Investigation of Related Impurities in Metadoxine by a Reversed Phase High Performance Liquid Chromatography Technique†

Krishnan SURESH BABU and Deivanayagam PARADESI†

Department of Chemistry, Faculty of Engineering and Technology, SRM Institute of Science and Technology, Kattankulathur, Pin Code: 603 203, Tamil Nadu, India.

† To whom correspondence should be addressed.

E-mail: paradesi77@yahoo.com
Abstract

A new reversed phase high performance liquid chromatography (RP-HPLC) method has been developed for the separation and identification of impurities present in metadoxine. Herein, we report that one of the impurities eluted from the metadoxine sample is 4-deoxypyridoxine hydrochloride (4-DPH). In HPLC analysis, the retention time (RT) of 4-DPH was observed at 13.5 min in both the reference and metadoxine samples and the relative retention time (RRT) was 1.71. The presence of 4-DPH in metadoxine sample was also confirmed by the chromatogram obtained by spiking 4-DPH standard into the sample. Furthermore, the elution and mass of impurity 4-DPH in metadoxine was proven by LC-mass spectroscopy studies. This method highlights the presence of another unknown impurity that has so far not been observed in earlier methods of metadoxine evaluation. Hence, the developed method achieved superior resolution between metadoxine and impurities and thereby facilitates the production of a purer metadoxine drug.
Introduction

Quality control is a crucial step in the manufacturing process of pharmaceutically important molecules. Since molecules generated in the pharmaceutical industry involve downstream consumption by humans, the purity of molecules plays a vital role. Hence, stringent methods are developed to test the quality of pharmaceutical products and their intermediates. Reversed phase high performance liquid chromatography (RP-HPLC) is a widely used tool to analyze products manufactured in the pharmaceutical industry.\(^1\) RP-HPLC is effectively used in the pharmaceutical industry to determine purity of products, analyze active pharmaceutical ingredients and develop impurity profiles for products.

In pharmaceutical industry, a drug impurity profile is mandatory for manufactured products.\(^2\)-\(^4\) Typically, drug impurity profiles consisting of both identified and unidentified impurities are developed using RP-HPLC. The RP-HPLC conditions for generating such an impurity profile are standardized to detect all the impurities present in the product. This process usually involves selecting an appropriate column, optimizing the mobile phase pH and identifying the ideal flow rate along with other factors such as the type of detector and column temperature. Once the conditions are established for a particular product and its impurity profile is standardized, the optimized RP-HPLC conditions can be used for quality control purposes.

Metadoxine (C\(_{13}\)H\(_{18}\)N\(_2\)O\(_6\)) is one of the best drugs for treating acute alcohol intoxication and alcohol related disorders. It is obtained by the salification of pyrrolidone carboxylic acid and pyridoxol. The structure of metadoxine is depicted in Fig. S1. Chemically
metadoxine is pyridoxine-pyrrolidone carboxylate, used as an antioxidant\textsuperscript{5,6} and is an approved drug in several European countries for treating chronic and acute alcohol intoxication.\textsuperscript{7-12} Metadoxine accelerates the clearance of alcohol from blood.\textsuperscript{13} It increases the rate of transformation of alcohol into acetaldehyde to be excreted in urine. It helps to prevent liver damage resulting from prolonged alcohol intake and supports to reverse fatty liver.\textsuperscript{14} 

The market for the drug metadoxine is considerably large since alcohol consumption is a causative factor in 4\% of all deaths worldwide. In general, the drug metadoxine is prescribed for liver transplantation, alcohol liver cirrhosis, alcohol addiction, alcohol intoxication, etc. RP-HPLC method development that can give a complete profile of the impurities present in manufactured metadoxine will be significant contribution to quality control process of this commercially important drug molecule. While reviewing the pharmacopeia and applying the related substances analysis, we found that there were no impurities that were separated from metadoxine. We suspected that some of the impurities may coelute or not be eluted by the existing method. The objective of the study is to improve upon the existing protocol, thereby identifying the unknown impurities existing in metadoxine. By considering the synthetic route of metadoxine, possible impurities may be traced out from its raw materials/intermediates.

Metadoxine is manufactured in two steps. In the first step, pyridoxine hydrochloride is converted into pyridoxine base and the second step yields metadoxine by treating the pyridoxine base with L-pyroglutamic acid. It is important from a pharmaceutical point of view to quantify both metadoxine and the other impurities present with in the final product. Developing a method to identify and quantify reactants will aid in the quality
control process of the drug molecule. A literature study revealed that several methods are available for the qualitative and quantitative analysis of metadoxine, including a UV spectrophotometric method, derivative spectroscopy method, and HPTLC methods. However, each of the methods available so far do not comprehensively provide the details of all the impurities present in the manufactured product metadoxine. This makes profiling of metadoxine for quality control processes susceptible to doubt. Keeping these facts in mind, we aimed to generate a new RP-HPLC method that will give a complete profiling of all the known and unknown impurities present in manufactured metadoxine. It is to be expected that the newly developed method should be of great help in identifying L-pyroglutamic acid and 4-DPH as well as any unknown impurities present in the drug metadoxine. Since, 4-DPH is a vitamin B₆ antagonist, it may be toxic to developing embryos subsequently it can have negative effects on collagen and elastin during development. The presence of this compound in a drug can produce vitamin B₆ deficiency, which suppresses the immune system.

**Experimental**

*Reagents and chemicals*

The metadoxine sample was kindly provided by Exeltis - Ordain Health Care Global Pvt Ltd. (India). 4-DPH (impurity B) was purchased from TCI Chemicals Pvt. Ltd. (India). The HPLC purity of 4-DPH was reported as 98.2 area % in the Certificate of Analysis. Potassium dihydrogen orthophosphate and orthophosphoric acid were acquired from Merck India Ltd. HPLC grade water was received from RANKEM.
Instrumentation

The Shimadzu HPLC system (Model: LC2010A) equipped with a UV detector and quaternary pump. The instrument has an auto injection capability and the injection volume was fixed at 5.0 μL for all standards and samples. LC solution software was used to integrate the chromatograms. A Hypersil BDS C-18 column (250 mm x 4.6 mm, 5μm) was used for the analysis and the column temperature was maintained at 30°C for the duration of analysis. The flow rate of mobile phase was set to 1.0 mL/min and UV detection was recorded at a wavelength of 210 nm. The HPLC column was conditioned with mobile phase for one hour prior to analysis. In order to determine the best conditions for resolving all the compounds present in addition to metadoxine, different mobile phase trials (with a pH range from 2.0 to 7.0) were carried out. The final chromatogram obtained with the newly developed method at pH 3.0 was compared with the existing method employing the same sample to conclusively prove good separation and better resolution.

Preparation of mobile phase

A quantity of 2.72 g of potassium dihydrogen phosphate (KH₂PO₄) was dissolved in 900 mL of HPLC grade water and the pH was adjusted to 3.0 using diluted ortho phosphoric acid solution followed by a final dilution to a volume of 1000 mL with HPLC water. The prepared buffer solution was filtered through a 0.45μm filter paper and degassed prior to use. The dilute ortho phosphoric acid solution was prepared by dissolving 3.5 mL of ortho phosphoric acid in 50 mL of water.

Standard and sample preparation

L-Pyroglutamic acid solution was prepared by dissolving 25.0 mg of standard substance
in 5 mL of diluent followed by sonication and dilution to 10 mL with diluent. A metadoxine sample solution was prepared by transferring 25.0 mg of accurately weighed metadoxine powder into a 10 mL flask dissolved in 5 mL of diluent followed by sonication and dilution with diluent. The stock solution of 4-DPH (impurity B of pyridoxine hydrochloride) was prepared by dissolving 25.0 mg of accurately weighed 4-DPH in 5 mL of diluent followed by sonication and further dilution to 10 mL with diluent. The diluted solution of 4-DPH was prepared by pipetting 3.0 mL of impurity B stock solution into a 50 mL standard flask containing a small quantity of diluent followed by dilution to the mark with diluent. The system suitability solution was prepared by dissolving 25.0 mg of metadoxine in small quantity of diluent followed by dilution to the mark in a 10 mL volumetric flask with a diluted 4-DPH solution. HPLC grade water was used as the diluent for the preparation of all test solutions.

Results and Discussion

Initially, the mobile phase of 0.06 M potassium dihydrogen orthophosphate at pH 7.0 adjusted with potassium hydroxide solution was used to generate the chromatogram of metadoxine sample. The HPLC chromatogram obtained by this method is depicted in Fig. S2. There was no appreciable separation between metadoxine and impurities. In general, the drug metadoxine is produced from L-pyroglutamic acid and pyridoxine hydrochloride. The expected impurities may be from the raw materials or byproducts, if there are any. Keeping this in mind, various mobile phase trials (in the pH range from 2.0 to 7.0) were carried out with 0.02M potassium dihydrogen orthophosphate. Among these mobile phase modifications, the pH 3.0 buffer system showed an excellent output for the isolation of the impurities.
First the diluent, Millipore water, was injected as a blank run to confirm the absence of any other peaks in the system due to background noise. Furthermore, the system suitability solution of the metadoxine sample spiked with 4-DPH was injected and their resolution was observed. The L-Pyroglutamic acid and metadoxine peaks separated well, but the unknown peak was merged with 4-DPH in all mobile phase conditions except the mobile phase system at pH 3.0. The results obtained by using the mobile phase systems at pH 4.0 and 5.0 were similar to the pH 2.0 system. The main peak was observed to split when using the mobile phase system at pH 6.0 and 4-DPH merged with the metadoxine peak.

The chromatograms of the metadoxine sample and 4-DPH standard obtained by modified pH 3.0 method are depicted in Fig. 1 and Fig. 2 respectively. Two impurities were observed in the metadoxine sample chromatogram at retention time of 13.5 and 15.7 min. The relative retention time (RRT) between the impurities and metadoxine were found to be 1.71 and 2.00 respectively. It was observed that the peak eluted at the retention time of 13.5 min matches with one of the impurities present in the metadoxine sample and belongs to 4-DPH. It can be concluded from both Fig. 1 and Fig. 2 that one of the impurities present in metadoxine is a known impurity i.e., 4-DPH. The HPLC chromatogram of L-pyroglutamic acid is displayed in Fig. S3. The HPLC chromatogram of system suitability solution and respective peak table are given in Fig. 3 and Table 1. Fig. 3 displays good resolution between (i) metadoxine and 4-DPH (R_S=4.7) and (ii) 4-DPH and an unknown impurity (R_S=1.5), since metadoxine exhibits significant relative retention time with the known and unknown impurities.

The LC-MS study was performed by using an Agilent 6460 instrument connected with
an Inertsil ODS-3V column (150x4.6 mm, 5 µm) maintained at 25°C. The flow rate of the mobile phase was set to 0.8 mL/min. Electrospray ionization mode was used to detect the impurities present in metadoxine sample. The details of the mass spectrometry conditions followed by preparation of the mobile phase and test solutions are provided in Supporting Information. The LC-MS chromatograms of the metadoxine sample and the 4-DPH impurity reference are displayed in Fig. 4 and Fig. 5, respectively. This LC-MS study also proves that one of the eluted impurities from the metadoxine sample is 4-DPH. The mass spectra of 4-DPH in the metadoxine sample and 4-DPH reference are provided in Fig. S4 and Fig. S5 respectively. The retention time and mass-to-charge ratio of 4-DPH in the reference material and metadoxine sample were clearly matched. The retention time of 4-DPH in the reference material and in the metadoxine drug sample were found to be 11.45 and 11.52 min, respectively.

The mass-to-charge ratio of the 4-DPH impurity in the metadoxine sample was found to be 154.20 amu, which matched the 4-DPH reference standard (m/z 154.10 amu). Both the m/z values of the standard and sample are in good agreement with the theoretical mass of 4-DPH (153.10 amu). The peak eluted at the retention time of 6.865 min in Fig. 5 is pyridoxine. Fig. S6 displays the mass spectrum of pyridoxine and the m/z value of pyridoxine was found to be 170.100. The mass spectrum of impurity eluted at the retention time of 12.496 min in Fig. 4 is presented in Fig. S7 which indicates the m/z value of 321.200 amu. The probable structure corresponded to this observed m/z value in metadoxine sample is pyridoxine dimer. Thus, the developed method is well suited for the investigation of impurities in metadoxine.
Conclusions

The drug metadoxine has several beneficial effects within living organisms. The newly developed RP-HPLC method is well suited to separate all the impurities present in metadoxine. The RRT between the impurities and metadoxine were found to be 1.71 and 2.00, which is considered to be good resolution (4.7 and 1.5) between the peaks. It can be concluded from the HPLC chromatograms of 4-DPH and the metadoxine sample that the impurity appeared at the RT of 13.5 min is well separated with an RRT of 1.71 and was confirmed to be 4-DPH by both HPLC and LC-MS studies. The m/z value of 4-DPH impurity in the metadoxine sample (154.20) matched with the m/z value of the 4-DPH reference standard (154.10). The other unknown impurity eluted at the RT of 15.8 min is also well separated from metadoxine with a RRT of 2.00. Hence, the reported RP-HPLC method is feasible, economical and can be used for related substance analysis of bulk metadoxine.

Acknowledgements

The authors are thankful to Mr. Mathialagan, Associate Director of Exeltis-India for providing a gift sample of the drug metadoxine. The authors are also thankful to Synthiya Research Labs Private Ltd., Pondicherry, India for providing the laboratory facility.
References

1. N. Kaul, H. Agarwal, B. Patil, A. Kakad and S. R. Dhaneshwar, *Chromatographia*, **2004**, 60, 501.

2. M. Saravanan, K. S. Kumar, P. P. Reddy and B. Satyanarayana, *Synth Commun.*, **2010**, 40, 1880.

3. B. Ramachandra, *Crit. Rev. Anal. Chem.*, **2017**, 47, 24.

4. T. V. Raghava Raju, S. Raja Kumar, A. Srinivas, N. Anil Kumar, I. Mrutyunjaya Rao and N. Someswara Rao, *J. Liq. Chromatogr. RT*, **2014**, 37, 498.

5. W. Wang, Y. Xu, C. Jiang and Y. Gao, *Curr. Med. Res. Opin.*, **2018**, 35, 261.

6. K. T. Suk, M. Y. Kim and S. K. Baik, *World J. Gastroenterol.*, 2014, **20**, 12934.

7. L. Leggio, G. A. Kenna, A. Ferrulli, W. H. Zywiak, F. Caputo, R. M. Swift and G. Addolorato, *Hum. Psychopharmacol. Clin. Exp.*, **2011**, 26, 554.

8. L. Vonghia, L. Leggio, A. Ferrulli, M. Bertini, G. Gasbarrini and G. Addolorato, *Eur. J. Intern. Med.*, **2008**, 19, 561.

9. G. Addolorato, C. Ancona, E. Capristo and G. Gasbarrini, *Int. J. Immunopathol. Pharmacol.*, **2003**, 16, 207.

10. M. M. C. Diaz, A. D. Martinez, V. V. Salcedo and C. C. Fuentes, *J. Int. Med. Res.*, **2002**, 30, 44.

11. L. S. Shpilenya, A. P. Muzychenko, G. Gasbarrini and G. Addolorato, *Clin. Exp. Res.*, **2006**, 26, 340.

12. I. Manor, J. H. Newcorn, S. V. Faraone and L. A. Adler, *Postgrad. Med.*, **2013**, 125, 181.
13. A. C. Childress and C. Tran. *Expert Opin. Investig. Drugs*, **2016**, 25, 463.

14. G. Mintziori and S. A. Polyzos, *Expert Opin. Pharmacother.* 2016, **17**(14), 1937.

15. G. Abirami, V. Vaidhyalingam, V. Niraimathi and A. Aruna, *Asian J. Chem.*, **2009**, 21, 1651.

16. P. Kumar, N. C. Joshi, A. Malik, N. Kaushik, A. Kushnoor and N. Gowda, *Anal. Chem. Ind. J.*, **2008**, 7, 311.

17. N. Kaul, H. Agarwal, B. Patil, A. Kakad and S. R. Dhaneshwar, *Il Farmaco.*, **2005**, 60, 351.

18. H. Qingchun, H. Chun, J. Yuzhu, L. Daping, L. Lichuan and H. Ting, Invention Patent Application - Application publication number CN 105566212A, **2016**, 05, 11.
Table 1  Time (min), Area, Height, RRT and Resolution of the peaks observed in the system suitability solution

| S. No | Time | Area   | Height  | RRT | Resolution | Name of the peak |
|-------|------|--------|---------|-----|------------|------------------|
| 1.    | 7.90 | 25463421 | 669829  | 1.00| -          | Metadoxine       |
| 2.    | 13.55| 2204177  | 40190   | 1.71| 4.70       | 4-DPH            |
| 3.    | 15.78| 227566   | 3559    | 2.00| 1.50       | Unknown impurity |

**Figure Captions**

Fig. 1  HPLC chromatogram of metadoxine by using modified mobile phase system at pH 3.0.

Fig. 2  HPLC chromatogram of 4-DPH.

Fig. 3  HPLC chromatogram of the system suitability solution.

Fig. 4  LC-MS study of the metadoxine sample.

Fig. 5  LC-MS study of the 4-DPH standard.
Fig. 1

Fig. 2
Graphical Index

| Peak Name        | RT  | RRT | Resolution |
|------------------|-----|-----|------------|
| Metadoxine       | 7.50| 1.00| -          |
| 4-DPH            | 13.55| 1.71| 4.70      |
| Unknown Impurity | 15.78| 2.00| 1.50      |

L-Pyrogulatamic acid

4-DPH

Time / min

Intensity / mV