Quantitative determination of aconitine in Aconitum chasmanthum and Aconitum heterophyllum from Kashmir Himalayas using HPLC
Neelofar Jabeen, Shakeel-u-Rehman, Khursheed A. Bhat, Mohd A. Khuroo and Abdul S. Shawl
1Indian Institute of Integrative Medicine (Br) (CSIR) Sanatnagar Srinagar, Jammu & Kashmir India 190005
2Department of Chemistry, University of Kashmir 190006

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ABSTRACT
The work presents the estimation of bioactive compound aconitine in two aconitum species (Aconitum chasmanthum, Aconitum heterophyllum) using a simple reversed phase HPLC-UV-DAD method. Peak heights were linear with relation to aconitine concentration with correlation coefficient >0.999. This assay is rapid, and highly reproducible. The extraction method described gives high yield of otherwise difficult to extract marker compound aconitine.

Key words: HPLC, aconitine, Aconitum chasmanthum, Aconitum heterophyllum.

INTRODUCTION
India has a unique position in the world where a number of traditional medicines are being practised. The Kashmir Himalaya, which is known as ‘paradise on earth’, is a rich repository of medicinal plants owing to its montane topography and diverse habitats.

The Genus Aconitum (Ranunculaceae) is widely distributed across North Asia and North America. 27 species of Aconitum are found in India. Aconitum chasmanthum and Aconitum heterophyllum are confined to western Himalayas including Kashmir and Pakistan. Both A. chasmanthum and A. heterophyllum are important medicinal plants used in the folk medicine of a number of South-East Asian countries. A. heterophyllum possesses potential immunomodulatory activity. In homeopathic system, it is used to relieve pain and promote a sense of calm.

Although aconites possess sedative, anodyne and anti-inflammatory properties, but in overdoses the drug and its derivatives are swift and act as fatal poisons and cause asphyxia in adults followed by death. Estimation of aconitine in different species of aconitum is given in (table 1). From table 1 it is clear that the amount of aconitine varies from species to species and with place of origin. Though the isolation of aconitine is reported from A. chasmanthum but there are no reports for its quantification. Until now there are no reports for the determination of aconitine in A. heterophyllum from Kashmir valley. Taking into consideration the fact that aconitine alkaloids are important bioactive secondary metabolites and simultaneously the main toxic compounds of Aconitum species with a narrow margin of safety between therapeutic and toxic dose, quality control of aconitum drugs/extracts is very necessary. In this direction we have carried out the estimation of aconitine in A. chasmanthum and A. heterophyllum using a simple and reliable HPLC method. The HPLC method used by Ohta et al. for the determination of aconitine alkaloids in blood and urine samples has been modified in the present studies. The mobile phase composition has been changed to meet the requirements for the development of completely resolved chromatogram.

MATERIALS AND METHODS

Chemicals and Reagents:
Aconitine, glycerol and trifluoroacetic acid (TFA) were purchased from Merck (India). All chemicals were of analytical reagent grade. Solvents (water, acetonitrile and methanol) were of HPLC grade and purchased from Ranbaxy Fine Chemicals Limited (Okhla, New Delhi).

Collection and authentication of plant material:
A. heterophyllum and A. chasmanthum were collected from the high altitudes of Kashmir. A. heterophyllum was collected from high reaches of Sonamarg and Gulpark and A. chasmanthum from two different eco-geographical zones (Gulmarg and Gurez) in August 2007. The plant species were identified at the Centre of Plant Taxonomy, University of Kashmir. The voucher specimen of both these species were deposited in the Herbarium of the Institute (IIM, Srinagar).

Chromatographic system:
The instrument used was Thermo finnigan HPLC system consisting of high pressure liquid chromatography pump (P 4000), an auto sampler (AS 3000), a column oven, a Diode array detector (UV 6000 LP), Vacuum membrane degasser (SCM 1000) and System Integrator (SN 4000). ChromQuest 4.0 software was used for data analysis and processing. The measurements were carried out on RP 18 column (250mm x 4.6mm; particle size 5mm; Merck, Germany) at 30°C.

Preparation of herbal extracts:
The rhizomes of A. heterophyllum and A. chasmanthum were thoroughly washed under running tape water to clean the plant material from the associated soil and dust particles and other extrinsic contamination. After proper washing the samples were air dried in hot oven below 45°C. The dried samples were chopped and subsequently powdered using pestle and mortar. 5.0g of each sample were extracted with 50mL HCl solution (0.05M) by sonication. The aqueous extract was partitioned with ethyl acetate (3x40mL) to remove the non-alkaloid components. The pH of the aqueous part was brought to 7 by addition of ammonia and extracted with CHCl3 (3x50mL). The solvent was removed under reduced pressure to furnish a semi-solid gum. 50mg from this alkaloid extract were dissolved in HPLC grade acetonitrile and the resulting solution was filtered through 0.45 mm filter membrane for HPLC analysis. The analysis was carried out using an isocratic solvent system consisting of a mixture of THF, 0.3% TFA (Water) and glycerol (25:73:0.2, v/v/v) and the flow rate was 1.0 mL/min.

Table 1: Percentage of aconitine reported in different species of Aconitum

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Aconitine (%)</th>
<th>Location</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. carniueuli (raw)</td>
<td>0.02±0.0003</td>
<td>Hokkaido</td>
<td>[14]</td>
</tr>
<tr>
<td>A. carniueuli (raw)</td>
<td>0.05±0.0042</td>
<td>Gumma</td>
<td>[14]</td>
</tr>
<tr>
<td>A. carniueuli (processed)</td>
<td>0.06±0.0023</td>
<td>China</td>
<td>[14]</td>
</tr>
<tr>
<td>A. carniueuli (processed)</td>
<td>0.05±0.0006</td>
<td>Hokkaido</td>
<td>[14]</td>
</tr>
<tr>
<td>A. carniueuli (processed)</td>
<td>0.03±0.0007</td>
<td>Gumma</td>
<td>[14]</td>
</tr>
<tr>
<td>A. carniueuli (processed)</td>
<td>0.06±0.0004</td>
<td>China</td>
<td>[14]</td>
</tr>
<tr>
<td>A. carniueuli</td>
<td>0.010</td>
<td>NR</td>
<td>[15]</td>
</tr>
<tr>
<td>A. heterophyllum</td>
<td>0.009</td>
<td>Sikkim</td>
<td>[12]</td>
</tr>
<tr>
<td>A. japonicum (processed)</td>
<td>0.007±0.0009</td>
<td>Niigata</td>
<td>[14]</td>
</tr>
<tr>
<td>A. kanezoffii</td>
<td>0.03±0.06</td>
<td>NR</td>
<td>[16]</td>
</tr>
<tr>
<td>A. sarocolicum</td>
<td>0.49</td>
<td>NR</td>
<td>[17]</td>
</tr>
</tbody>
</table>

NR: Not reported

*Corresponding author.
Khursheed A Bhat
Scientist
Indian Institute of Integrative Medicine (CSIR)
Sanatnagar, Srinagar-190005
Jammu & Kashmir India

Preparation of stock solutions and calibration of standard curve: Stock solution of Aconitine (1mg/mL) was prepared in acetonitrile and stored in refrigerator at 0°C. The stability of stock solution was monitored and no change in concentration was observed. Working solutions were prepared by diluting the stock solution with mobile phase.

RESULTS AND DISCUSSION

Though a number of HPLC methods are reported in literature18,19 but the following assay system, comprising an acidic mobile phase, was found most suitable for the typical chromatogram development in the present studies. A non-acidic solvent system failed to elute aconitine from HPLC column. Aconitine was first injected individually to record its UV spectrum. The mobile phase consisted of a mixture of THF, 0.3% TFA(Water) and glycerol (25:73:02, v/v/v) and the flow rate was 1.0 mL/min. Optimal chromatographic conditions were obtained after testing different mobile phase compositions with reversed phase C18 column. An isocratic elution using a mixture of THF, 0.3% TFA in H2O and glycerol in 25:73:02 ratio resulted into the complete resolution of different components present in the plant extract. The flow rate of mobile phase was 1.0 mL/min and the detection of marker compound was done at 235 nm (?max) using diode array detector. The processed plant extract was injected to evaluate the resolution of different components present in the extract. A suitable, reliable, rapid and simple HPLC method was established for efficient separation of various components in the crude plant extract. A typical chromatogram with smooth baseline was obtained using the isocratic elution of processed plant extract (fig 2c). The identification of the marker compound from the plant extract was established by comparison of the UV spectra and retention time with that of authentic standard. Peak heights were linear with relation to aconitine concentration (fig 2a). The correlation coefficient >0.999 was obtained. The relative standard deviation (RSD) for 8 repetitions was 0.9%. This indicated the preciseness of HPLC method. In a typical experiment measured volume of standard aconitine solution was added to a sample solution containing 70 µg/mL aconitine. After extraction and HPLC analysis, the concentration of final solution was determined. Recovery was calculated by comparing the concentration of final solution determined through HPLC with the given concentration. The recoveries for aconitine were 93.1%, 93.3% and 94.8% at the concentration of 400, 250 and 100 µg/mL respectively (Table 2). The high recoveries thus obtained reflect the suitability of the extraction method as well as the stability of sample solutions. Had the extraction process been inefficient, the recoveries as given in table 2 would not have been possible.

Table 2: Recovery of aconitine from plant extract

<table>
<thead>
<tr>
<th>Spiked conc. (µg/mL)</th>
<th>Recovery</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>94.8</td>
<td>93</td>
</tr>
<tr>
<td>250</td>
<td>93.3</td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>93.1</td>
<td></td>
</tr>
</tbody>
</table>

Using the above mentioned HPLC conditions the quantity of aconitine in four accessions of two Aconitum species have been determined (Table 3). The HPLC results show that A. chesmanthum possesses higher levels of aconitine as compared to A. heterophyllum.

Table 3: Amounts of aconitine in A. chesmanthum and A. heterophyllum.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Plant species</th>
<th>Accession code</th>
<th>Site of collection</th>
<th>Aconitine % Extract</th>
<th>% Plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aconitum chesmanthum</td>
<td>AcC-01</td>
<td>Gurez Razdani pass</td>
<td>0.81</td>
<td>0.0320</td>
</tr>
<tr>
<td>2</td>
<td>Aconitum chesmanthum</td>
<td>AcC-02</td>
<td>Gulmarg</td>
<td>0.78</td>
<td>0.0310</td>
</tr>
<tr>
<td>3</td>
<td>Aconitum heterophyllum</td>
<td>AcH-03</td>
<td>Gulmarg</td>
<td>0.08</td>
<td>0.0018</td>
</tr>
<tr>
<td>4</td>
<td>Aconitum heterophyllum</td>
<td>AcH-04</td>
<td>Sonamarg</td>
<td>0.07</td>
<td>0.0014</td>
</tr>
</tbody>
</table>

AcC-01 accession shows highest aconitine concentration as compared to other three accessions. The different aconitine concentrations of two accessions of A. heterophyllum can be attributed to their occurrence in two different eco-geographical zones.

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REFERENCES


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