Directly coupled CZE-NMR and CEC-NMR spectroscopy for metabolite analysis: paracetamol metabolites in human urine

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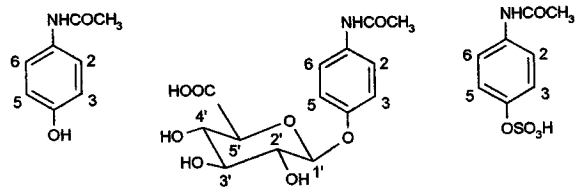
Direct coupling of NMR spectroscopic detection with both capillary zone electrophoresis (CZE) and capillary electrochromatography (CEC) was applied to the separation of metabolites of the drug paracetamol in an extract of human urine. Continuous-flow CZE-NMR and CEC-NMR allowed the detection of the major metabolites, the glucuronide and sulfate conjugates of the drug and the endogenous material hippurate. Identification of these substances was achieved by examination of individual rows of the NMR chromatogram and this also gave estimates of the detection limits. For CEC-NMR, spectra were also obtained in the stopped-flow mode including a two-dimensional TOCSY NMR experiment which afforded confirmatory evidence for paracetamol glucuronide. Characterisation of drug metabolites using NMR spectroscopy is therefore possible with nanolitre sample volumes.

Direct coupling of HPLC with NMR spectroscopy is now a routine commercially available technique which has proved useful in many areas of analytical chemistry, particularly for drug metabolite identification,[1,2] natural product derivatives[3–5] and peptide libraries.[6] The information content of such studies has been extended by the further parallel coupling with mass spectrometry.[7,8] More recently, extensions to the method have included capillary HPLC-NMR[9,10] and preliminary results using capillary zone electrophoresis (CZE)-NMR.[11–13] Overviews of NMR spectroscopy directly coupled with separation methods have recently been published.[14,15]

Capillary electrophoretic methods are well suited for the separation of charged species and therefore these techniques have a wide applicability in metabolic studies. Very recently, developments in CZE-NMR and capillary electrochromatography (CEC)-NMR have been reported using model compounds.[16] This methodology has now been evaluated in the important area of drug metabolite analysis using an extract of human urine containing the metabolites of the analgesic paracetamol in order to assess the feasibility of the techniques. A preliminary account of part of this work has been published.[17]

The metabolism of paracetamol is well established and has been studied extensively using NMR spectroscopy.[18,19] It is known that the two major metabolites are the glucuronide and sulfate conjugates of the phenolic hydroxyl group. The structures of paracetamol and the two major metabolites are shown here. More recently, the metabolism of paracetamol has been used as a model system to investigate the usefulness of both HPLC-NMR[20] and HPLC-NMR-MS technology.[8]



(1) (2) (3)

Experimental

The sample comprised 1.5 mL of human urine from a healthy male volunteer who had ingested 1 g of paracetamol with urine collection 3 h later. Partial purification of the metabolites was obtained by passing the urine down a 500 mg C_{18} solid phase extraction cartridge (Bond Elut, Jones Chromatography, Hengoed, UK). The cartridge had previously been conditioned with methanol (5 mL) and 0.1 M HCl (1 mL). The bulk of the polar endogenous organic material and salts was removed by washing with 0.1 M HCl (1 mL). The fraction containing the paracetamol metabolites was washed off with 100% methanol and evaporated to dryness. The material was reconstituted in 30 μL of MeOD–D_2O (1 + 2) for the CZE separation and into 90 μL of D_2O for the CEC separation.

Conventional CZE separation was carried out using an HP^{3D}CE instrument (Hewlett-Packard, Waldbronn, Germany) and buffer conditions were investigated to provide optimum separation. In this case, a diode-array UV detector set at 254 nm

was used with a buffer of 20 mM sodium formate at pH 7 and an applied voltage of 10 kV employing a 75 μm id fused silica capillary of length 38.5 cm.

For direct coupling to NMR, a modular CZE System 100 (Grom, Herrenberg, Germany) and a Sykam (Gilching, Germany) pump were used. The arrangement for the CZE-NMR experiments used a capillary of 80 μm id and 2 m total length with a 1 m effective length to the detection cell. This detection unit was inserted directly into a glass tube around which the NMR detector coil was constructed as described previously.[16] The detection volume for NMR detection was approximately 400 nL comprising a length of 9 mm. For CEC-NMR experiments, a 250 μm id, 360 μm od capillary of length 20 cm was coupled directly to the detection capillary. This was packed with Gromsil ODS 0 AB 5 μm (Grom) and separation was effected using a 2 mM borate buffer–acetonitrile eluent (80 + 20) with an applied voltage of 20 kV and a pressure of 14 bar. An 8 nL sample was used for CZE-NMR and an injection volume of 500 nL was used for CEC-NMR. Performance details of the CZE-NMR and CEC-NMR instruments have been given earlier.[16]

In directly coupled CZE-NMR and CEC-NMR experiments we used a Bruker AMX-600 NMR spectrometer operating at 600.13 MHz and 300 K for ^{1}H NMR spectroscopy. For continuous-flow CZE-NMR experiments, 70 increments were collected using accumulations of 64 FIDs per row into 8K data points and a spectral width of 6024 Hz. A further relaxation delay of 0.8 s was used to avoid saturation. The data were multiplied by an exponential factor equivalent to a line broadening of 5 Hz, zero-filled by a factor of 2 and then Fourier transformed. For continuous-flow CEC-NMR, the NMR parameters were identical except that the relaxation delay was increased to 1 s and only eight scans per increment were acquired. In addition, using CEC-NMR, a stopped-flow NMR spectrum was obtained for the paracetamol glucuronide metabolite for which a two-dimensional total correlation (TOCSY) spectrum was also acquired. For the TOCSY spectrum, a total of 144 FIDs were accumulated for each of 256 increments into 4096 data points using a spectral width of 4716 Hz and an acquisition time of 0.44 s. The data were multipled by apodisation functions of 3 Hz in both domains and zero-filled prior to Fourier transformation.

Results

The direct coupling of HPLC with NMR spectroscopy is now well established but only recently have studies been reported concerning the direct coupling of capillary HPLC with NMR spectroscopy and the coupling of CZE with NMR.[11,12,16] The experimental set-up used here was reported by Pusecker *et al.*[16] The injection device of the CZE system was located directly under the magnet. The requisite volume of liquid was loaded electrokinetically on the CZE capillary or by applying pressure to the CEC column. The CEC column was coupled to the detection capillary by a PTFE tube outside the NMR probe. By this means it was possible to switch from CZE to CEC without removing the NMR probe or dismantling the detector assembly.

Using this approach, the separation of paracetamol metabolites from an extract of human urine was attempted. Initially, conventional CZE separation with UV detection was used (conditions as given in the Experimental section) in order to optimise the separation conditions. The UV-detected separation is shown in Fig. 1 and two major peaks not normally found in control urine were detected at 3.6 and 4 min (I and II). Also, a third component gave a sharp peak at 4.9 min (III).

The optimised separation conditions were then used in a directly coupled CZE-NMR experiment with detection of the NMR response carried out in the continuous-flow mode. Thus, successive ^{1}H NMR spectra were collected for 94 s accumulations for 110 min. The result, which is viewed as a contour plot with CZE separation time on the vertical axis and the NMR chemical shift on the horizontal axis, has been shown previously.[17] The individual rows taken from the continuous-flow CZE-NMR experiment are shown in Fig. 2.

A number of peaks are spread throughout the contour plot and these arise from the singlet resonance of formate from the buffer at δ8.4, residual water in the D_2O buffer at δ4.7 and a small amount of glycine which remained from earlier use of a glycine-containing buffer at δ3.5. In addition, at separation times of 49 and 75 min, sets of peaks related to paracetamol can be observed. The peak eluting at 49 min can be assigned to paracetamol glucuronide (**2**) based on the aromatic proton chemical shifts of δ7.2 (H2, H6) and δ6.8 (H3, H5) and the additional peaks from the protons of the glucuronide moiety at δ4.2 (H5′) and δ3.8 (H2′, H3′ and H4′). The *N*-acetyl methyl group singlet signal is seen at δ2.1. The resonance from the anomeric H1′ proton of the glucuronide ring is below the threshold in the contour plot but is just visible on examination of the appropriate individual row in the NMR chromatogram as shown in Fig. 2(a). Also, the compound eluting at 75 min shows paracetamol-related resonances with aromatic proton peaks at

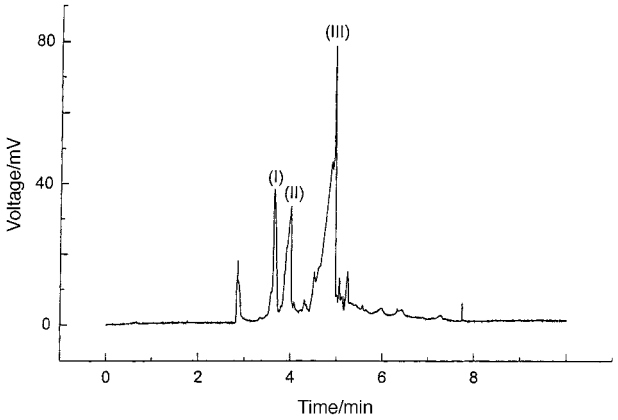


Fig. 1 CZE separation of the human urine extract using UV detection at 254 nm.

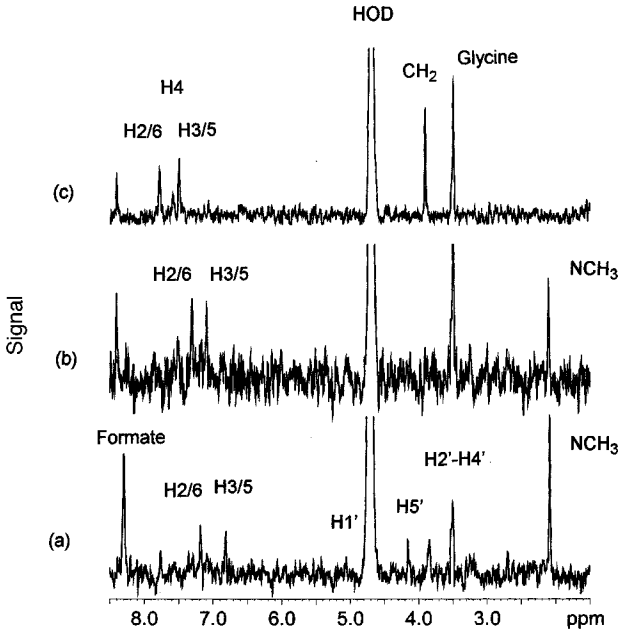


Fig. 2 Single rows extracted from the continuous-flow CZE-NMR chromatogram shown earlier:[17] (a) paracetamol glucuronide (**2**); (b) paracetamol sulfate (**3**); and (c) the endogenous material hippurate.

δ7.3 and δ7.1 consistent with paracetamol sulfate (**3**). This also displays the *N*-acetyl singlet at δ2.1 as expected and the spectrum taken from the continuous-flow run is shown in Fig. 2(b). Finally, a third component is detected in the continuous-flow CZE-NMR experiment at a time of 93 min and this is consistent with the endogenous compound hippurate (the glycine conjugate of benzoate) found in human urine at millimolar concentrations. This shows the expected aromatic proton resonances at δ7.8 (H2, H6), δ7.6 (H4) and δ7.5 (H3, H5), together with a singlet at δ3.9 from the glycyl methylene group as also shown in Fig. 2(c).

Directly coupled CEC-NMR spectroscopy was also achieved using the same sample. The result using the conditions described in the Experimental section has also been shown earlier.[17] This was again a continuous-flow experiment with the NMR data being collected in real time as the separation occurred. The CEC-NMR result is similar to that from the CZE-NMR experiment with the glucuronide conjugate (**2**) eluting first at 39 min, followed by the sulfate (**3**) at 45 min and then hippurate at 55 min. The NMR signal-to-noise ratio is much higher in the CEC-NMR experiment reflecting the increased amount of sample in the NMR detector.

The individual ^{1}H NMR spectra taken from the appropriate rows of the on-flow NMR chromatogram can be assigned as follows. The ^{1}H NMR spectrum corresponding to paracetamol glucuronide (**2**) has resonances from H2/H6 at δ7.4, from H3/H5 at δ7.1 and the glucuronide signals at δ5.2 (H1′), δ4.2 (H5′) and δ3.7 (H2′, H3′ and H4′) and the *N*-acetyl methyl singlet at δ2.2. Similarly, the ^{1}H NMR spectrum of paracetamol sulfate (**3**) has the aromatic proton signals at δ7.3 (H2/H6) and δ6.9 (H3/H5) and the acetyl methyl resonance at δ2.2. Finally, the spectrum of hippurate has aromatic proton resonances at δ7.8 (H2/H6), δ7.6 (H4) and δ7.5 (H3/H5) with the glycyl methylene signal at δ4.2.

The separation was repeated and the flow was halted at the retention time corresponding to the glucuronide metabolite (**2**). This allowed the measurement of a spectrum with a much improved signal-to-noise ratio. This is shown in Fig. 3. By measuring the spectrum in the stopped-flow mode it was possible to acquire more transients and hence improve the signal-to-noise ratio and digital resolution. This allowed the resolution of spin–spin coupling patterns which further confirmed the 1,4-substitution pattern of the aromatic ring and the identity of the glucuronide ring. In addition, a two-dimensional TOCSY spectrum was acquired. This required an overnight data collection period and is also shown in Fig. 3. The normal spectrum is represented as contours along the diagonal and cross-peak contours indicate those protons which are coupled to each along an unbroken chain of couplings. Hence the coupling between the aromatic protons is clearly observable as is the spin coupling connectivity within the glucuronide ring (both shown by dotted lines).

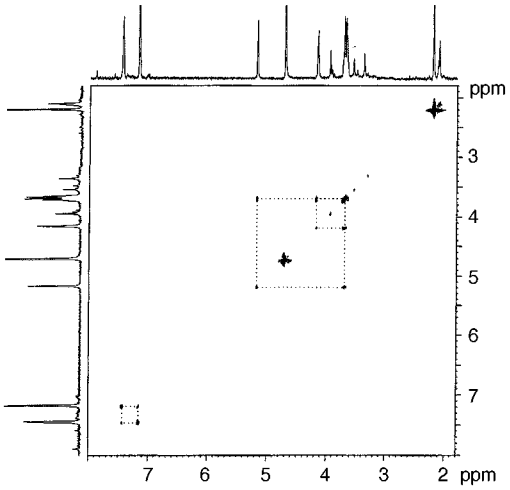


Fig. 3. Stopped-flow CEC-NMR spectra of paracetamol glucuronide (**2**): the one-dimensional ^{1}H NMR spectrum is shown vertically and horizontally and the two-dimensional ^{1}H–^{1}H TOCSY NMR spectrum is shown as a contour plot. The one-dimensional NMR spectrum is also plotted vertically for convenience.

For CZE-NMR, based on the metabolite concentration in the urine and given the low NMR signal-to-noise ratio shown in Fig. 2, it is estimated that approximately 10 ng of each metabolite were detected. The CEC-NMR spectra showed a much better signal-to-noise ratio, because of the possibility of sample preconcentration and a higher loading capacity. Here the NMR signal-to-noise ratio is much higher and leads to the extrapolation that detection limits in the low nanogram range are also possible. As a consequence, CEC-NMR is likely to become a useful technique for the identification of drug metabolites. Although CZE-NMR remains a technically difficult procedure, it has potential for the identification of small, by conventional NMR standards, amounts of analytes.

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