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ARTICLE *in* ANALYTICAL CHEMISTRY · JANUARY 1997

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Combined HPLC, NMR Spectroscopy, and Ion-Trap Mass Spectrometry with Application to the Detection and Characterization of Xenobiotic and Endogenous Metabolites in Human Urine

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The direct coupling of HPLC with NMR spectroscopy has been extended by splitting the HPLC eluent after conventional UV detection and sending part to a NMR spectrometer and part to an ion-trap mass spectrometer in a "triple-hyphenated" HPLC−NMR−MS system. Combined UV, ^{1}H NMR, and positive-ion electrospray MS detection was achieved in the continuous-flow mode using whole human urine from a subject dosed with acetaminophen. By means of HPLC−NMR−MS, the structural information available from the complementary spectroscopic techniques provided rapid confirmation of the identity of the acetaminophen glucuronide and sulfate metabolites, together with a number of endogenous metabolites. In particular, the HPLC−NMR−MS approach allowed the unequivocal identification of phenylacetylglutamine in human urine, an endogenous metabolite not previously observed in ^{1}H NMR spectra of urine because of extensive overlap with resonances from other metabolites. The analytical advantages and complementarity of NMR and MS techniques in direct hyphenation with HPLC are discussed. The new technique of HPLC−NMR−MS will provide the scope for more comprehensive and fully automated analysis of biofluids and other complex mixtures than was previously available from single hyphenation of these instruments.

The recent development of practical, directly coupled HPLC−NMR systems has enabled the direct analysis of complex mixtures of both synthetic and biological origins.[1−3] In particular, there have been many effective applications of this technology for the analysis and structural characterization of both drug-related and endogenous metabolites in biological fluids and cell extracts.[4−11] Several different NMR-active nuclei can be used to monitor chromatographic processes, including ^{1}H, ^{2}H, ^{31}P, and ^{19}F.[9−11] In principle, other nuclei could also be used, such as ^{13}C NMR for labeled metabolites, but in most cases the majority of the structural information obtainable from HPLC−NMR has been derived from ^{1}H NMR spectral measurements, with other nuclei providing a useful NMR-active handle or "radiophore" with which to determine the retention time of the label in the complex mixture.[9−11] In certain circumstances, the ^{1}H NMR spectrum is insufficient on its own to provide information that will fully characterize a metabolite. This is obviously the case where analytes contain functional groups that are deficient in protons or where the protons can readily chemically exchange with the solvent, the signals thus being broadened beyond detection.[12] Such problems arise with certain common classes of drug metabolite, e.g., ether sulfate conjugates of hydroxyl groups formed during phase II metabolism. These problems would also preclude the direct detection of phosphates and N-oxides by ^{1}H NMR, and, of course, the presence of heteroatoms with poor or nonexistent magnetic properties (such as chlorine) are also "NMR-silent". NMR spectroscopy cannot provide direct information on molecular weight, and therefore it is often used in conjunction with mass spectrometry (MS). One possible solution to such problems in the development of a "universal" detector structure characterization package for HPLC-based separations is the further direct hyphenation of the HPLC−NMR system to a mass spectrometer to give an HPLC−NMR−MS system. The practicality of this

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S0003-2700(96)00646-4 CCC: $12.00

configuration has recently been shown by Pullen et al.[13] for the analysis of a mixture of three related triazoles present in a simple solution in similar proportions. For the analysis of biological matrices such as biofluids, the triple configuration needed for effective metabolite characterization needs careful selection and interfacing to overcome some of the mutual incompatabilities of HPLC−NMR and HPLC−MS systems, in particular the selection of eluents. In general, HPLC−NMR methodologies are robust in cases where the solvent signals can be effectively supressed and do not interfere with the signals of the analytes. In the case of HPLC−MS, the primary requirement is that the analytes ionize effectively so that they can be accelerated through the mass spectrometer. Ionization propensities vary considerably according to solvent, spectrometer type (ionization source), and complex matrix effects. NMR detection is largely immune to variations in the matrix except in cases where paramagnetic ion concentrations are high, which is rare in biological samples.

In the present study, we show for the first time the hyphenation of an HPLC−NMR system with a parallel on-line ion-trap multipole mass spectrometer, applied to the separation and characterization of drug and endogenous metabolites in a complex biological matrix, i.e., human urine.

The metabolism of acetaminophen has been used as a test sytem because the metabolism of this substance in humans is well documented,[14,15] and we have also recently used it to evaluate directly coupled HPLC−NMR alone.[16] Acetaminophen is metabolized principally to glucuronide and sulfate conjugates, which are excreted predominantly in the urine, but a number of other minor metabolites are also formed.[14,15] The structures of acetaminophen and its sulfate and glucuronide conjugates are given in Figure 1. Urine also contains a variety of endogenous metabolites, and we wished to investigate the utility of direct characterization of these using HPLC−NMR−MS.

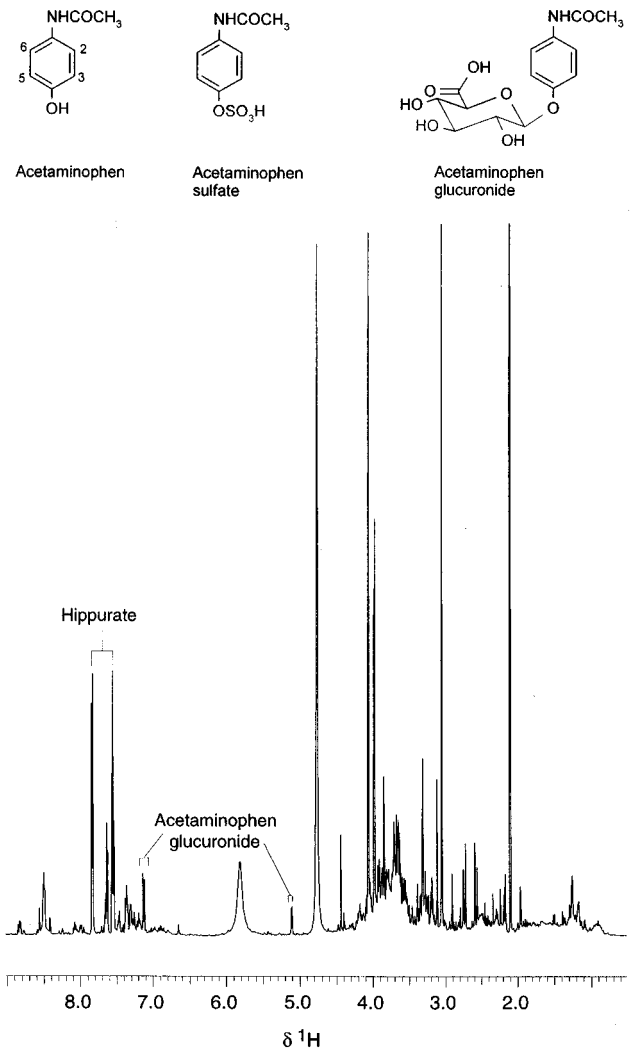

Figure 1. 500 MHz ^{1}H NMR spectrum of the whole human urine used for HPLC−NMR−MS analysis. Assignments are as marked.

EXPERIMENTAL SECTION

Human urine was obtained from a healthy male volunteer 0−4 h after the oral administration of 500 mg of acetaminophen. The urine (5 mL aliquot) was freeze-dried and reconstituted in 1 mL of D_2O/acetonitrile-d_3 (95:5 v/v). Typically, 50 μL of this sample was injected onto the HPLC column. The HPLC instrument was a Hewlett-Packard 1050 series chromatograph connected to a Bruker BPSU-12 collector using a Waters Symmetry C18 Spherisorb column (3.9 mm × 150 mm) with 3 μm particles. Separation was effected at 24 °C using a flow rate of 1.0 mL/min with gradient elution, starting at 100% D_2O (containing non-deuterated trifluoroacetic acid) at pH 2/0% acetonitrile-d_3, increasing to 50% acetonitrile-d_3/50% D_2O over 30 min, and holding at this proportion for a further 10 min. Chromatographic detection was by UV at 210 nm, by ^{1}H NMR spectroscopy, and by mass spectrometry. The chromatography was controlled by Bruker Chromstar software. Following UV detection of the chromatographic fractions, the eluent was split in a ratio of approximately 95:5, with transfer times of 29 s to the NMR probe and 45 s to the MS inlet. The major proportion was directed to the flow probe of the NMR spectrometer, with a cell volume of 120 μL. The NMR spectra were measured at 500.13 MHz using a Bruker DMX-500 spectrometer. For the continuous-flow HPLC detection, successive NMR spectra were acquired automatically, with each spectrum comprising the summation of eight transients with an acquisition time of 0.48 s into 4096 data points. The residual water resonance was suppressed using a selective sinc pulse applied for six cycles of 100 ms each, thereby giving a total recycle time of 1.08 s. The data were zero-filled by a factor of 4 and multiplied by a line-broadening function of 0.6 Hz to improve the signal−noise ratio before Fourier transformation. Chemical shifts were referenced to acetonitrile-d_2 at δ 2.0 The minor proportion (5%) of the eluent was simultaneously directed to the inlet of a Finnegan MAT LCQ ion-trap multipole mass spectrometer. Ionization was by positive-ion electrospray, and mass spectra were acquired in real time, up to mass m/z = 600. In addition, tandem experiments utilizing MS−MS studies to identify fragment ions were carried out in the positive-ion mode.

RESULTS

The 500 MHz ^{1}H NMR spectrum of the whole human urine is shown in Figure 1. This complexity of biofluid NMR spectra is such that, on horizontal and vertical expansion, several thousand

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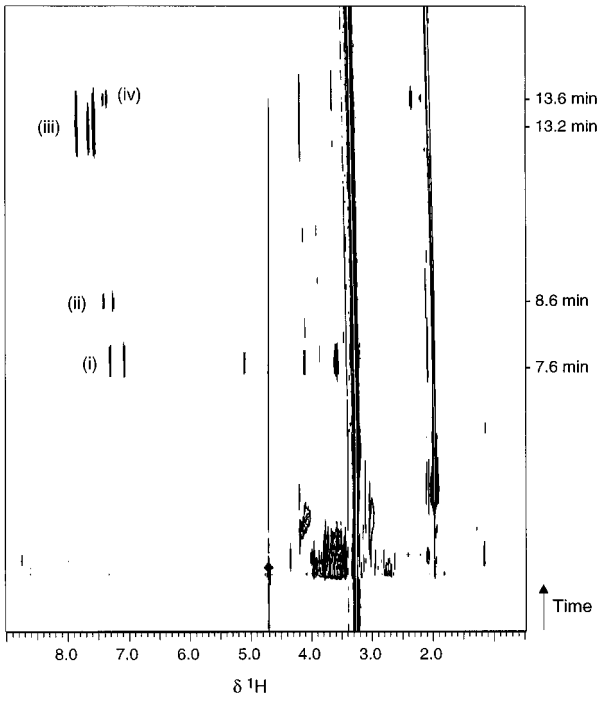

Figure 2. 500 MHz ^{1}H NMR continuous-flow-detected chromatogram of human urine following administration of acetaminophen. The horizontal axis represents ^{1}H NMR chemical shifts, and the vertical axis denotes retention time. The eluting peaks are shown as contours.

peaks are observed from hundreds of endogenous metabolites.[17] In addition, in this case, the urine contained acetaminophen metabolites, and these are expected to comprise principally the phenolic glucuronide and the sulfate conjugates.[14,15] However, only certain NMR resonances of the glucuronide metabolite were clearly visible, as indicated. This sample was subjected to a continuous-flow HPLC−NMR study in which ^{1}H NMR spectra were accumulated rapidly as the HPLC eluent flowed through the NMR probe. As well as obtaining clean NMR spectra and mass spectra for the acetaminophen metabolites, a number of endogenous species were also detected. Figure 2 shows the continuous-flow HPLC−NMR chromatogram obtained from human urine after the subject had been dosed with acetaminophen. A corresponding control urine was not measured, as the principal metabolites of acetaminophen have been characterized using HPLC−NMR alone.[16] In continuous-flow HPLC−NMR, the ^{1}H NMR chemical shifts are on the horizontal axis, the chromatographic retention time is on the vertical axis and the NMR intensity data are visualized as a contour plot. The line at about δ 4.7 arises from residual HDO in the D_2O solvent and has been suppressed by the use of selective irradiation at this point. The band at about δ 2.0, which changes its chemical shift as the eluent composition changes, arises from residual acetonitrile-d_2 in the acetonitrile-d_3 solvent. Normally in HPLC−NMR, acetonitrile itself would have been used, but in this case acetonitrile-d_3 was employed in order to alleviate detection difficulties for the N-acetyl groups of acetaminophen metabolites. The large band at about δ 3.3, which also changes its chemical shift as a function of time, is due to methanol contamination from an unknown source. However, this had no effect on the interpretation of the results. Many of the endogenous components in urine are highly polar and have very

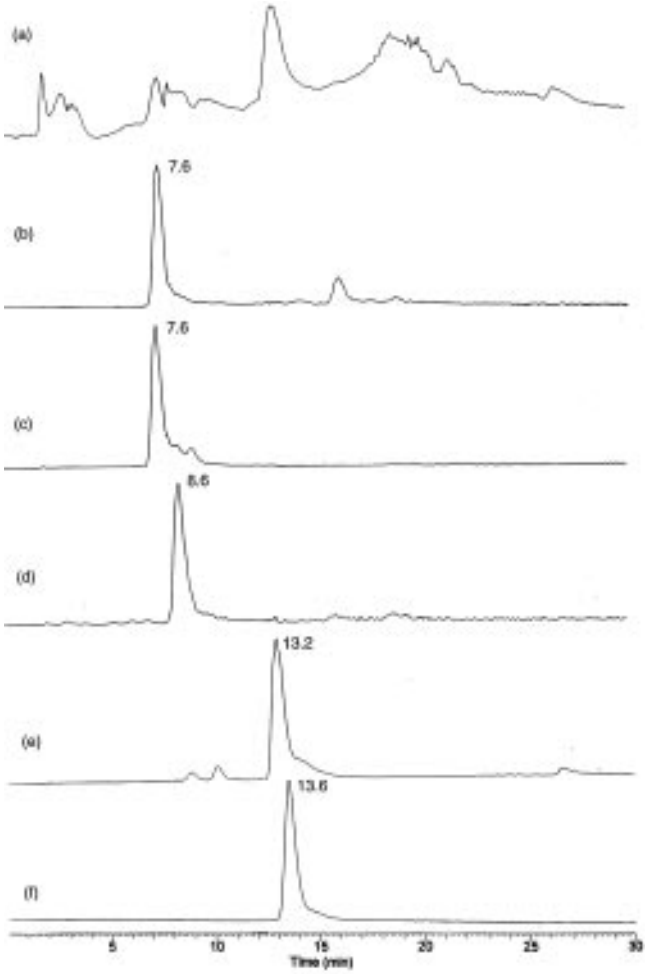

Figure 3. Continuous-flow MS chromatogram of human urine following administration of acetaminophen using electrospray ionization in positive-ion mode: the vertical scale is relative intensity. (a) Total ion current trace. (b−f) Reconstructed ion chromatograms for specific ions: (b) m/z = 334, (c) 155, (d) 235, (e) 183, and (f) 270.

short retention times. These give rise to the series of bands which appear at the start of the chromatogram.

Figure 3 shows the MS results obtained concurrently with the NMR data. Figure 3a is the total ion current (TIC) in positive-ion mode. From this are extracted MS chromatograms based on selected ions, and these are shown in Figure 3b−f. Since fully deuterated solvents were used for the HPLC, all species detected have all exchangeable hydrogens exchanged for deuterium. Thus, the expected m/z values now become those due to $[M + D]^+$, where M includes all exchangeable hydrogen-to-deuterium substitutions.

Following the initial elution of the polar endogenous metabolites, a number of other components are eluted, and the first of these appears at t_r = 7.6 min (labeled i in Figure 2) and can be shown to be acetaminophen glucuronide. Thus Figure 4a shows the ^{1}H NMR spectrum obtained by extracting the row in the NMR chromatogram which corresponds to this retention time. The aromatic protons appear as an AA′BB′ spin system at the previously characterized chemical shifts of δ 7.30 (H2,H6) and 7.07 (H3,H5). The glucuronide moiety H1′ proton gives a doublet at δ 5.11, H5′ has a resonance at δ 4.10, and the other glucuronide protons appear in a complex band near δ 3.6. The singlet from the N-acetyl group at δ 2.07 is visible because of the use of deuterated solvent. The identity of the glucuronide is confirmed

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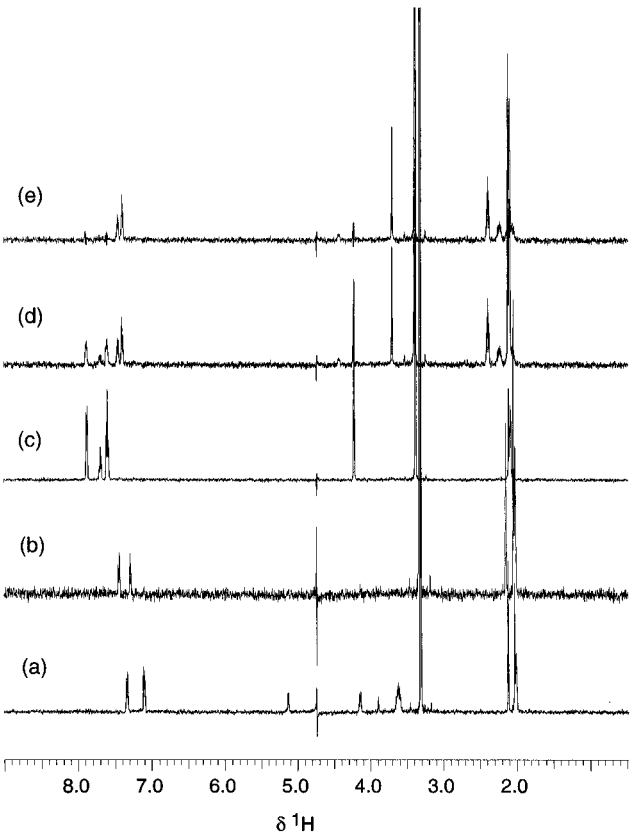

Figure 4. 500 MHz ^{1}H NMR spectra obtained by extracting appropriate rows from the continuous-flow NMR chromatogram shown in Figure 2: (a) $t_r = 7.6$ min, acetaminophen glucuronide; (b) 8.6 min, acetaminophen sulfate; (c) 13.2 min, hippurate; (d) 13.6 min, phenylacetylglutamine and hippurate; and (e) 13.6 min with subtraction of hippurate resonances.

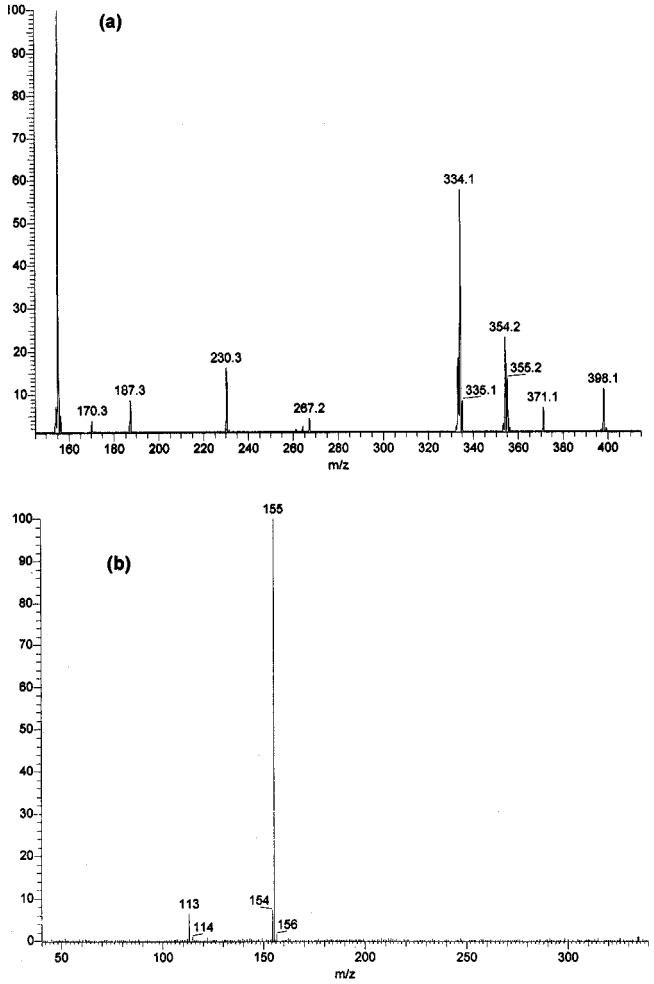

Figure 5. (a) Positive-ion mass spectrum of acetaminophen glucuronide obtained during continuous-flow HPLC−NMR−MS detection and (b) the MS−MS spectrum of the $m/z = 334$ ion. Adducts $(M + ND_4)^+$ and $(M + ND_4 + ACN\text{-}d_3)^+$ are seen at $m/z = 354$ and 398, respectively. There is evidence from the multiplet nature of some of the peaks of incomplete deuteration of exchangeable hydrogens.

by the MS chromatogram corresponding to $m/z = 334$, which also appears at $t_r = 7.6$ min (Figure 3b). This corresponds to the molecular ion $[M + D]^+$ for the glucuronide with, as expected, the NH group, three OH groups, and the COOH group all deuterated. Additional confirmation of the acetaminophen-related nature of this component is given by the MS chromatogram shown in Figure 3c, corresponding to $m/z = 155$, and which is the ion for acetaminophen itself caused by fragmentation of the glucuronide. Finally, the glucuronide identity was further confirmed by an MS−MS experiment obtained during a second chromatographic elution in which the fragment ions of the $m/z = 334$ ion were detected. The MS is given in Figure 5a and shows an ion at $m/z = 334$ corresponding to the glucuronide with fully deuterated exchangeable hydrogens. In addition, ions are seen for adducts to the glucuronide, and the multiplet nature of all of these peaks indicates incomplete deuteration of exchangeable hydrogens. The MS−MS arising from the $m/z = 334$ ion is shown in Figure 5b, indicating the loss of the glucuronic acid moiety to give acetaminophen itself at $m/z = 155$. There is a further loss from the acetyl group of 42 (ketene) to give $m/z = 113$.

At a retention time of 8.6 min, a second acetaminophen-related material was eluted, as can be seen in Figure 2 (labeled ii). The NMR spectrum corresponding to this row of the NMR chromatogram is shown in Figure 4b. This shows the AA′BB′ NMR pattern at shifts of δ 7.41 and 7.25 consistent with the known shifts for the H2,H6 and H3,H5 protons, respectively, of acetaminophen sulfate. Although the singlet from the N-acetyl group is observed at δ 2.12, no other diagnostic resonances are available to confirm the identity of the sulfate. However, in the concurrent MS data, the MS chromatogram corresponding to $m/z = 235$ (i.e., the molecular ion of the sulfate conjugate, taking into account deuteration) also gives a retention time of 8.6 min. Detailed analysis of this chromatographic peak using an MS−MS scan to detect the fragment ions of the $m/z = 235$ ion (acetaminophen sulfate $[M + D]^+$ plus two hydrogen-to-deuterium substitutions) gave ions at $m/z = 155$ corresponding to loss of SO_3 and a further loss of 42 (ketene) from the N-acetyl group to give $m/z = 113$. This is a good example of the complementarity of the NMR and MS approaches, where neither technique can provide unambiguous structural characterization.

Extracting the NMR spectrum (labeled iii in Figure 2) corresponding to a retention time of 13.2 min gave the spectrum in Figure 4c. This shows, as expected, resonances from the residual acetonitrile-d_2 and HDO used as HPLC eluents at δ 2.07 and 4.70, respectively. In addition, a peak at δ 3.34 arises from the methanol contamination of the HPLC column. The remaining peaks arise from the endogenous substance hippurate, with the aromatic protons H2,H6 at δ 7.85, H3,H5 at δ 7.55, and H4 at δ7.65. The methylene protons from the glycyl conjugation appear as a singlet at δ4.19. Additional evidence for this structure was provided by the concurrent directly coupled MS chromatogram, showing the

ion at $m/z = 183$ at the same retention time. This corresponds to the $[M + D]^+$ ion, allowing for two hydrogen-to-deuterium substitutions caused by the use of D_2O as eluent.

The NMR spectrum shown in Figure 4d is that taken at a retention time of 13.6 min (labeled iv in Figure 2). At this retention time, there is evidence of the presence of two components, one of which is hippurate. The spectrum shown in Figure 4e has been enhanced by subtraction of a proportion of the hippurate spectrum shown in Figure 4c. The peaks that remain are consistent with the structure of phenylacetylglutamine. Thus, the phenyl group protons give rise to the peaks at δ7.42 (H3,H5) and 7.34 (H2,H4,H6). The methylene group protons appear as a singlet at δ3.65, while the glutamine resonances give characteristic patterns at δ4.39 (α-CH), 2.34 (γ-CH2), and 2.20 and 2.00 (nonequivalent β-CH_2 protons). Confirmation of the identity of phenylacetylglutamine was provided by the concurrent MS data, which indicated a molecular ion at $m/z = 270$ which corresponded to the $[M + D]^+$ ion, taking into account that the ion contains four deuterium atoms caused by exchange of the NH and OH protons with deuterium from the D_2O eluent. The use of an MS−MS scan gave fragment ions which support the structure, and these are seen at $m/z = 250$ (loss of D_2O or ND_3), 222 (loss of CO), 152 (loss of phenylacetyl), and 132 (loss of D_2O/ND_3 and phenylacetyl).

Phenylacetylglutamine has been shown to be a constituent of human urine and blood plasma and to be elevated in the plasma of uremic patients. Its levels and identity have been based largely on HPLC assays using authentic standard material for verification and on gas chromatography/mass spectrometry determinations following derivatization.[18−22] For the first time, NMR and MS have simultaneously provided complementary information for the molecular structure of phenylacetylglutamine. This approach demonstrates the excellent separation abilities of HPLC with the comprehensive structural capabilities of NMR spectroscopy and the high sensitivity and potential quantitative nature of mass spectrometry. Although we have made no attempt to quantify the levels of phenylacetylglutamine in this urine, through the use of appropriate internal standard substances, the MS methodology will allow this in principle.

The example of acetaminophen metabolites used here to demonstrate the coupling of both NMR and MS to HPLC might have been solved using HPLC−MS−MS alone. However, it is well known that, in general, NMR and MS yield complementary information, with NMR spectroscopy being particularly successful for distinguishing isomers. Moreover, the NMR spectrometer provides a very specific detector for the HPLC and, in particular, shows that the glucuronide conjugate is, indeed, the β-1-isomer as expected.

We have demonstrated previously the detection limits of directly coupled HPLC−NMR spectroscopy.[23] For a one-dimensional ^1H NMR spectrum, this is about 100 ng for a 16 h data collection period. The diversion of 5% of the HPLC eluent to a mass spectrometer will not alter this limit appreciably.

In summary, therefore, HPLC−NMR−MS is an efficient new technique for the identification of components in complex mixtures, and the two on-line detectors are complementary in providing unequivocal structural identification for both expected compounds and for unknown substances.

ACKNOWLEDGMENT

We thank St. Peter's Research Trust for financial support (to PJDF) and A. Nicholls for computational expertise.

Received for review July 2, 1996. Accepted October 3, 1996.[⊗]

AC9606463

[⊗] Abstract published in *Advance ACS Abstracts*, November 15, 1996.

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