SHORT PAPER

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Measurement of 4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl by high performance liquid chromatography–electrospray ionisation mass spectrometry†

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Reverse phase high performance liquid chromatography coupled to electrospray ionisation mass spectrometry and tandem mass spectrometry has allowed quantitative measurement of a stable nitroxide, 4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl (4-hydroxy-tempo), the latter with high sensitivity.

Keywords: 4-hydroxy- 2,2,6,6-tetramethyl-piperidine-N-oxyl (4-hydroxy-tempo)

4-Hydroxy-tempo (**A**, Fig. 1) belongs to a group of nitroxide radicals that are used predominantly as spin-labels for electron paramagnetic resonance (EPR) spectroscopy studies.[1,2] A similar group of nitroxide radicals, pyrroline-N-oxyl derivatives, are major products when relatively unstable free radicals are captured by 'spin-traps' such as 5,5-dimethyl-1-pyrroline-N-oxide (DMPO)[3] and 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO).[4] EPR measurements provide useful information about the environment of the nitroxide, more specifically the extent of the hydration of the N–O group, and correlation times (in the region 10^{-6}–10^{-11}s) particularly relevant to many biological systems.[1–2,5] However, only limited structural data is obtained for nitroxides by EPR, since the electron spin tends to be localised on the N-O group. In a recent report, a range of stable nitroxide derivatives was characterised by electrospray–ionisation mass spectrometry (ESI-MS).[6] In the present study, 4-hydroxy-tempo has been measured quantitatively by both ESI-MS and ESI tandem mass spectrometry (MS/MS) in multiple reaction monitoring (MRM) mode, the latter technique demonstrating particularly high sensitivity for the compound.

A commercially available sample of 4-hydroxy-tempo was introduced into the mass spectrometer via the integrated HPLC apparatus. The initial on-line HPLC step was found to be important since it separated out a number of impurities contained within the sample (data not shown). In accordance with previous observations, a major ion at *m/z* 172 was found in the ESI mass spectrum, attributed to the molecular ion (M^+, **B**, Fig. 1) and resulting from one-electron oxidation of the nitroxide.[6–8] In addition, both singly and doubly protonated species were also detected, *m/z* 173 ($MH^{+\cdot}$) and *m/z* 174 ($MH_2^{+\cdot}$) respectively. Figure 2 shows a plot of ion intensity (*m/z* 172) versus amount of 4-hydroxy-tempo (over a range 250 pg – 50 ng on column). M^+ demonstrates good linearity over this range ($R^2 = 0.9994$). Approximately 150 pg on column represents the limit of detection for 4-hydroxy-tempo using ESI-MS. In an attempt to improve the sensitivity of the method ESI with triple quadrupolar mass spectrometry (in MRM mode) was employed. A major product ion, suitable for MRM and derived from the molecular ion, was detected at *m/z* 140. Not observed previously,[6] it is believed to correspond to net loss of two methane molecules from M^+ (*i.e.* M-32, **C**, Fig. 1). Optimisation of the voltage of the collision cell allowed quantitation of 4-hydroxy-tempo by detection in MRM mode of the transition *m/z* 172 – 140. A 150-fold improvement in sensitivity (1 pg on column) over HPLC-ESI-MS was observed. The limit on sensitivity for the former is probably background signal from solvent molecule aggregation, with the latter in MRM mode giving a significant reduction in such "noise" and thus generating an apparent overall increase in sensitivity.[9]

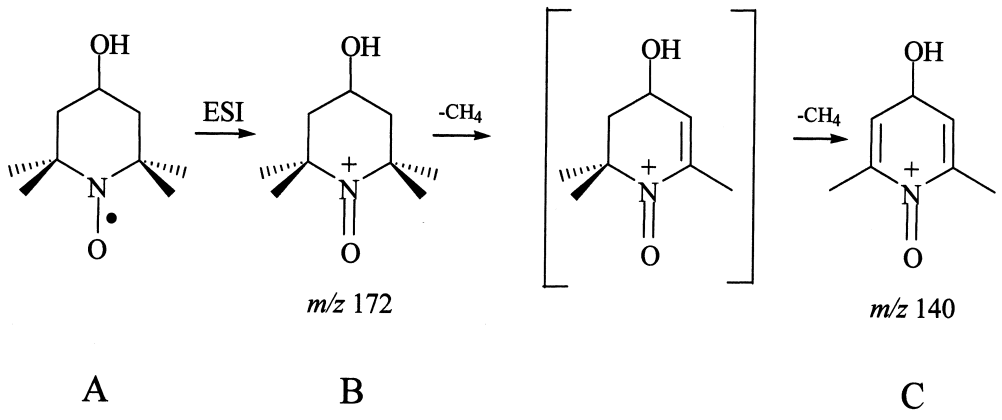

Fig. 1 Suggested mechanism for the formation of a fragment at *m/z* 140 (C) in the ESI-MS/MS spectrum of 4-hydroxy-tempo (A), generated from the molecular ion (B).

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† This is a Short Paper, there is therefore no corresponding material in *J Chem. Research (M).*

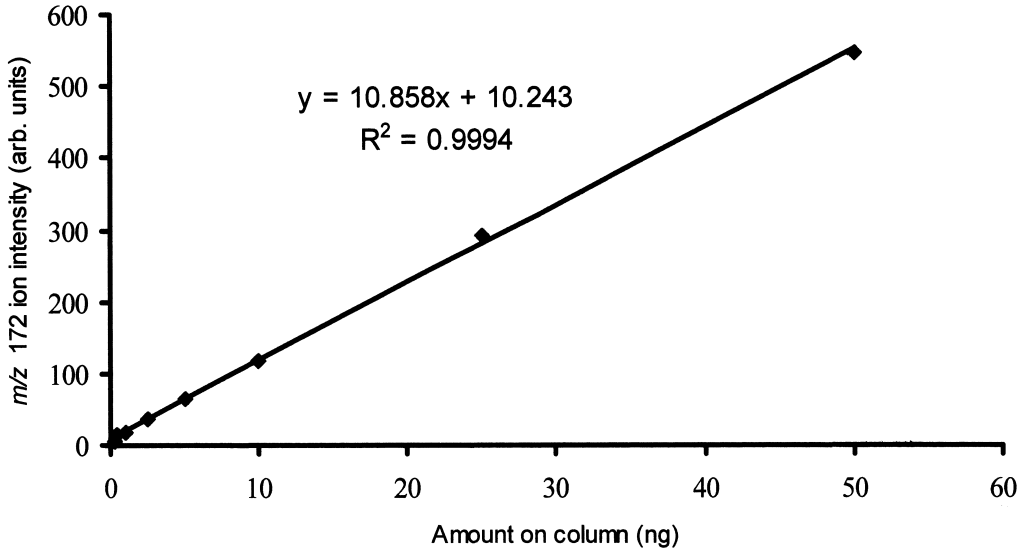

Fig. 2 Plot of ion intensity of B (*m/z* 172) versus amount of 4-hydroxy-tempo on column.

This result demonstrates that electrospray ionisation mass spectrometry, particularly tandem mass spectrometry, can detect stable nitroxides with high sensitivity. A good linear calibration curve ($R^2 = 0.9994$) was obtained over approximately three orders of magnitude demonstrating the quantitative capability of the techniques for these compounds. Thus, it is reasonable to conclude that HPLC-ESI-MS(MS) when applied to nitroxides, such as piperidine- and pyrroline-N-oxyl derivatives, will provide useful quantitative data that is both complimentary and supplementary to that of EPR spectroscopy.

Experimental

Solutions of 4-hydroxy-tempo were injected onto a C8 HPLC column (2.1 × 100 mm) at a flow rate of 300 µl/min. HPLC separations were carried out using a Waters Alliance 2795 system. The mobile phase initially consisted of approx. 95% water, 5% methanol and 0.1% formic acid. After 1 minute the methanol content was increased rapidly to 95%, and then after 5 minutes decreased to 5%. ESI-MS and ESI-MS/MS studies were conducted on Q-tof Ultima (time-of-flight) and Quattro Ultima (triple-quadrupole) mass spectrometers respectively (Micromass UK Ltd.). Nitrogen was used as the nebulising, drying, and cone gas. The flow rate was approximately 1000l/h for the nebulising and drying gas and 66l/h for the cone gas. For MS/MS measurements, M (*m/z* 172) was initially selected in the first quadrupole and the ion passed through a collision cell (collision gas argon) set at $2.5e^{-4}$ mTorr. In MRM mode the third quadrupole was set up to monitor the product ion at *m/z* 140. The source temperature was 150°C and the cone voltage 30V.

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