Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 36 (2004) 477–482
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Superheated water chromatography-nuclear magnetic resonance spectroscopy and mass spectrometry of vitamins

Orapin Chienthavorn[a], Roger M. Smith[a,*], Shikha Saha[a], Ian D. Wilson[b], Brian Wright[b], Stephen D. Taylor[b], Eva M. Lenz[b]

[a] *Department of Chemistry, Loughborough University, Loughborough, Leics LE11 3TU, UK*
[b] *AstraZeneca, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK*

Received 29 April 2004; accepted 18 July 2004
Available online 27 August 2004

Abstract

The water-soluble vitamins, pyridoxine, riboflavin, and thiamine, were separated by reversed-phase liquid chromatography using hot or superheated water as the mobile phase and were detected using a range of detectors, including ultraviolet and fluorescence spectroscopy and mass spectrometry. By using deuterium oxide as the eluent, direct on-line nuclear magnetic resonance spectra could be obtained with minimal spectral interference from the mobile phase. Some of the compounds showed deuterium exchange of alkyl-protons when separated at high temperatures.

Keywords: Vitamin; Riboflavin; Superheated water; HPLC; LC-NMR

1. Introduction

There has been an increasing interest in the application of superheated water as a solvent-free mobile phase for reversed-phase liquid chromatography [1–5], as an alternative to conventional organic-water eluents. Separations have been demonstrated with a number of analytes, including phenols [1,5], antioxidants [2], analgesics [6], barbiturates [7], and sulphonamides [8]. Most of the separations have been carried out using polystyrene-divinyl benzene (PS-DVB) or PBD-zirconia [5] columns at temperatures varying from 100 to 220 °C and pressures up to 30 bar. The pH of the eluent can also be controlled to increase selectivity [8].

The absence of an organic mobile phase offers particular advantages in detection, as there is virtually no background signal in spectroscopic detection and both UV and fluorescence detection can be employed down to short wavelengths [1,7]. In addition, by using deuterium oxide as the mobile phase, it is possible to carry out on-line NMR spectroscopy with negligible interference from the mobile phase [6], which potentially has considerable advantages in the analysis of metabolites and biochemicals where structural identification is required. Normally, for LC-NMR, with conventional organic-aqueous mobile phases, care must be taken to suppress the signal due to the organic solvent as the signal can overload the data system or the mobile phase components have to be replaced by their deuterated equivalents (deuteromethanol or deuteroacetonitrile). Previous studies have demonstrated the use of superheated deuterium oxide in the LC-NMR (and LC-NMR-MS) of barbiturates [6], analgesics [7] and of sulphonamides (when a specific deuterium exchange reaction of a methyl group occurred) [9] and in the identification of the components of an extract of ginger [10]. Even more complex arrangements of spectroscopic detectors have proved to be possible including the application of on-line LC-IR-UV (DAD)-NMR-MS to drugs [11] and ecdysteroids present in plant extracts [12]. More recently, the combination of LC using superheated water with ICP-MS has also been demonstrated [13].

* Corresponding author. Tel.: +44 1509222563; fax: +44 1509223925.
E-mail address: R.M.Smith@lboro.ac.uk (R.M. Smith).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2004.07.023

The present study extends this approach to the superheated or hot water separation with NMR spectroscopic and MS detection of the water-soluble vitamins, which are of interest because of their complexity and reports of thermal instability.

2. Experimental section

2.1. Chemicals

Pyridoxal hydrochloride was obtained from May and Baker (Dagenham, England), riboflavin from BDH Chemicals (Poole, England), thiamine hydrochloride from Acros Organic (Geel, Belgium) and 4-methyl-5-thiazole-ethanol from Sigma–Aldrich (Gillingham, England). Deuterium oxide was of 99.9% purity and was purchased from Fluorochem (Old Glossop, England).

2.2. Superheated water chromatograph

The superheated water chromatographic system [1] consisted of an LC-10AD Shimadzu pump (Kyoto, Japan), which delivered water or D_2O at 1.0 ml min^{-1} to the column through a preheating coil made of a 100 cm × 0.01 in i.d. stainless steel tubing. The column and preheating coil (1 m × 0.01 in) were placed in a gas chromatographic oven (Series 104, Pye Unicam, Cambridge UK), whose temperature was controlled by a programmer/controller (Series 104, Pye Unicam, Cambridge UK). The samples were injected using a Rheodyne 7125 valve (Cotati, USA) fitted with a 20 μl sample loop that was mounted outside the oven. The analytes were separated on either a PLRP-S (PS-DVB) (150 mm × 4.6 mm, Polymer Laboratories, Church Stretton UK) or a 5 μm Novapak C_{18} (150 mm × 4 mm, Waters, Milford, USA) column. A set of copper cooling fins (3 cm × 12 cm × 0.05 mm) was attached to the tubing connecting the column to a Jasco UV/Visible detector (Model 870, Tokyo Japan) operating at 254 nm. The pressure at the outlet of the column was held above the boiling point of the water by either a Jasco back pressure regulator set at 30 bar (Model 880/81, Tokyo, Japan) or a restrictor coil of PEEK tubing (3 m × 0.13 mm i.d.). The chromatograms were collected on a Model 3396A integrator (Hewlett–Packard).

In initial studies, an SFM 25 fluorescence detector (Kontron, Zurich, Switzerland) fitted with an LC flow cell was placed after the UV detector before the back-pressure regulator. The detector was operated at an excitation wavelength of 300 nm and an emission wavelength 430 nm. The signal was collected on a Model 3394 integrator (Hewlett–Packard).

2.3. LC-NMR spectroscopy

For the NMR studies, the fluorescence detector was replaced by a Rheodyne 7125 injection valve, which could be used as a switching valve so that the flow was directed either to a back pressure regulator (or a restrictor coil of 3 m × 0.13 mm i.d. PEEK tubing) or via 3 m × 0.13 mm i.d. of PEEK tubing to the flow cell of a Bruker DRX500 NMR spectrometer (Bruker Biospin Ltd.) operating at 500.13 MHz (^{1}H) with a detection cell volume of 120 μl [7]. For stop-flow HPLC-^{1}H NMR experiments, depending on analyte concentration, 240–10,000 free induction decays (FIDs) were collected over a spectral width of 8278 Hz into 16,384 data points using an acquisition of 0.99 s using the NOESYPRESAT pulse sequence (Bruker Biospin Ltd.). The residual water resonance was suppressed using pre-irradiation during the relaxation delay of 2.0 s and mixing period of 0.10 s. The delay time between UV detector and NMR spectroscopic detector was approximately 33 s. The thiamine spectrum acquired in the continuous-flow mode was collected using the following parameters: the spectrum was acquired with 16 FIDs into 8192 data points over a spectral width of 8278 Hz, resulting in an acquisition time of 0.53 s. Water suppression was achieved during the relaxation delay of 0.5 s and the mixing time of 100 ms by application of the NOESYPRESAT pulse sequence.

2.4. LC-NMR-MS

A T-piece junction was placed in the tubing leading to the NMR spectrometer 30 cm before the magnet. The flow was split through a second 3 m × 0.13 mm length of i.d. PEEK tubing to a Quattro LC mass spectrometer (Micromass Ltd, Altrincham, Cheshire, UK) fitted with a Z spray source running two cone voltages of 25 and 60 V in positive electrospray mode. At 25 V, the mass range was scanned from 80 to 450 amu over 1 s, with a 0.1 s interscan delay. At 60 V, the mass range 35 to 450 amu was scanned, also over 1s with a 0.1 s interscan delay. The capillary was at 3.45 kV and the source block temperature was maintained at 80 °C. The desolvation temperature was 150 °C. The nebuliser gas flow was 80 l/h, and the desolvation gas flow was 564 l/h.

3. Results and discussion

The configuration of a superheated water chromatograph is similar to that of a conventional isocratic HPLC system [1]. A gas chromatographic oven is used to heat the column and a preheating coil is placed between the injector and the column to ensure temperature control. Conventional HPLC UV or fluorescence detector cells are used, as the pressure required to maintain the superheated water as a liquid is less than 50 bar at 200 °C. The system back-pressure is controlled so that the water in the column is held in the liquid state either by an electronically controlled back-pressure regulator or by a coil of capillary PEEK tubing. The pressures employed are not critical as the water under these conditions is barely compressible and changes in pressure have no effect on retention. The eluent is cooled before it reached the spectroscopic detectors to reduce refractive index fluctuations. Most of the work with superheated water has used polystyrene divinyl benzene (PS-DVB) columns because of

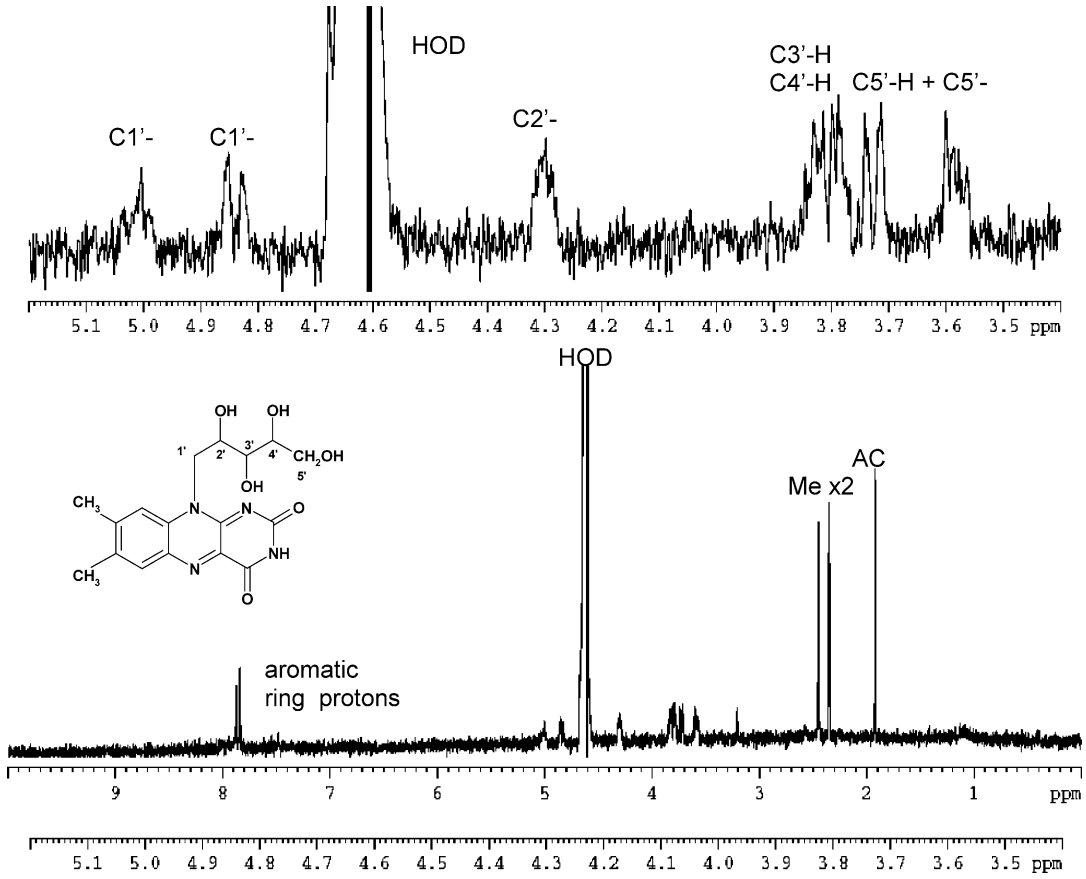

Fig. 1. On-line stop-flow ^{1}H NMR spectrum of riboflavin. Separation conditions: column, PS-DVB; column temperature, 200 °C; mobile phase, deuterium oxide at 1 ml min^{-1}, back-pressure 15 kg cm^{-2}.

their greater thermal stability compared to ODS-bonded silica columns.

The vitamins under study are well known for their instability towards heat and light. Hence, their stability at the higher temperature conditions required for superheated water chromatography was of interest. Suitable separation conditions, such column type, pH of eluent and elution temperature were selected for each vitamin to provide a reasonable retention time and resolution.

3.1. Pyridoxine

Pyridoxine (vitamin B_6) is highly water soluble and in conventional HPLC on an ODS column, typical conditions for the separation of pyridoxine hydrochloride are 0.1 M phosphate buffer at pH 2.15 with 1.25 mM octanesulphonic acid and 3% methanol [14]. When pyridoxine was examined with superheated water at 180 °C on a PS-DVB column without pH control or the use of an ion-pair reagent, the analyte eluted as a single peak with a short retention time (2.94 min) and could be readily detected using UV spectroscopic and fluorimetric (317 nm excitation, 376 nm emission) detectors. There was no evidence of analyte degradation and the separation confirmed that fluorimetric detection was achievable following a superheated water separation.

3.2. Riboflavin

There have been many reports of isocratic and gradient separations of riboflavin (vitamin B_1) by HPLC on ODS bonded reversed-phase columns, with mobile phases typically containing 13–25% acetonitrile in the mobile phase at pH 2.7–4 [15–18]. When riboflavin was examined in the present study on a PS-DVB column using unbuffered superheated water as the eluent, it was eluted at 200 °C with a retention time of 3.78 min (k = 2.78) and could be detected by both UV and fluorescence (excitation 450 nm, emission 530 nm) detectors. Although the solubility of riboflavin in water is low, there was no evidence of precipitation or loss of the analyte when the eluent was cooled post-column before detection. The fluorescence detector gave a linear response from 5 to 24 ng and an LOD of 3.6 ng on column. The sensitivity was comparable to the value of 0.5 ng, reported for an optimised separation on an ODS-silica column using acetonitrile-pH 4 buffer (13:77) as the eluent [16].

Using deuterium oxide at 200 °C as the eluent with the PS-DVB column, on-line ^{1}H NMR spectra could be obtained in the stop-flow mode using an injection of 10 μl of a 1 mg/ml sample solution. The spectrum (Fig. 1) largely matched with that reported for riboflavin in acidic deuterium oxide [19]. It contained a dominant, broad peak at 4.6 ppm for the residual

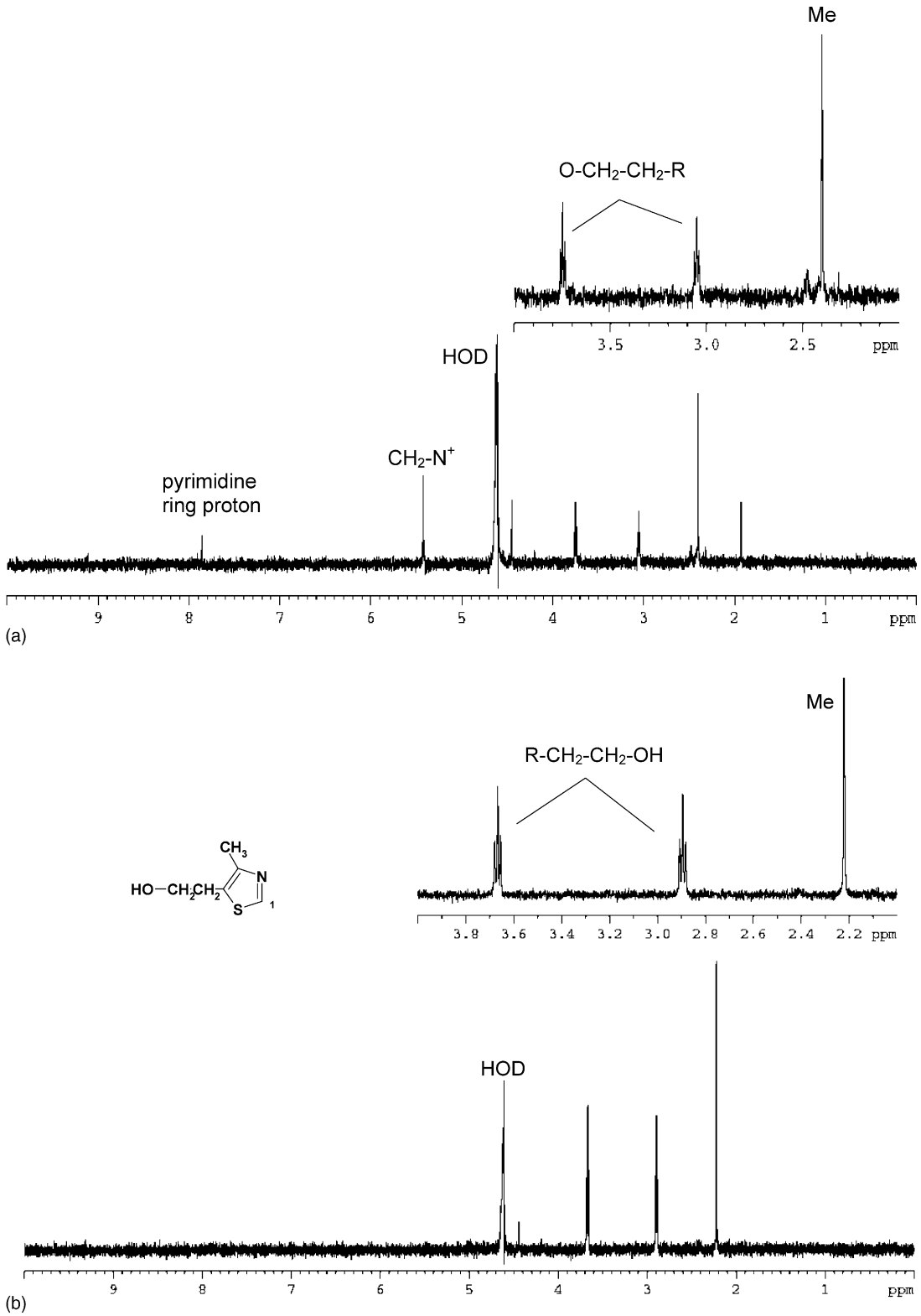

Fig. 2. On-line stop-flow ^{1}H NMR spectrum of peaks (t_R = 3.77 min and 8.55 min) in the chromatogram of thiamine separated on a PS-DVB column at 160 °C using a deuterium oxide phosphate buffer at pH 3.0. (a) Peak at 3.77 min. (b) Peak at 8.55 min attributed to 4-methyl-5-thiazole-ethanol.

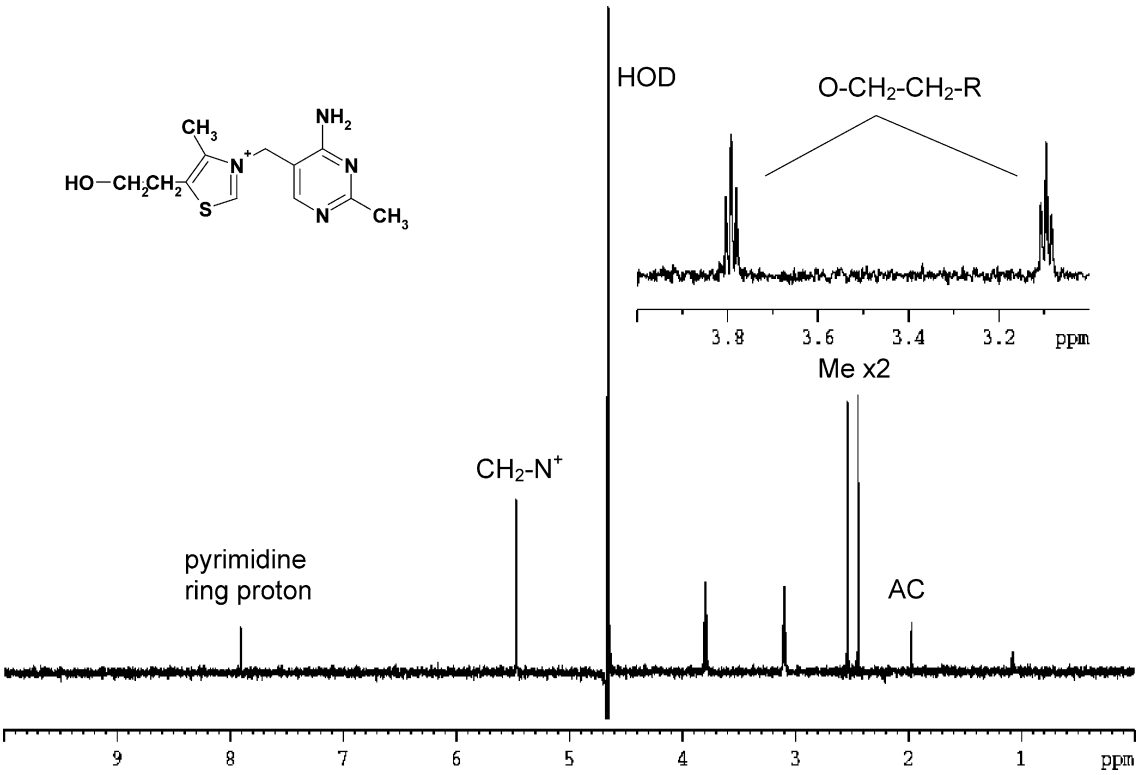

Fig. 3. On-line continuous-flow ^{1}H NMR spectrum of peak at t_R = 2.00 min in the chromatogram of thiamine separated on a PS-DVB column at 50 °C using a deuterium oxide phosphate buffer at pH 3.0.

water (labelled as HOD) contained in the D_2O mobile phase; a peak at 1.95 ppm, which was attributed to a residual trace of acetonitrile in the instrument from earlier studies; and a further contaminant peak at 3.2 ppm, which was assigned to a trace of residual methanol. The singlets at 2.35 and 2.45 ppm were assigned to the aryl methyl groups, and the singlets at 7.85 and 7.9 ppm to the aromatic ring protons. A complex envelope of signals was observed in the region of 3.4–5.2 ppm. By analogy with reported spectra of riboflavin and its derivatives [20–22], the resonances at 4.30, 3.83 and 3.78 ppm corresponded to the C-2′, C-3′ and C-4′ protons on the ribityl side chain, respectively. The multiplets at 3.58 and 3.72 ppm were attributed to the non-equivalent geminal C-5′ protons. Similarly, the multiplet at 5.01 ppm and the 'doublet' at 4.85 ppm (J = 12.8 Hz) were assigned to the C-1′ protons.

3.3. Thiamine

Examination of thiamine (vitamin B_1) was expected to prove difficult in superheated water as it is reported to be thermally and pH sensitive, and to decompose readily generating 4-methyl-5-thiazole ethanol and up to 60 other components [23–25]. On examination at 160 °C on a PS-DVB column using a deuterium oxide phosphate buffer (pH 3), UV spectrophotometric detection showed a broad tailing peak at 3.77 min and a second smaller tailing peak at 8.55 min. The ^{1}H NMR and MS spectra of these peaks were then obtained on-line and compared with the spectrum of a pure standard of thiamine in deuterium oxide under ambient conditions.

The peak at 3.77 min, captured in stop-flow mode, gave a ^{1}H NMR spectrum (Fig. 2a) which showed similarities to that of the thiamine standard, however, only one of the expected two methyl signals could be detected at 2.4 ppm. It also lacked the signal for the C-1 proton, adjacent to the thiazole quaternary nitrogen atom, which is observed at 9.7 ppm in the thiamine standard spectrum. Yet, the remaining protons were clearly present, comprising the pyrimidine ring proton (a singlet at 7.85 ppm), the singlet at 5.4 ppm for the CH_2–N^+-protons and the triplets at 3.75 and 3.05 ppm representing the –O–CH_2–CH_2–R-side chain. A small peak at 4.4 ppm was identified as a solvent impurity. In a previous study, the LC-NMR analysis of sulphamethazine, in superheated deuterium oxide at 160 °C, highlighted deuterium exchange of the methyl-protons on a pyrimidine ring [9]. Hence, the loss of the signal for one of the methyl groups, as observed in this study, suggests that deuterium exchange had also occurred here.

The mass spectral results of this peak were somewhat inconclusive as scanning through the chromatographic peak, ions at *m/z* 144 and 145 were detected initially, followed by ions at *m/z* 136, 137, 146, 147, and a cluster of ions at *m/z* 270–272. It was thus evident that the peak was not homogeneous, as the spectrum varied with increasing retention time. Direct injection of thiamine in deuterium oxide at pH 3, at ambient temperature, gave a molecular ion at *m/z* of 265 and fragment ion at *m/z* of 144, which corresponds to the molecular

ion of the parent and the thiazole ring, respectively, following exchange of the hydroxyl hydrogen with deuterium. The cluster of ions at *m/z* 270–272 in the superheated water separation suggests multiple deuterium exchange of five to seven protons.

The second smaller peak, eluting at 8.55 min, gave a stop-flow ^{1}H NMR spectrum (Fig. 2b), which suggested it was the thermal decomposition product, 4-methyl-5-thiazole-ethanol. Again, the spectrum lacked the signal for the C-1 proton, reported at 8.5 ppm in the standard spectrum of 4-methyl-5-thiazole-ethanol [26]. When a sample of the thiazole-standard was re-chromatographed under the same analysis conditions (160 °C), it gave a sharp peak with a similar retention time (8.27 min), with the corresponding ^{1}H NMR spectrum identical to that obtained in Fig. 2b. The on-line analysis of the thiazole-standard by LC-MS produced a molecular ion at an *m/z* of 147, corresponding to deuterium exchange of the hydroxyl- and C1-protons, and the presence of a deuterium adduct. Direct injection at ambient temperature produced a molecular ion at *m/z* of 146, confirming that an additional deuterium exchange had occurred in the heated LC system.

The tailing of the thiazole peak (8.55 min) at the higher temperature implied that continuous thermal decomposition was occurring during chromatography.

Comparison with previously reported separations of thiamine salts suggested that the superheated water conditions might be using unnecessarily high temperatures. For example, at ambient temperature thiamine had a relatively short retention time of about 5 min using 0.1 M perchloric acid–water (8:1) [27] as the mobile phase on a PS-DVB column.

Therefore, a repeat separation at 50 °C was carried out to test this hypothesis, again, using the PS-DVB column and deuterium oxide at pH 3.0. In this case, thiamine eluted as a single sharp peak after 2 min. No peak was observed for 4-methyl-5-thiazole-ethanol, suggesting that thiamine was stable under these conditions. The continuous-flow ^{1}H NMR spectrum of the thiamine peak (Fig. 3) now closely resembled that of a reference spectrum of thiamine in deuterium oxide at ambient temperature. Both included the signals for the two methyl groups at 2.45 and 2.55 ppm, and displayed the singlets for the pyrimidine ring proton (at 7.9 ppm) and the CH_2–N^+-protons (at 5.5 ppm), as well as the triplets at 3.8 and 3.1 ppm for the HO–CH_2–CH_2–R side-chain. However, the signal corresponding to the C-1 proton, expected at 9.6 ppm, was again absent from the spectrum, suggesting that the deuterium exchange was still occurring on-column even at 50 °C.

4. Conclusions

These studies demonstrated that hot or superheated water can be used to chromatograph complex, thermally labile, natural products, such as vitamins, provided that appropriate care is taken to ensure that the analyte is matched to the column type and that the minimum temperature required for elution is used. When detection is carried out by on-line ^{1}H NMR and mass spectrometry, using D_2O as the eluent, the potential for deuterium exchange must be taken into account when interpreting the spectra.

Acknowledgements

The authors thank the Government of Thailand for a studentship to O.C. and the Government of Bangladesh for a studentship to S.S.

References

[1] R.M. Smith, R.J. Burgess, J. Chromatogr. A 785 (1997) 49–55.
[2] D.J. Miller, S.B. Hawthorne, Anal. Chem. 69 (1997) 623–627.
[3] Y. Yang, A.D. Jones, C.D. Eaton, Anal. Chem. 71 (1999) 3808–3813.
[4] B.A. Ingelse, H.G. Janssen, C.A. Cramers, J. High Resolut. Chromatogr. 21 (1998) 613–616.
[5] B. Yan, J. Zhao, J.S. Brown, J. Blackwell, P.W. Carr, Anal. Chem. 72 (2000) 1253–1262.
[6] R.M. Smith, O. Chienthavorn, I.D. Wilson, B. Wright, Anal. Commun. 35 (1998) 261–263.
[7] R.M. Smith, O. Chienthavorn, I.D. Wilson, B. Wright, S.D. Taylor, Anal. Chem. 71 (1999) 4493–4497.
[8] O. Chienthavorn, R.M. Smith, Chromatographia 50 (1999) 485–489.
[9] R.M. Smith, O. Chienthavorn, S. Saha, I.D. Wilson, B. Wright, S.D. Taylor, J. Chromatogr. A 886 (2000) 289–295.
[10] S. Saha, R.M. Smith, E. Lenz, I.D. Wilson, J. Chromatogr. A 991 (2003) 143–150.
[11] D. Louden, A. Handley, S. Taylor, I. Sinclair, E. Lenz, I.D. Wilson, Analyst 126 (2001) 1625–1629.
[12] D. Louden, A. Handley, R. Lafont, S. Taylor, I. Sinclair, E. Lenz, I.D. Wilson, Anal. Chem. 74 (2002) 288–294.
[13] C. Smith, B.P. Jensen, I.D. Wilson, F. Abou-Shakra, D. Crowther, Rapid Commun. Mass Spectrom. 18 (2004) 1487–1492.
[14] J. van Schoonhoven, J. Schrijver, H. van den Berg, G.R.M.M. Haenen, J. Agric. Food Chem. 942 (1994) 1475–1480.
[15] F. Valls, M.T. Sancho, M.A. Fernández-Muiño, M.A. Checa, J. Agric. Food Chem. 47 (1999) 1067–1070.
[16] K. Johansen, P.O. Edlund, J. Chromatogr. 506 (1990) 471–479.
[17] C. Andres-Lacueva, F. Mattivi, D. Tonon, J. Chromatogr. A 823 (1998) 355–363.
[18] I.N. Papadoyannis, G.K. Tsioni, V.F. Samanidou, J. Liq. Chromatogr. Relat. Technol. 20 (1997) 3203–3231.
[19] G. Williamson, D.E. Edmondson, Meth. Enzymol. 122 (1986) 240–248.
[20] Spectral Data Base System. http://www.aist.go.jp/RIODB/SDBS/ Entry 2254. (accessed 16/4/2004).
[21] A.M. Edwards, A. Saldaño, C. Bueno, E. Silva, S. Alegría, Bol. Soc. Chil. Quím. 45 (2000) 423–431.
[22] F.J. Fraiz, R.M. Pinto, M.J. Costas, M. Ávalos, J. Canales, A. Cabezas, J.C. Cameselle, Biochem. J. 330 (1998) 881–888.
[23] S. Ebel, M. Herboth, A. Werner-Busse, Fres. Z. Anal. Chem. 320 (1985) 724–725.
[24] J.M. Ames, O. Hincelin, A. Apriyantono, J. Sci. Food Agric. 58 (1992) 287–289.
[25] M. Güntert, J. Brüning, R. Emberger, M. Köpsel, W. Kuhn, T. Thielmann, P. Werkhoff, J. Agric. Food Chem. 38 (1990) 2027–2041.
[26] C.J. Pouchert, J. Behnke, The Aldrich library of 13C and 1H FT NMR spectra, The Aldrich Chemical Company, Milwaukee, Wisconsin, 1993.
[27] Polymer Laboratories, 2001–2002 Catalogue, Issue 2, Church Stretton, UK.