Isolation and quantification of pinitol in *Argyrolobium roseum* plant, by $^1$H-NMR

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**KEYWORDS**

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**Abstract**

Chemical investigations on ethanolic extract of *Argyrolobium roseum* led to the isolation of Pinitol as the major constituent of the plant. Pinitol is chemically known as 3-O-methyl-D-Chiro-inositol and has been found to possess anti-diabetic activity. It helps in the regeneration of beta cells, present in the areas of the pancreas called as islets – of Langerhans. These cells make and release insulin, a hormone which controls the level of glucose in the blood. Pinitol was isolated from the ethanolic extract of the plant and a sensitive & reliable method, based on Proton Nuclear Magnetic Resonance (PNMR), was developed and used as an analytical tool for quantification and identification of this relatively UV insensitive compound in the alcoholic extract of the plant. The method involves the use of pyrazinamide (an anti-tuberculosis drug), as a reference. Validation of the method was carried out by preparing a known concentration of an artificial mixture of pinitol and pyrazinamide. The recovery of pinitol in the mixture was in the range of 98.5–101.3%. Pinitol in pure form was isolated from the ethanolic extract of *A. roseum* by repeated column chromatography over silica gel followed by crystallization in methanol. Pinitol isolated from the plant was identified on the basis of $^1$H-NMR, $^{13}$C-NMR, DEPT (45°, 90° and 135°) experiments and mass spectral data. The method was successfully applied for the quantitation of pinitol in various extracts of the said plant.

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1. Introduction

Pinitol (Fig. 1) or 3-O-methyl D-Chiro-inositol, has been reported to possess antidiabetic and hypoglycaemic activities [1,2]. It has been claimed that the compound possesses insulin like effects. The studies have revealed that D-pinitol can act like insulin to improve glycaemic control in hypoinsulinaemic STZ-diabetic mice [3]. Inositol phosphoglycans are potentially important post-receptor mediators of insulin action. Pinitol
has been found to mediate certain actions of insulin [4]. Lot of work has been reported in the literature on pinitol and its analogues [5–8]. The compound has shown significant hypoglycaemic and antidiabetic activities in normal and alloxan–induced diabetic albino mice and is free from acute toxicity. Screening of *Argyrolobium roseum* for its chemical constituents led to the isolation of pinitol (Fig. 1) as the major constituent of the plant. This is a virgin plant, and no reference regarding its chemical constituents was found in the literature. As pinitol showed very poor response to the HPLC-UV (DAD) method, we decided to quantify this compound in the ethanol extract of the plant by Proton Magnetic Resonance Spectroscopy while using pyrazinamide (Fig. 2) as a reference compound.

Proton Magnetic Resonance (PNMR) spectroscopy is becoming of great importance [9–16] in the determination and quantitative analysis of natural products, food products and drug metabolites. The plant extract is a biogenetic cocktail and some of the components in this biogenetic cocktail show poor response to commonly used Diode Array, UV/VIS and Fluorescent detectors. There is strong demand for non-chromatographic alternatives in qualitative / quantitative assessment of the reference compounds. Quantitative NMR [17] is a versatile tool used for simultaneous detection and quantification of the compounds along with impurities. NMR involves direct determination and is non destructive in nature. It requires fewer cleanups as compared to HPLC and HPLC-MS and NMR signals are visible even in the complex matrices. $^1$H-NMR, being more sensitive than $^{13}$C-NMR is therefore, more versatile for routine analysis with respect to time and cost. The quantitative NMR concept is being developed with a focus on its potential in the quantification and quality control of the reference compounds. The quantitative – NMR concept offers a way to assess the purity of the natural product in a single analytical step, without the need of performing multiple analyses, while still offering the option to retain the substance.

Many times NMR information is used simply for qualitative analyses, but the desire to obtain quantitative information from NMR is growing rapidly, especially for high throughput techniques like combinatorial chemistry. This seems to arise from the difficulties encountered in performing accurate quantification with mass spectrometric techniques, especially for samples still bound to the resin beads during the solid phase organic synthesis. NMR data can be very quantitative as long as certain precautions are observed. Primary importance is the need to ensure that complete relaxation ($T_1$) has taken place, but the other important considerations include the need to use broad band excitation, to generate flat baselines, to minimize spectral overlap and nOe interferences. Internal standards are also an important aspect of quantification. There appears to be no one perfect internal standard. Some standards are too volatile to give reliable signals (for example TMS) while other internal standards are too involatile to be removed from the sample after measurement (like TSP). Standards have been shown to interact at times with the glass sample container. A good standard should exhibit minimum spectral overlap with the signals of interest. In this article, we report a direct determination and quantitation of pinitol in *A. roseum* extract by means of its NMR signal using pyrazinamide as internal standard.

2. Experimental

2.1. Plant material

The plant material was collected from the hilly terrains of the Jammu region of Jammu and the Kashmir (India) State. The specimen voucher of the plant was deposited in the Herbarium division of the institute.

2.2. Solvents

All the solvents, such as pyridine-d$_5$, methanol-d$_4$ and D$_2$O used for identification as well as for quantification were from Sigma–Aldrich, Bangalore (India), whereas HPLC grade Methanol for ESI-MS analyses (Rankem make) was from Ranbaxy chemicals Ltd. (Mohali, Punjab, India). Pyrazinamide was received as a gift from a private Indian based pharmaceutical company.

2.3. Isolation and characterization of pinitol

Dried and coarsely powdered plant material (500 g) was kept in 95% ethanol (2 L) in a percolator for 15 h at ambient temperature. The solvent was drained off, and the process was repeated four times. All the drained off portions (4 × 2 L) were combined, filtered and finally evaporated under diminished pressure to yield a residue (36 g). The residue was triturated successively with hexane (4 × 500 ml) to remove the non-polar part of the extract. The residue (26 g) left behind after trituration was subjected to column chromatography on silica gel (520 g, 60–120 mesh) using ethyl acetate as the eluting solvent. The polarity of the mobile phase was increased by gradual addition of methanol. The fractions, eluted in ethyl acetate: methanol (1:1), exhibited a similar pattern on TLC, were pooled and evaporated under vacuo to yield residue (9.5 g). The residue was crystallized in hot ethanol to yield 5.2 g of pure compound that was analysed for C$_{18}$H$_{24}$O$_6$, m.p. 184–185°C, [α]$_{D}^{20}$ = +61.5 (C: 0.27, H$_2$O), $^1$H-NMR (400 MHz Fig. 3), pyridine-d$_5$ δ 4.80–4.85 (4H, m, H-2, H-3, H-4, and H-5) 4.18–4.23 (1H, t, J = 6.0 Hz, H-1), 4.65–4.71 (1H, t, J = 6.0 Hz, H-6), 3.97 (3H, s, OMe). When $^1$H NMR was taken in D$_2$O, MeOD and DMSO the signal resonating for methoxyl protons of pinitol was observed at δ 3.46, 3.51 and
3.42, respectively (Fig. 4A–C). 13C-NMR (200 MHz), pyridine-d5: δ 71.297 (C-1), 70.98 (C-2), 83.40 (C-3), 72.34 (C-4), 70.80 (C-5), 71.44 (C-6), and 58.61 (OCH3). In all DEPT 13C experiments, i.e. 90°, 45°, and 135°, all the carbons exhibited upward (+ve) signals indicating the presence of only –CH and –CH3 carbons. As there was no downward (−ve) signal in DEPT 45° 13C-NMR experiment, the presence of a CH2 group in the molecule is ruled out. ESI-MS (+ve mode) exhibited a molecular ion peak at m/z 217 [M+Na]+ (Fig. 8), pentaacetate. 1H-NMR (CDCl3): δ 5.20–5.45 (m, 5H, H-1, H-2, H-4, H-5, & H-6), 3.67 (t, 1H, J = 10 Hz, H-3), 3.50 (s, 3H, OCH3), 2.19 (s, 6H, 2XOAc), 2.12 (s, 3H, OAc), 2.08 (s, 3H, OAc), 2.02 (s, 3H, OAc). ESI-MS +ve mode at m/z 426.6 [M+Na]+, MS2 at m/z 366.6 [426-CH3COOH]+. 1H-NMR, 13C-NMR and MS data confirmed the structure of the compound as pinitol.

Pyrazinamide (Fig. 5): 1H-NMR (400 MHz, Fig. 4), D2O: 9.05 (s, 1H, H-3), 8.67 (d, 1H, H-5) and 8.62 (d, 1H, H-6).

2.4. Instrumentation

NMR spectra were recorded in pyridine-d5 (Aldrich 99.9% D) at room temperature using Bruker Avance DPX-400 (400-MHz) Fourier Transform (FT) NMR Spectrometer equipped with 5-mm multinuclear inverse probehead with a Z-shielded gradient. Spectra were recorded with a spectral width of 12019.2 Hz, with 32 k data points (td), ns = 16, dt = 1 s, 0:1; middle of the 1H spectra. For quantification the pulse programmes were taken from the Bruker software library. 1D Processing was done by with additional zero filling to 64 k to perform a base line correction on the FID before the Fourier transformation. The phase of the spectrum was also accurately adjusted to perform the baseline correction on the spectrum. It was ensured that the integral limits are far enough apart to give a complete integration.

2.4.1. MS conditions

Pure Pinitol solution (200 ppm) concentration was prepared in MS grade water and filtered through 0.2 μm filter before injecting into the MS system. MS experiments were performed on Agilent 6540 LC-Q-T of Mass spectrometer. Positive mode ESI was used for ionization of the compound. Agilent 6540 UHD Accurate-Mass detector was used with AJS ESI (Agilent Jet Stream) as source. Agilent Mass Hunter Qualitative analysis (B.04.00) software was used for data analysis and Agilent Mass Hunter workstation software was used for data acquisition. The gas temperature was maintained at 325 °C, drying gas flow 9 L/min, nebulizer 40 psi, sheath gas flow 9 L/min, sheath gas temp. 350 °C, V cap voltage 3500V, Nozzle voltage 1000 V, fragmentor voltage 135V and the acquisition range was set between 150 and 250 m/z to get good MS spectra.

2.5. Preparation of samples for quantification (NMR)

All the standards, i.e. pure pinitol (Fig. 1) and pyrazinamide (Fig. 2), were weighed accurately and prepared in deuterated water (2 mg/ml). These solutions were mixed together to give a working solution of concentration 1 mg/ml each of pinitol (isolated and purified) and pyrazinamide in the mixture, and finally the mixture was used in recovery studies for validation purposes. Similarly the solutions (5 mg/ml) of different extracts were prepared in D2O, and to these extract solutions, pyrazinamide solution of known concentration was added for quantification purposes. Before quantification a 1H NMR spectrum of the mixture of Pinitol and Pyrazinamide was taken (Fig. 6) to identify the marker signals for quantification studies. The methoxyl signal in pinitol, integrating for three protons, at δ 3.46, and a downfield proton, (H-3) at δ 9.05, of pyrazinamide were considered for quantification.

3. Results and discussion

In 1H NMR spectroscopy the signal area is normally proportional to the number of nuclei contributing to the signal, provided that saturation is avoided. It is, therefore, possible to use the integrals of 1H NMR spectra for quantitative determinations. The area of the signals is more reliable for the quantitative analysis of a mixture of a few components. This is not true...
in the case of complex mixture spectra, since the high number of peaks sometimes induces signal overlap.

The most important parameter in quantitative NMR is the adjustment of parameters in such a manner that they allow, at the same time, identification of the components of a mixture. Hence, the parameters which allowed a valuable qualitative analysis were maintained. The integrals of $^1$H NMR spectra were used for quantitative determinations because the integrals give the relative measure of the number of resonating nuclei. Accurate integrations can be obtained if the relaxation delay is 5 times longer than the $T_1$ [18–19]. Modern instruments allow for extremely accurate integration of the peak area. In q-NMR analysis of a mixture, integral accuracy of 99% is required, and the experimental conditions were set to obtain

**Figure 4** (A) 400 MHz NMR of pinitol in D$_2$O. Marker Signal taken for quantification. (B) 400 MHz NMR of Pinitol in MeOD. (C) 400 MHz NMR of pinitol in DMSO.
this level of accuracy, Lower S/N per unit was compensated by longer time averaging. Integrals give a relative measure of the number of resonating nuclei. Accurate integrals and the quantitative determination is carried out by taking into account the ratio of the average intensities of selected signals belonging to the analysed component with respect to that of a reference. Then the mass of an unknown component is calculated. The following formula applies in $^1\text{H}$ NMR quantification.
In our case \( G_{pi} \) and \( G_{pz} \) are the parts by weight of pinitol and pyrazinamide, respectively, and \( F_{pi} \) and \( F_{pz} \) are the areas of signal of –OCH\(_3\) (\( \delta \) 3.46) group of pinitol and one downfield H (\( \delta \) 9.05) of pyrazinamide. \( N_{pi} \) and \( N_{pz} \) are the number of resonating nuclei which cause the signals, in our case 3 resonating nuclei (protons of –OCH\(_3\)) of pinitol and 1 resonating nucleus of pyrazinamide have been taken into consideration. \( M_{pi} \) and \( M_{pz} \) are the molecular masses of the pinitol (194 g/mole) and pyrazinamide (123 g/mole) respectively. Using the measured integrals, the software itself calculates the amount of unknown component if the amount of internal standard is known. In our case the amount of pyrazinamide is known, and the amount of pinitol has been determined in different batches of the extracts of \textit{A. roseum}. Extracts of \textit{A. roseum} are soluble in water and NMR spectra were taken in D\(_2\)O (Fig. 7). The results of the studies have been detailed in \textbf{Table 1}.

### Table 1  Concentration (%W/W) of pinitol in plant \textit{Argyrolobium roseum}.

<table>
<thead>
<tr>
<th>Batch</th>
<th>Amount% (pinitol)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch-1</td>
<td>1.28 ± 0.012</td>
<td>2.13</td>
</tr>
<tr>
<td>Batch-2</td>
<td>1.38 ± 0.018</td>
<td>2.89</td>
</tr>
<tr>
<td>Batch-3</td>
<td>1.31 ± 0.021</td>
<td>3.66</td>
</tr>
</tbody>
</table>

For each sample 64 scans were recorded using the parameters described in quantification.

4. Conclusions

The NMR method is easy to implement for quantification and could probably be adapted to assay such types of compounds where HPLC methods are not feasible. For quantification purposes the three methoxyl protons of pinitol at \( \delta \) 3.46 and one downfield proton of pyrazinamide resonating at \( \delta \) 9.05 were considered (Fig. 6). Both the signals for these two compounds were not in the very crowded region of the spectrum. Moreover, these signals were separated by large chemical shifts which explain the reason why these signals were considered for quantification purposes. Pyrazinamide is a stable internal standard which exhibits a sharp singlet in an uncrowded region of the spectrum. There was no change in the quantification values even after 24 h of measuring these values. The method described here allowed the quantification of pinitol, a UV insensitive compound. It may be expected that this method could be useful in the analysis of pinitol in complex mixtures. Our purpose of using pyrazinamide as an internal standard in the studies was that both pinitol and pyrazinamide are soluble in water and the marker signals taken for quantitation in both the compounds were separated by larger \( \delta \) values in the \textsuperscript{1}H spectrum.

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### References


