Pergamon

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 3, pp. 419–424, 1994
Elsevier Science Ltd
Printed in Great Britain
0731-7085/94 $6.00 + 0.00

Short Communication

Improvement in the characterization of minor drug metabolites from HPLC–NMR studies through the use of quantified maximum entropy processing of NMR spectra

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Keywords: *HPLC–NMR; quantified maximum entropy; metabolism; paracetamol.*

Introduction

In biochemical applications of NMR spectroscopy, it is sometimes necessary to compromise on the data acquisition regime, both in terms of achieved signal-to-noise ratio and in the spectral digital resolution, in order to preserve sample viability or because of the time scale of events being monitored. Likewise, in coupled HPLC–NMR applications, it is possible that such less than optimum acquisition conditions are necessary because of the desire to measure NMR spectra on-flow or because extended stopped-flow experiments, which can take several hours of data accumulation, can cause band-broadening and hence loss of chromatographic resolution. Under such circumstances, it is possible that advantages in terms of information content may be realized if the data is processed after acquisition. A standard approach in NMR spectroscopy is to apply weighting functions to the NMR free induction decay (FID) before Fourier transformation in order to improve the signal-to-noise ratio or the spectral resolution [1]. However, a probabilistic method called quantified maximum entropy has recently been developed. This can operate directly on the raw frequency domain spectrum making use of any prior knowledge and producing a solution containing the minimum structure consistent with the raw data [2]. Here the use of quantified maximum entropy data processing is demonstrated to improve the characterization of a minor metabolite of paracetamol found in human urine which had been separated and detected in an HPLC–NMR system [3].

Experimental

The details of the HPLC–NMR study of human urine to characterize the metabolism of paracetamol have been reported elsewhere [3]. The ^{1}H-NMR data were acquired using a Bruker AMX-500 spectrometer as described previously [3] and were processed with the quantified maximum entropy software, MemSys5 [4] on a SUN SPARCstation 2 fitted with an I860 accelerator board. The input linewidth and lineshape used in the data processing were optimized for the whole spectrum by following the output diagnostics of the MemSys5 software and adjusting these parameters to give the result with maximum probability.

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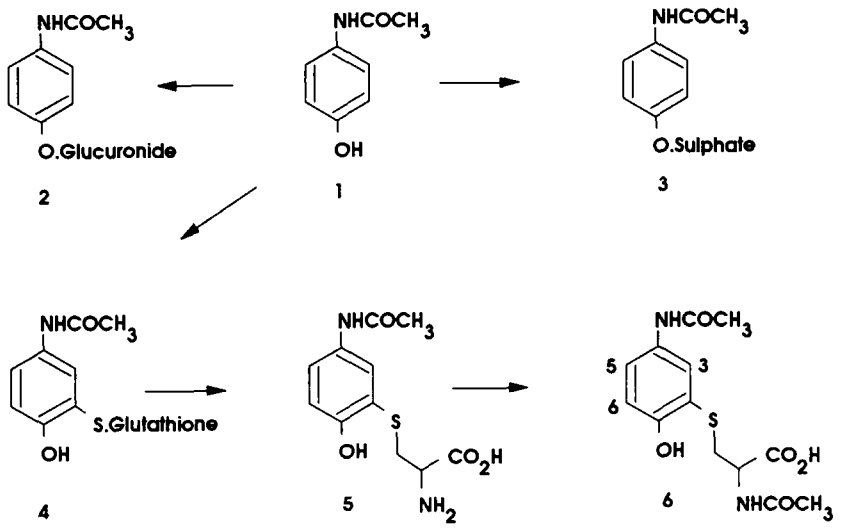

Figure 1
The metabolic transformations of paracetamol.

Results

The metabolic fate of paracetamol has been well characterized by conventional techniques and is illustrated in Fig. 1. The N-acetyl-cysteinyl conjugate (structure **6**) is a minor metabolite resulting from the metabolism of the adduct formed between glutathione and a reactive metabolite of paracetamol (see Fig. 1). The detection and identification of this minor metabolite of paracetamol in untreated human urine by NMR spectroscopy has been reported by Bales *et al.* [5] who used two-dimensional NMR at 400 MHz together with a synthetic standard compound to confirm the signal assignments.

This compound has also been detected in human and rat urine using coupled HPLC–NMR spectroscopy at 500 MHz with D2O/acetonitrile gradient elution and double NMR solvent suppression [3] and, with the use of a FID weighting function, it was possible to show that the signals were consistent with the structure **6**. Figure 2(a) shows the ^{1}H-NMR spectrum assigned to **6** measured in stopped-flow mode during the HPLC–NMR study. The N-acetyl signals appear at about δ2.05 and are not shown, being partially obscured by the suppressed acetonitrile solvent resonance. This spectrum has been processed with no line-broadening function and indicates the quality of the raw data. Figure 2(b) shows the same data processed with a line-broadening of 1.95 Hz, an estimate of the measured linewidth from Fig. 2(a), the so-called optimum filter which maximizes the signal-to-noise ratio. Important coupling constant information is heavily damaged by this process at the expense of the signal-to-noise improvement [1]. Figure 2(c) shows the result using the MemSys5 software obtained from the optimized input linewidth of 2.1 Hz and lineshape (100% Lorentzian).

Figure 3(a) shows an expansion of the raw data result for the methylene proton resonances of **6** plus signals arising from an endogenous substance which co-elutes with **6**. Figure 3(b) is the optimum MemSys5-processed result and Fig. 3(c) is this result convolved with the optimum lineshape function derived from the program diagnostics, this being essentially a match to the experimental spectrum without the noise.

Figure 4 shows the results on the individual proton resonances for **6**. From the quantitative nature of the algorithm it is possible to derive values for the chemical shifts and coupling

Table 1
^{1}H-NMR parameters for structure **6**

Proton	Chemical shift (δ)	Coupling splittings (Hz)
3	7.49	2.47 ± 0.04
5	7.29	2.5 ± 0.2; 2.6 ± 0.2 8.7 ± 0.2; 8.7 ± 0.2
6	6.96	8.7 ± 0.1
α	4.46	3.9 ± 0.5; 4.6 ± 0.4 7.2 ± 0.5; 7.9 ± 0.4
β	3.45*	4.3 ± 0.2; 4.5 ± 0.3 14.2 ± 0.3; 14.4 ± 0.3
β′	3.23*	8.0 ± 0.2; 8.0 ± 0.2 13.9 ± 0.2; 19.9 ± 0.2

Errors quoted are taken from the MemSys5 software and represent 1 standard deviation.
*, Assignments may be reversed.

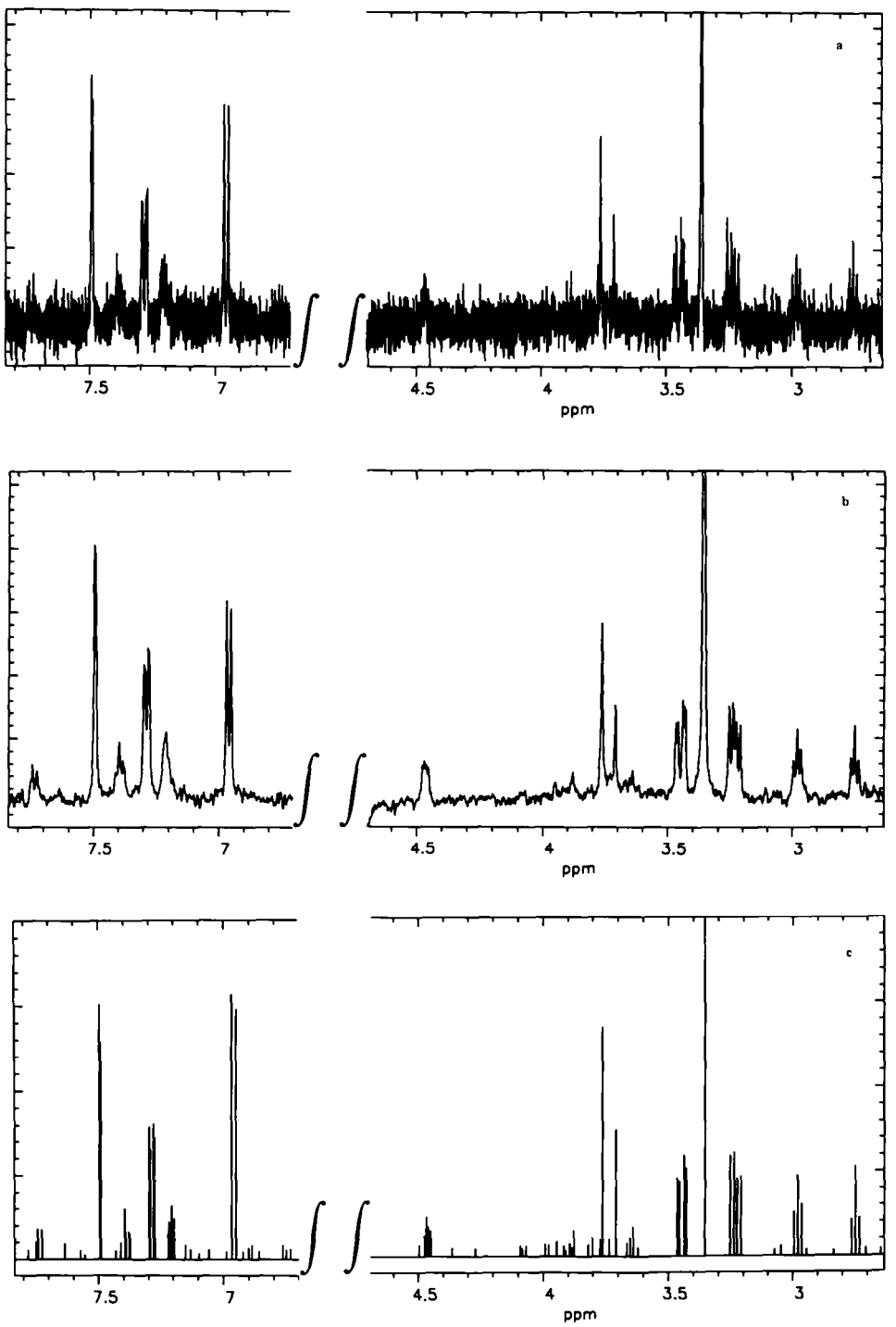

Figure 2
The 500 MHz ^{1}H-NMR spectrum of **6** measured in stopped-flow HPLC–NMR mode. (a) No FID weighting, (b) optimum filter of 1.95 Hz, (c) MemSys5 result.

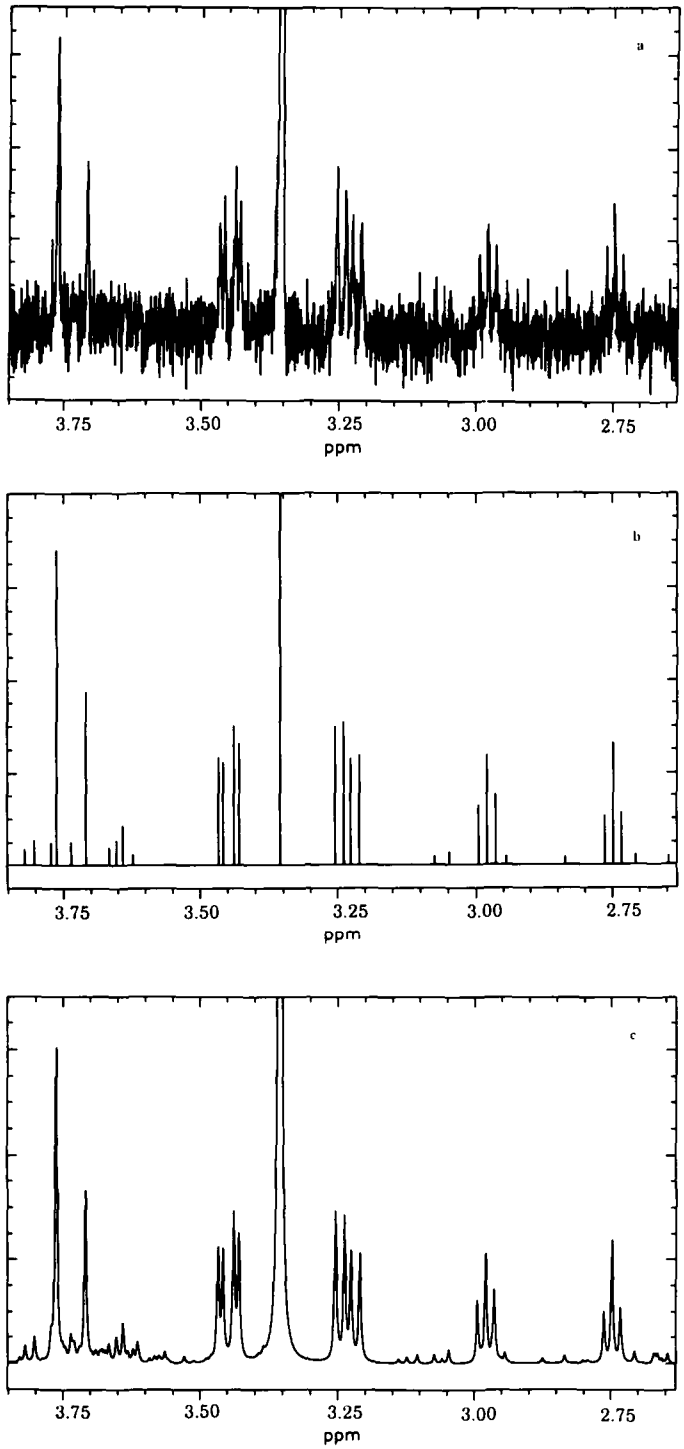

Figure 3
Partial 500 MHz ^{1}H-NMR spectrum, which includes the ββ′-CH_2 signals from **6**. (a) No FID weighting, (b) MemSys5 result, (c) MemSys5 result convolved with the optimum linewidth.

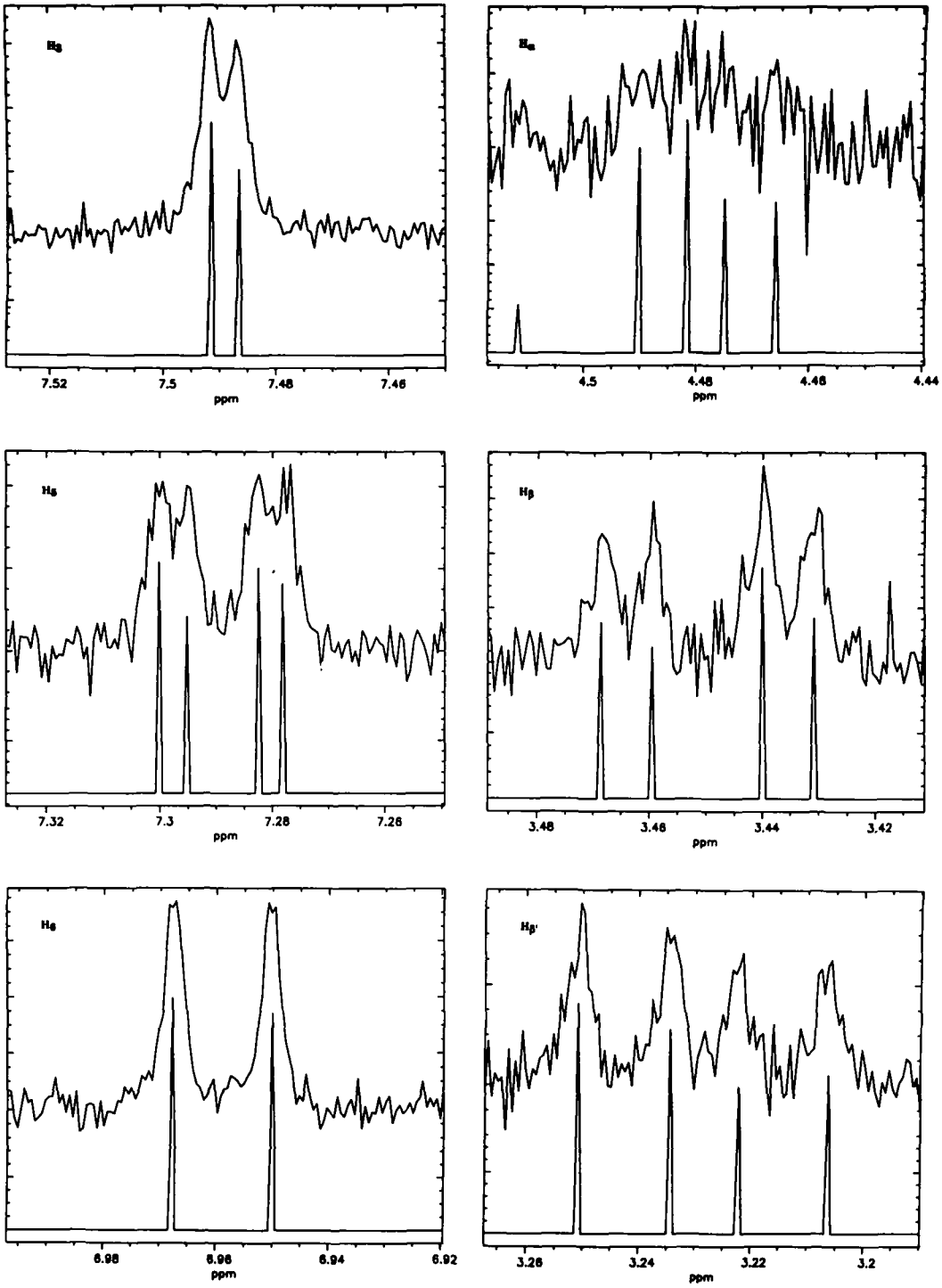

Figure 4
Expansions of the 500 MHz NMR spectrum of **6**. Individual proton resonances, showing raw data and MemSys5 result.

constants together with an estimate of the precision of the latter and these are listed in Table 1. These parameters provide a firm confirmation of the identity of **6**. The utility of the methodology is particularly apparent in the case of the resonance for the cysteinyl α proton which has its intensity reduced because of the suppression of the adjacent water resonance. At this lower signal-to-noise ratio, it proved difficult by conventional means to unambiguously determine the coupling constants.

Conclusions

Post-acquisition processing of high resolution NMR spectra using the quantified maximum entropy method promises to have significant benefits in situations where compromises on data gathering have to be made. This could include the study of unstable species, coupled HPLC–NMR spectra as shown here, or *in vivo* NMR spectroscopy where it is desirable to limit the animal/patient scanning time to be as brief as possible.

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[*Received for review 24 June 1993; revised manuscript received 20 August 1993*]