in mustard seed oil as per classical Ayurvedic text [5, 6].

Samples of the same formulations in triplicate, manufactured by three different reputed ayurvedic drug manufacturers were collected from retail pharmacies in Indore, Madhya Pradesh, India.

2.3. Preparation of Standard Solutions. The stock solution of 1 mg/mL in methanol was prepared after keeping the purity of reference aconitine into consideration. Solution was filtered through Whatman filter paper (no. 1). Aliquots of stock solution were diluted to 5 mL using methanol to obtain working standards in concentration range from 15 to 90 µg/mL.

2.4. Chromatographic Conditions. The mobile phase consisted of acetonitrile: aqueous 0.01 M ammonium bicarbonate buffer, adjusted to pH 9.6 using ammonia solution (65 : 35% v/v) at a flow rate of 1 mL/min. Before use, the mobile phase was degassed by an ultrasonic bath and filtered using 0.4 µm membrane filter. Separation was performed at room temperature on HPLC system having a pump (Shimadzu LC 10AT_{VP}) with 20 µL Rheodyne injector, Phenomenex Luna C₁₈ (5 µm × 25 cm × 4.6 mm i.d) column,

and SPD-10 A_{VP} photodiode array (PDA) UV-Visible detector set at 238 nm and equipped with CLASS-VP software (Shimadzu, Kyoto, Japan).

2.5. HPLC Analysis of Ayurvedic Taila Preparations. Oil formulations (10 gm) were homogenated using homogenizer (Scientific instruments ltd, Indore) using methanol (50 mL) in proportion of 1 : 5, w/v at 50°C for 20 min. The mixture was centrifuged at 2000 rpm for 20 min at 4°C and the supernatant was collected. The residue was resuspended in methanol and the extraction was repeated five more times similarly. The supernatants were pooled and concentrated under vacuum at room temperature and made up to a volume of 20 mL using methanol. The extracts were filtered through 0.45 µm filter and HPLC was performed under the conditions optimized for the reference compound. The amount of aconitine was quantified using calibration curves plotted with the reference compound.

2.6. Validation of Method

(a) Calibration Graph (Linearity of the HPLC Method). The calibration curve was obtained at 6 concentration levels of aconitine standard solutions (15–90 µg/mL). The solutions (20 µL) were injected into the HPLC system (*n* = 6) with the chromatographic conditions previously given. The linearity was evaluated by the least-squares regression method.

(b) Limits of Detection and Quantification. For determination of the limit of detection (LOD) and the limit of quantification (LOQ) different dilutions of the standard solution of aconitine were analyzed using mobile phase as the blank. The LOD and LOQ were determined on the basis of signal-to-noise ratio until the average responses were approximately 3 and 10 times the responses of the blank, respectively.

(c) Accuracy (Recovery). The accuracy of the methods was determined by calculating recovery of aconitine by the standard addition method. Known amounts of standard solution of aconitine (at three levels 50%, 100%, 150%) were added to prequantitated sample solutions. The amount of aconitine was estimated by applying values of peak area to the regression equations of the calibration graph. Five replicate samples of each concentration level were prepared.

(d) Method Precision (Repeatability). The precision of the instruments was checked by repeatedly injecting and analyzing (*n* = 6) standard solutions of aconitine (45 µg/mL). The results are reported in terms of relative standard deviation (RSD).

(e) Intermediate Precision (Reproducibility). The intraday and interday precision of the proposed method were determined by analyzing standard solution of aconitine at 3 different concentrations (15, 45, and 90 µg/mL) three times on the same day and on three different days. The results are reported in terms of RSD.

(f) *Solution Stability and Mobile Phase Stability.* Solution stability in the assay method was evaluated by leaving test solutions of sample and reference standard in tightly capped volumetric flasks at room temperature in the dark for 24 h. The same sample solutions were assayed every 6 h interval in the study period. Mobile phase stability was studied by assaying the freshly prepared sample solutions against freshly prepared reference standard solutions at 6 h intervals up to 24 h. Mobile phase was prepared and kept constant during the study period. The relative standard deviation (RSD) of the assay of aconitine was calculated for the study period during mobile phase and solution stability experiments.

2.7. *Statistical Analysis.* The statistical analysis was performed using Microsoft Excel 2003.

3. Result and Discussion

The literature revealed that methanol is preferred for extraction of aconitine from *Aconitum chasmanthum* [10–14]. The same was used for extraction of aconitine from oil formulations. It is advantageous as the base oil in all the selected ayurvedic oil formulations was immiscible in this solvent. The immiscibility of oil in solvent will help in reducing number of interfering components in further chromatographic development. Multiple extractions were carried out to ensure complete extraction.

3.1. *Development of the HPLC Method.* The method development and selection of a suitable mobile phase involved several trials because of the complexity of the chemical composition of the herbals and the affinities of the components towards various solvents. The proportions of the organic and aqueous phases were adjusted to obtain a simple assay method with a reasonable run time and suitable retention time. Further optimization of mobile phase was performed based on resolution, asymmetric factor, and theoretical plates obtained for aconitine. Different mobile phases were tried like methanol: acetonitrile: water (45:45:10) which gave broad peak for aconitine. Combination of acetonitrile: methanol (10:90) was tried which gave broad and tailed peak. A mixture of methanol: water (60:40) was tried, which gave unresolved peak at retention time 48.9 min. Under optimized conditions HPLC with C18 column and UV detector at 238 nm using mobile phase (acetonitrile: aqueous 0.01 M ammonium bicarbonate buffer, adjusted to pH 9.6 using 30% ammonia solution (65:35%v/v)) gave well-resolved symmetric band for aconitine from its oil formulation (Figure 1). The resolution was found to be 1.7. Retention time was found to be around 42 minutes and aconitine appeared on chromatogram at 42.54 minutes. Retention time of aconitine was found to be of 42.54 minutes.

The method consumes less volume of HPLC solvents. When the same drug solution was injected 6 times, the retention time of the drug was found to be the same (Figure 2).

3.2. *Validation of Method.* The calibration curve was prepared by plotting the peak area against aconitine concentration; it was found linear in the range of 15–90 $\mu\text{g/mL}$. The

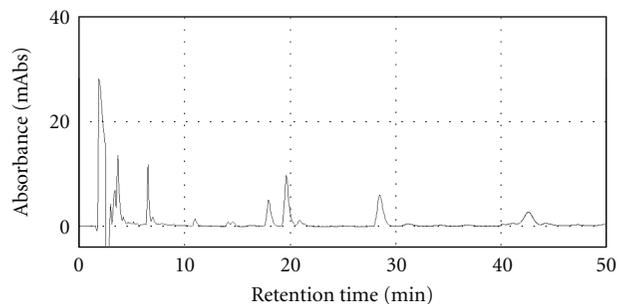


FIGURE 1: HPLC chromatogram of a 20 μL injection of 40 $\mu\text{g/mL}$ reference aconitine at 238 nm.

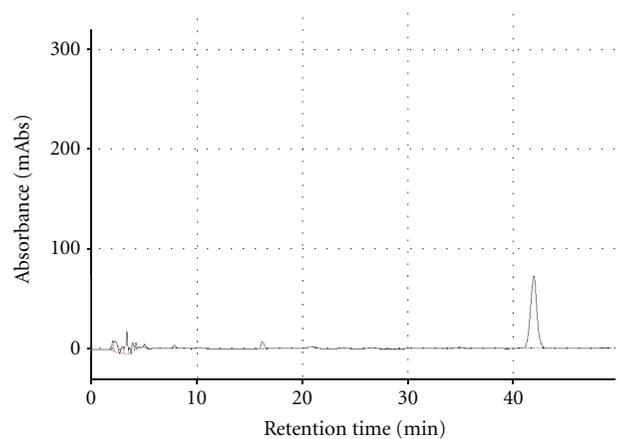


FIGURE 2: HPLC chromatogram of a 20 μL injection of the sample of taila preparation at 238 nm.

TABLE 1: Summary of validation parameters and system suitability parameters.

Parameters	Observations \pm % RSD ($n = 06$)
Retention time (min)	42.54 \pm 0.33
Tailing factor	1.1 \pm 0.1
Resolution	1.7 \pm 0.23
Theoretical plates	3213 \pm 0.42
Linearity range ($\mu\text{g/mL}$)	15–90
Correlation coefficient (r^2)	0.9989
Regression equation	$y = 71.5x - 24.5$
Limit of detection ($\mu\text{g/mL}$)	0.03
Limit of quantification ($\mu\text{g/mL}$)	0.1
Repeatability (% RSD, $n = 6$)	0.55–1.68

regression equation was found as $y = 71.5x - 24.5$ ($r^2 = 0.9989$), showing excellent linearity (Figure 3). The method was validated in terms of precision, repeatability, accuracy, and other validation parameters (Table 1). The repeatability of the HPLC method and the intermediate precisions for intraday and interday variations are given in Table 2. The LOD value was found to be 0.03 $\mu\text{g/mL}$, which is the concentration that yields a signal-to-noise (S/N) ratio of 3/1. The LOQ value under the described conditions was 0.1 $\mu\text{g/mL}$

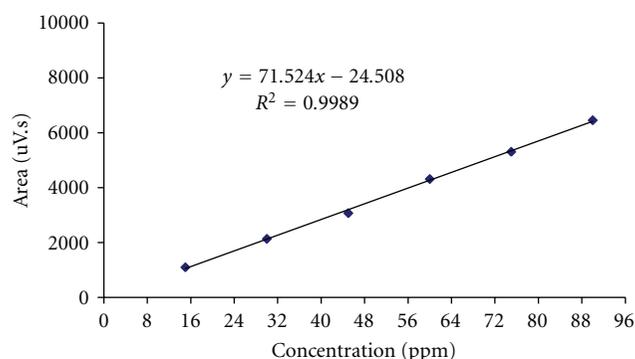


FIGURE 3: Calibration curve of reference aconitine in blank oil sample matrix at 238 nm.

TABLE 2: Precision studies data for aconitine.

Concentration ($\mu\text{g/mL}$)	Intraday		Interday*	
	Peak area \pm SD	% RSD*	Peak area \pm SD	% RSD*
15	1101.6 \pm 16.7	1.52	1112.0 \pm 6.3	0.57
45	3059.9 \pm 29.5	0.96	3063.0 \pm 29.5	0.96
75	5282.5 \pm 42.3	0.80	5292.6 \pm 47.8	0.90

*Relative standard deviation (% RSD, $n = 3$).

with an S/N ratio of 10:1. This confirmed the sensitivity for quantitation of aconitine in *taila* preparations. Recovery values from 97.7 to 99.9% indicate excellent accuracy of the method (Table 3). The RSD values of assay of aconitine during solution stability, mobile phase stability and robustness studies (Table 4) were within 2.0%. The data obtained in both experiments proves that the sample solutions and mobile phase used during assay were stable up to 24 h.

3.3. HPLC Analysis of Ayurvedic Taila Preparations. Quantitative estimation of aconitine in polyherbal oil formulations given in Table 5 revealed variation in its content in different brands, which indicates the need of standardization of raw material used and uniformity in method of manufacturing to be followed by different ayurvedic manufacturers. The method developed here does not require separation of unsaponifiable matter for quantification as reported for some active ingredients in oil formulation. Oil extract can be directly used for analysis. Avoidance of long and tedious step therefore makes this method more amendable to the high-throughput screening.

4. Conclusion

A method for analysis of *Aconitum chasmanthum* using aconitine as analytical reference in polyherbal oil formulation was developed. Proposed method does not require tedious steps of saponification for separation of fatty acid which are the major interfering component in analysis of oils. Further the method does not require any chemical transformation of active moiety aconitine and it is analyzed as such. The method was found to be simple, precise, specific,

TABLE 3: Accuracy study of the proposed method for aconitine.

Amount detected in sample ($\mu\text{g/mL}$)	Amount of standard drug added ($\mu\text{g/mL}$)	Amount of drug recovered ($\mu\text{g/mL}$)*	% Recovery*
<i>Varnaraksasa taila</i>			
28.3	0	27.6 \pm 0.4	97.7 \pm 1.6
28.3	14	41.7 \pm 0.2	98.6 \pm 0.5
28.3	28	56.2 \pm 0.3	99.9 \pm 0.6
28.3	42	69.2 \pm 0.4	98.5 \pm 0.5
<i>Vipritmalla taila</i>			
32.2	0	31.8 \pm 0.2	98.7 \pm 0.6
32.2	16	47.9 \pm 0.4	99.4 \pm 0.2
32.2	32	65.4 \pm 0.3	101.8 \pm 0.5
32.2	48	80.4 \pm 0.5	100.2 \pm 0.7

*Mean value \pm relative standard deviation (% RSD, $n = 3$).

TABLE 4: Results of robustness studies.

Parameter	% RSD peak area
Mobile phase composition	1.22
Amount of mobile phase	0.83
Temperature	0.92
Chamber saturation time	0.71
Chamber dimensions	0.43

TABLE 5: Aconitine content found in various *taila* preparations.

Sample	Brand	Aconitine content in oil (mg/100 gm)
<i>Varnaraksasa taila</i>	1	0.27 \pm 0.003
	2	0.28 \pm 0.005
	3	0.26 \pm 0.004
<i>Vipritmalla taila</i>	1	0.31 \pm 0.05
	2	0.32 \pm 0.07
	3	0.32 \pm 0.08

*Mean \pm relative standard deviation (RSD, $n = 3$).

sensitive, and accurate. It can be used for routine quality control of polyherbal oil formulations containing *Aconitum chasmanthum*.

Acknowledgments

The authors are thankful to School of Pharmacy, DAVV University, Indore (Madhya Pradesh) and Director, SICART, V. V. Nagar (Gujarat) for providing analytical facilities.

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