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Directly coupled HPLC-NMR and HPLC-MS approaches for the rapid characterisation of drug metabolites in urine: application to the human metabolism of naproxen

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Abstract

High resolution nuclear magnetic resonance (NMR) spectroscopy is a very powerful tool for the structural identification of xenobiotic metabolites in complex biological matrices such as plasma, urine and bile. However, these fluids are dominated by thousands of signals resulting from endogenous metabolites and it is advantageous when investigating drug metabolites in such matrices to simplify the spectra by including a separation step in the experiment by directly-coupling HPLC and NMR. Naproxen (6-methoxy-α-methyl-2-naphthyl acetic acid) is administered as the S-enantiomer and is metabolised in vivo to form its demethylated metabolite which is subsequently conjugated with β-D-glucuronic acid as well as with sulfate. Naproxen is also metabolised by phase II metabolism directly to form a glycine conjugate as well as a glucuronic acid conjugate at the carboxyl group. In the present investigation, the metabolism of naproxen was investigated in urine samples with a very simple sample preparation using a combination of directly-coupled HPLC-^{1}H NMR spectroscopy and HPLC-mass spectrometry (MS). A buffer system was developed which allows the same chromatographic method to be used for the HPLC-NMR as well as the HPLC-MS analysis. The combination of these methods is complementary in information content since the NMR spectra provide evidence to distinguish isomers such as the type of glucuronides formed, and the HPLC-MS data allow identification of molecules containing NMR-silent fragments such as occur in the sulfate ester.

Keywords: Rapid analyses; Naproxen; Drug metabolism; Biofluids; HPLC-NMR; HPLC-MS

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1. Introduction

The non steroidal anti inflammatory drug, Naproxen (6-methoxy-α-methyl-2-naphtyl acetic acid), given orally, is nearly fully absorbed and is excreted almost exclusively in the urine in the form of metabolites, with only a few percent of the parent compound excreted unchanged [1,2]. Naproxen is metabolised in vivo in humans to form its demethylated phase I metabolite DM-naproxen and several phase II metabolites,-the glycine conjugate and the ester glucuronide. DM-naproxen is also glucuronidated and both the ether and the ester glucuronide has been proposed (see Fig. 1). It has been reported that both glucuronides were formed in liver microsomes from rats [1]. Because of the variety of Phase I and II metabolites of naproxen excreted in the urine, this compound was chosen as a model compound for the present investigations, where a rapid approach to characterisation of drug metabolites in urine samples is described.

When drug metabolites are analysed in biological fluids directly using ^{1}H nuclear magnetic resonance (NMR), there are thousands of peaks in the NMR spectrum resulting from all the endogenous compounds present. However, the NMR spectra of biofluids can be simplified by directly coupling HPLC with NMR. If the metabolic pattern of the compound being investigated is very complex, directly-coupled HPLC-NMR is obviously advantageous as metabolites can be separated and then their structures can be elucidated individually, in the stop-flow HPLC-NMR mode applying 2D experiments if necessary or, if the concentration of the metabolites are high enough, in the continuous flow mode [3–8]. Directly coupled HPLC-NMR analysis has been shown in several cases to be very successful for the analysis of very complex metabolic problems [8–13]. If the metabolites be-

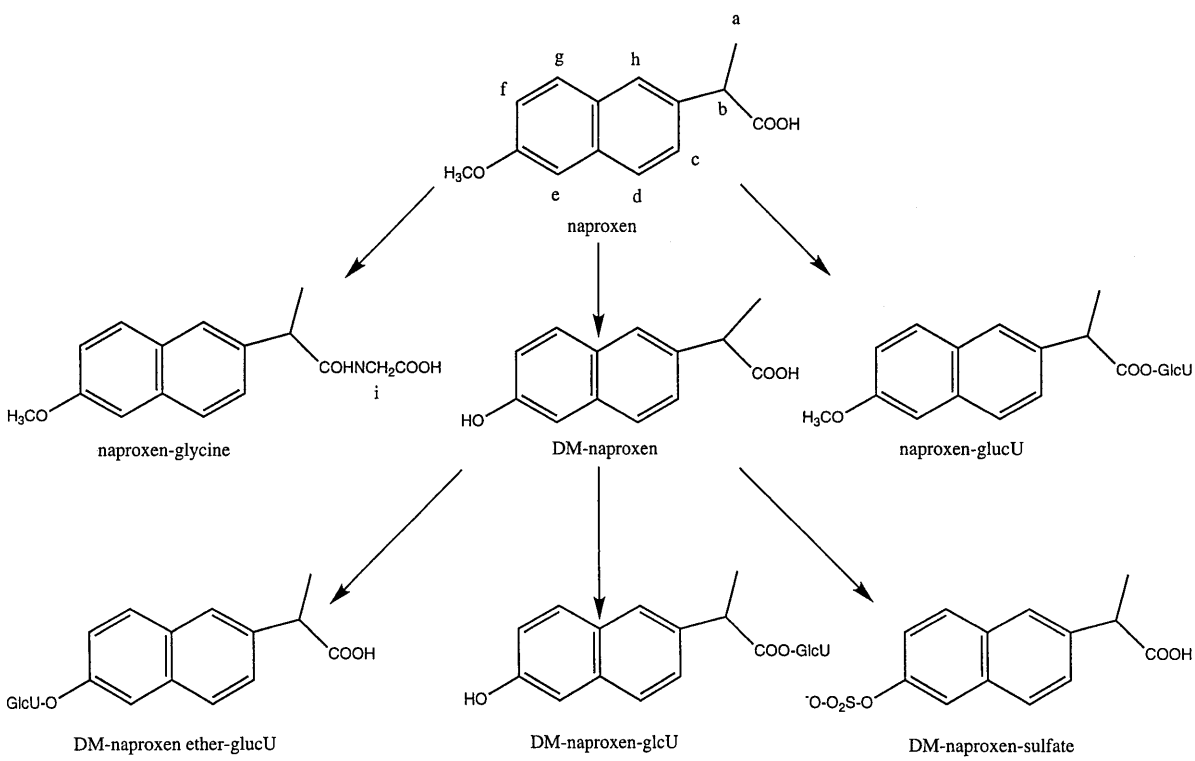

Fig. 1. Metabolic profile of naproxen (6-methoxy-α-methyl-2-naphthyl acetic acid). a–i represents the numbering scheme applied for the ^{1}H nuclear magnetic resonance (NMR) analysis of the metabolites of naproxen.

ing investigated contain certain functional groups such as sulfate esters that have no protons, then NMR analysis is insufficient since NMR spectroscopy does not provide any information from such moieties. Mass spectrometry (MS) can provide such information on the basis of molecular weights and characteristic fragmentation patterns, and in the present investigations a combination of directly-coupled HPLC-^{1}H NMR spectroscopy and HPLC-MS was used in order to unambiguously identify all the known metabolites of naproxen in a urine samples obtained from a human volunteer. In addition, the study describes a rapid approach for obtaining structural information about the metabolism of a urinary-excreted drug using a very simple sample preparation step.

The buffers that are usually used for directly-coupled HPLC-NMR analysis contain either potassium phosphate or trifluoroacetic acid (TFA). If HPLC-MS is to be applied in addition to HPLC-NMR, then neither of these buffers are optimal since they are known to suppress ionisation in the MS. As a consequence, the use of ammonium formate buffer was therefore investigated.

2. Experimental section

2.1. Chemicals

HPLC solvents were of analytical grade and obtained from Riedel De Hahn (Germany). All other chemicals were of analytical chemical grade and purchased from Aldrich (UK.). Daprox tablets (containing naproxen) were kindly donated by Nycomed (Copenhagen, Denmark). Reference compounds of naproxen and its phase I metabolite DM-naproxen as well as the two phase II metabolites the naproxen glycine conjugate and the DM-naproxen-*O*-sulfate were synthesised as described earlier [1].

2.2. Chromatography

The HPLC system used for the directly-coupled HPLC-NMR experiment consisted of a Hewlet Packard 1050 series chromatograph (Palo Alto, CA) where the variable wavelength UV-detector was operated at 232 nm. The chromatographic system was connected to a Bruker BPSU-12 collector (Rheinstetten, Germany) and the chromatography was controlled by Bruker Chromstar software. The BPSU-12 collector was connected to the HPLC-NMR flow-probe via an inert polyether(ether)ketone capillary (0.25 mm I.D.). The chromatographic data were collected using the Chromstar HPLC data system. The HPLC system used for the HPLC-MS experiment consisted of a ContraMETRIC 4100 MS series (Thermo Separation products, Riviera Beach, FL) the variable wavelength UV-detector was operated at 232 nm. Data were collected using the Finnigan MAT LCQ data system (San Jose, USA).

The analytical method (Method I) used for the continuous-flow HPLC-NMR experiment employed a reversed phase Waters Symmetry™ (Milford, USA) C18 column (3.9 × 150 mm I.D., 5 μm particles): the chromatographic conditions were simple 0.1% TFA in D_2O and acetonitrile-d_3 in a linear gradient moving from 2% acetonitrile to 50% acetonitrile in 35 min with a flow rate of 0.8 ml/min. For the stopped-flow HPLC-NMR and the HPLC-MS studies, a second method (Method II) was used with a reversed-phase chromatographic column (4.6 × 120 mm I.D., 5 μm particles): Spherisorb ODS-2 (Deeside, UK.) with a flow-rate of 1 ml/min. Here, eluent A consisted of 5% acetonitrile in ammonium formate (20 mM), eluent B consisted of 21% acetonitrile in ammonium formate (20 mM). The linear gradient system used initially 10% B:90% A which was increased to 58% B:42% A over 10 min. For the stop-flow HPLC-NMR analysis ammonium formate was dissolved in D_2O.

2.3. Sample preparation

Urine samples were collected 0–3 or 4–6 h after intake of 500 mg naproxen. The urine samples were diluted 1:3 with acetonitrile and centrifuged at 9000 × *g* for 10 min. The injection volume was 20 μl. For the directly-coupled HPLC-NMR experiments, 5 ml samples were freeze dried and dissolved in 1 ml acetonitrile-d_3/H_2O 3:1 (v/v) and then centrifuged at 9000 × *g* for 10 min. The injection volume was 50 μl.

2.4. NMR and directly-coupled HPLC-NMR spectroscopy

The HPLC-NMR data were acquired using a Bruker DMX-500 MHz spectrometer equipped with a ^{1}H flow probe (cell of 3 mm I.D., with a volume of 120 μl). ^{1}H NMR spectra were obtained in the 'stop-flow' mode at 500.13 MHz. In order to suppress the solvent signals, the one dimensional (1D) ^{1}H NMR spectra were collected using a NOESYPRESAT pulse sequence [6]. This is a 1D version of the nuclear Overhauser effect spectroscopy (NOESY) pulse sequence and in the present case it was used for suppression of the residual water and the acetonitrile signals. Free induction decays (FIDs) were collected into 32 K computer data points with a spectral width of 16025.64 Hz, 90° pulses were used with an acquisition time of 1.01 s. Prior to Fourier transformation, an exponential apodisation function was applied to the FID corresponding to a line broadening of 1.0 Hz. Sixty four scans were accumulated in the three first spectra, whereas the fourth eluting peak required 800 scans in order to obtain an appropriate signal-to-noise ratio. The continuous-flow HPLC-NMR data were similarly obtained at 500.13 MHz. The residual water signal was suppressed using a shaped sinc pulse applied for five cycles of 100 ms each, thereby giving a total recycle time of 0.9 s. FIDs were collected into 4 K computer data points with a spectral width of 5000 Hz, the acquisition time was 0.4 s and the spectra were acquired by accumulation of 16 scans, 192 spectra in total were collected during the chromatographic run time. Prior to Fourier transformation, an exponential apodisation function was applied to the FID corresponding to a line broadening of 1.0 Hz.

2.5. NMR spectra of reference compounds

The 1D NMR data of the purified metabolites were acquired using a Bruker AMX-400 spectrometer (Rheinstetten, Germany) ^{1}H NMR spectra were obtained at 400.14 MHz. FIDs were collected into 32 K computer data points with a spectral width of 5050.5 Hz, 90° pulses were used with an acquisition time of 2.58 s and the spectra were acquired by accumulation of 16 scans. Prior to Fourier transformation an exponential apodisation function was applied to the FID corresponding to a line broadening of 0.3 Hz. Two dimensional COSY and NOESY experiments were performed on the purified metabolites of naproxen in order to improve signal assignment of the aromatic region. The parameters for the COSY experiments were as follows: the number of scans per increment was 16, the spectral width was 5881.62 Hz, and 512 increments were performed in the F1 dimension. The FIDs were collected into 1 K computer data points. The relaxation delay between successive pulses was 1.5 s. The parameters for the NOESY experiments were as follows: the number of scans per increment was 32, the spectral width was 4901.96 Hz, and 512 increments were performed in the F1 dimension. The FIDs were collected into 1 K computer data points, the delay between successive pulses was 2 s and the mixing time was 0.53 s. The data were zero-filled by a factor of 2 prior to Fourier transformation.

All NMR spectra of the purified glucuronides were recorded in D_2O/CD_3CN 1:1 (v/v).

2.6. HPLC-MS

Mass spectra were obtained on a Finnigan MAT LCQ instrument (San Jose, USA) using atmospheric pressure chemical ionisation in the negative mode. The evaporation temperature was 450°C, the capillary temperature was 150°C with -8 V applied and the sheath gas flow (N_2) was set to 40 (arbitrary units). The Aux Gas Flow Rate was 10 U, the discharge Current was 7 μA, the capillary voltage was 48 V and the Tube Lens Offset was -8 V. All data were collected in the full scan mode (190–600 m/z).

3. Results and discussion

Two separate studies have been undertaken of the identification of naproxen metabolites in human urine. Initially, a continuous-flow HPLC-NMR study of urine collected 0–3 h after administration of the drug was performed and

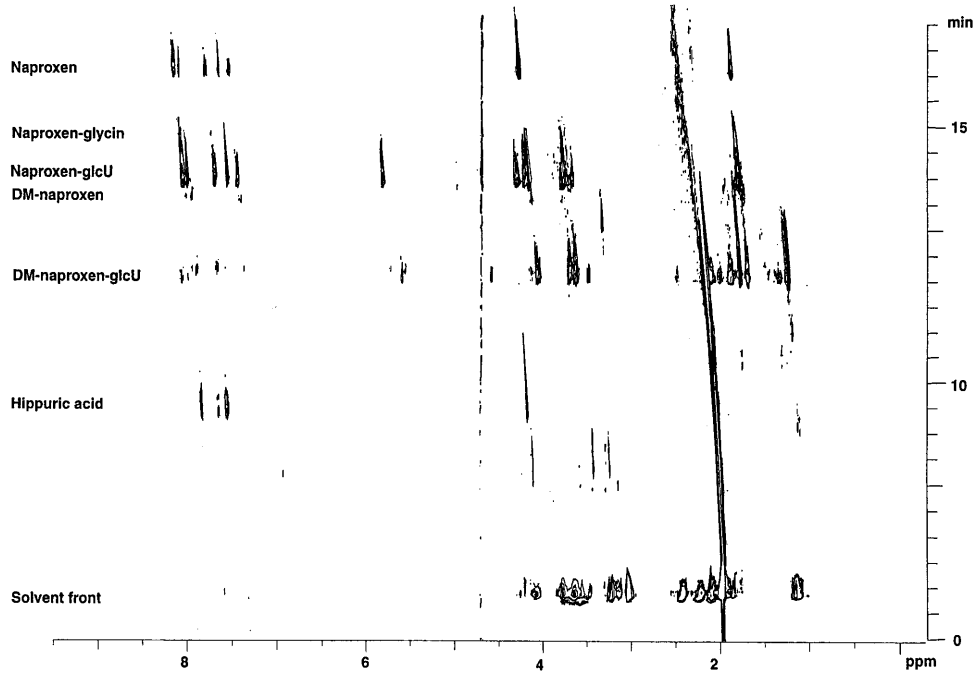

Fig. 2. The nuclear magnetic resonance (NMR) chromatogram obtained by continuous-flow 500 MHz HPLC-NMR of a urine sample containing metabolites of naproxen (6-methoxy-α-methyl-2-naphthyl acetic acid). The sample was obtained 0–3 h after intake of 500 mg naproxen. The band observed at δ4.8 arises from residual HDO in the solvent and has been suppressed by saturation. The band observed at δ2 arises from acetonitrile in the solvent and has been suppressed by saturation. For metabolite identification see Fig. 1. The analytical method (I) used is given in Section 2.

then a more detailed HPLC-NMR and HPLC-MS study of urine collected 4–6 h after naproxen administration was carried out.

The continuous-flow HPLC-NMR experiment was performed applying the chromatographic Method I to a urine sample obtained 0–3 h after intake of 500 mg naproxen. The result of this experiment is shown in Fig. 2. The ^{1}H NMR chemical shift values are presented along the horizontal axis and the chromatographic separation time along the vertical axis. Because an acetonitrile gradient was applied there is a shift in the chemical shift values during the experiment.

Initially the solvent front elutes and secondly after 8.5 min one of the major constituents of urine, namely hippuric acid, elutes. After about 12 min the metabolites of naproxen start to elute. The first metabolite is a glucuronic acid conjugate and from the resonance of the anomeric proton it is confirmed as an ester glucuronide (δ5.76). Also, the signal from the methoxy group is missing and it is thus the β-1-*O*-acyl glucuronide of the demethylated metabolite DM-naproxen. The next compound to elute has no glucuronide resonances but corresponds to the demethylated metabolite itself. Although not easily seen in Fig. 2, further data processing allowed the extraction of the full 1D NMR spectrum and by inspection it was clear that this compound eluting after 13.5 min was the demethylated metabolite of naproxen. The major metabolite elutes next and is the ester glucuronide of naproxen. On the tail of this peak, it is possible to detect the glycine conjugate and finally the parent compound elutes after 16.5 min.

In order to investigate lower level metabolites and to evaluate the time course of metabolite excretion, the metabolites of naproxen found in urine samples, obtained 4–6 h after oral intake of 500 mg naproxen by a human volunteer, were separated and identified by directly-coupled

stopped-flow ^{1}H HPLC-NMR and HPLC-MS experiments. A reversed phase chromatographic system was developed that separated all known metabolites of naproxen (chromatographic Method II). The chromatogram obtained using UV-detection is presented in Fig. 3. Ammonium formate was used as buffer because of the intention to carry out a HPLC-MS experiment in parallel with the HPLC-NMR experiment. The formate ions give rise to a singlet at δ8.3 in the ^{1}H NMR spectra and the buffer system is therefore applicable in the present investigations as neither naproxen or its metabolites have resonances near this δ-value. The peaks in the chromatogram shown in Fig. 3 were identified by the stopped-flow HPLC-NMR experiment. The flow was stopped on each of the peaks indicated and as many scans as necessary could therefore be acquired in order to obtain an appropriate signal-to-noise ratio. The individual 1D ^{1}H NMR spectra obtained from these investigations are presented in Fig. 4, where only the spectral region corresponding to δ3.5–8.2 is shown.

One potential problem occurred during the stopped-flow HPLC-NMR experiment, namely that the concentration of the major metabolite, naproxen ester glucuronide, was so high in the urine samples that the chromatographic system was overloaded with respect to this metabolite and resonances from this molecule can be seen in the NMR spectra of the other metabolites. However it is still possible to identify all the metabolites present because of the high structural information content of ^{1}H NMR spectra and the signals originating from naproxen ester glucuronide can therefore easily be identified. This problem did not occur during the HPLC-MS experiments as more dilute samples were injected into the chromatographic system.

The assignments in the NMR spectra obtained by the HPLC-NMR experiment were based on the chemical shift values obtained by acquiring 2D NMR experiments of the reference compounds available. The chemical shifts of naproxen and the phase I and phase II metabolites of naproxen obtained in the directly-coupled HPLC-NMR experiment are summarised in Table 1.

Characteristics of the NMR spectra resulting from naproxen and its metabolites is that the aromatic protons of naproxen have resonances in

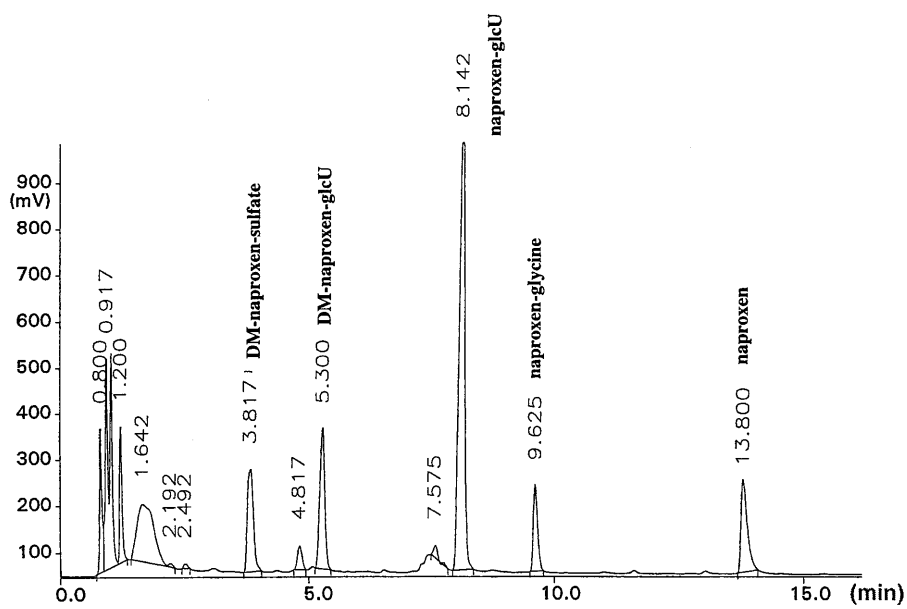

Fig. 3. The resulting chromatogram of naproxen (6-methoxy-α-methyl-2-naphthyl acetic acid) and its metabolites obtained using UV-detection by analysing a urine sample collected 4–6 h after intake of 500 mg naproxen. For identification of peaks, see Fig. 1. The chromatographic method (II) is given in Section 2.

Table 1
^{1}H nuclear magnetic resonance (NMR) chemical shifts (δ) of naproxen (6-methoxy-α-methyl-2-naphthyl acetic acid) and the phase I and phase II metabolites of naproxen[a]

δ (multiplet pattern)	Naproxen	Naproxen-glycine conjugate	Naproxen ester glucuronide	Desmethyl-naproxen	Desmethyl-naproxen glucuronide	Desmethyl-naproxen sulfate
a	1.48 (d)	1.47(d)	1.54(d)	1.61(d)	1.53(d)	1.50(d)
b	3.85 (q)	3.89 (q)	4.03(q)	3.98(q)	4.03(q)	3.94(q)
c	7.41 (dd)	7.39 (dd)	7.44 (dd)	7.50 (dd)	7.41 (dd)	7.50 (dd)
d	7.77 (d)	7.69 (d)	7.80 (d)	7.79 (d)	7.72 (d)	7.88 (d)
e	7.27 (s)	7.18 (s)	7.30 (s)	7.31 (s)	7.20 (s)	7.82 (s)
f	7.15 (dd)	7.12 (dd)	7.17 (dd)	7.27 (dd)	7.13 (dd)	7.43 (dd)
g	7.77 (d)	7.78 (d)	7.80 (d)	7.86 (d)	7.80 (d)	7.90 (d)
h	7.71 (s)	7.69 (s)	7.75 (s)	7.80 (s)	7.73 (s)	7.82 (s)
i	–	3.86 (dd)	–	–	–	–
1′	–	–	5.48 (d)	–	5.49 (d)	–
OCH_3	3.88 (s)	3.89 (s)	3.89 (s)	–	–	–

[a] For proton key (a–i) see Fig. 1, 1′ is the anomeric proton of glucuronic acid conjugates. Coupling pattern: d, doublet; dd, double doublet; s, singlet; q, quartet.

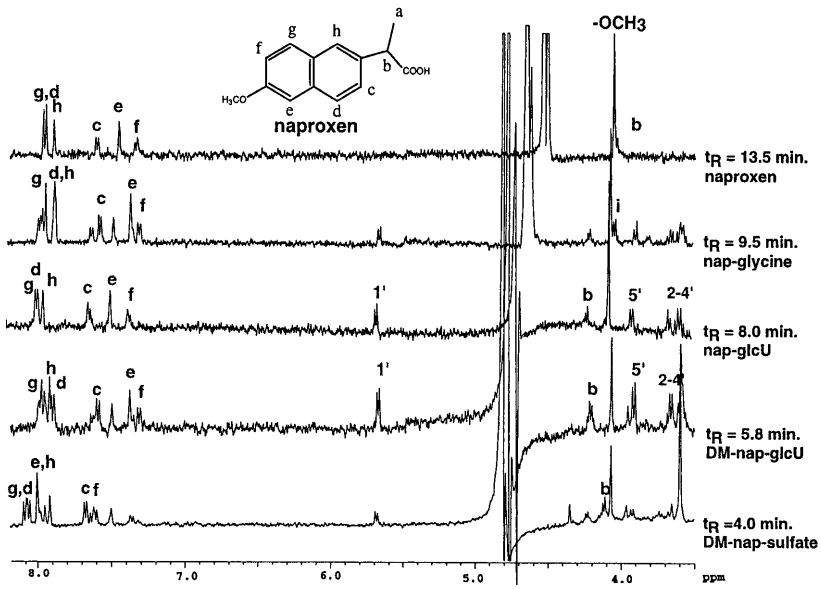

Fig. 4. The individual one dimensional (1D) ^{1}H nuclear magnetic resonance (NMR) spectra obtained from the 500 MHz directly-coupled stop-flow HPLC-NMR experiment. For metabolite identification see Fig. 1. i is the CH_2-group in the glycine moiety of the glycine conjugate of naproxen (6-methoxy-α-methyl-2-naphthyl acetic acid), 1′–5′ are the protons in the glucuronic acid moiety of the glucuronic acid conjugates where 1′ is the anomeric proton.

the region δ7–8 and shifts are observed of the signals in this region as a consequence of demethylation and conjugation, especially for the signal from the e-proton. The methoxy group has a resonance at ∼δ4, and this signal is lost as a consequence of demethylation. The methyl group (a) in the propionic acid part of the molecule has a resonance just to high frequency of δ2 and this signal shifts slightly to higher frequency as a result of conjugation, especially ester glucuronide formation. The single proton (b) next to the methyl group has a resonance at δ4.2, and this signal shifts also to higher frequency as a consequence of ester glucuronide formation.

The first eluting peak in the chromatogram (Fig. 3) is the sulfate ester. The results obtained from the HPLC-NMR experiment and the HPLC-MS experiment are shown in Fig. 5. In the NMR spectrum, it is possible to subtract the signals that corresponds to naproxen ester glucuronide and show by this means that there are no additional glucuronide moiety protons in the spectrum. However the e-proton in the aromatic ring is deshielded by 0.5 ppm relative to the e-proton in naproxen itself, indicating that conjugation has taken place at the hydroxyl group. The MS spectrum acquired on the same peak in the parallel HPLC-MS experiment proved that this is the sulfate ester of demethylated naproxen (molecular ion with $m/z = 295.1$).

The second eluting peak is the glucuronide of the demethylated metabolite of naproxen. It is not the ether glucuronide as the e-proton is shifted to low frequency. The glucuronide proton NMR resonances are superimposed on those of naproxen ester glucuronide. The integral values, however, prove the presence of two glucuronides. The MS data agrees with the assignment as a glucuronic acid conjugate of the demethylated naproxen since a molecular ion with $m/z = 391.6$ was observed.

The third eluting metabolite is naproxen ester glucuronide. It is, as expected, the β-1-*O*-acyl glucuronide which is identified by the doublet occurring at δ5.48 in the NMR spectrum which results from the anomeric proton in the glucuronic acid moiety. No changes were observed in the aromatic region of the NMR spectrum rela-

tive to that of naproxen itself. The a and b protons are, however, shielded because of glucuronidation at the carboxylic acid group. The MS data proves that this is the glucuronic acid conjugate of naproxen because the molecular ion is observed at $m/z = 405.7$.

The fourth metabolite is identified as the glycine conjugate (see Fig. 6). The c,e,f-proton chemical shifts are to low frequency by about 0.2 ppm and the glycine conjugate is characterised by the two doublets near δ3.86 ppm arising from the CH_2-group (i) in which the protons are diastereotropic and therefore give rise to a typical AB-pattern. Again the molecular ion from the MS experiment proves that this metabolite is the glycine conjugate. The ion with a mass m/z = 376.7 is the molecular ion of the glycine conjugate in association with a formate ion from the buffer. Finally, the last eluting peak also contains naproxen itself.

4. Conclusions

Both a stop-flow HPLC-NMR experiment and a continuous-flow HPLC-NMR experiment were performed in the present study but with different chromatographic methods as TFA was used as a buffer in the continuous-flow experiment. The urine samples used were collected at different time points for the continuous-flow experiment the urine samples were collected from 0 to 3 h after intake of naproxen whereas for the stop-flow experiment the urine samples were collected 4–6 h after intake of naproxen. The elution order of the metabolites of naproxen is the same in the two chromatographic systems. However DM-naproxen is only observed in the continuous-flow experiment, possibly because of the earlier collection time point of the urine sample. The sulfate ester is only observed in the stop-flow experiment

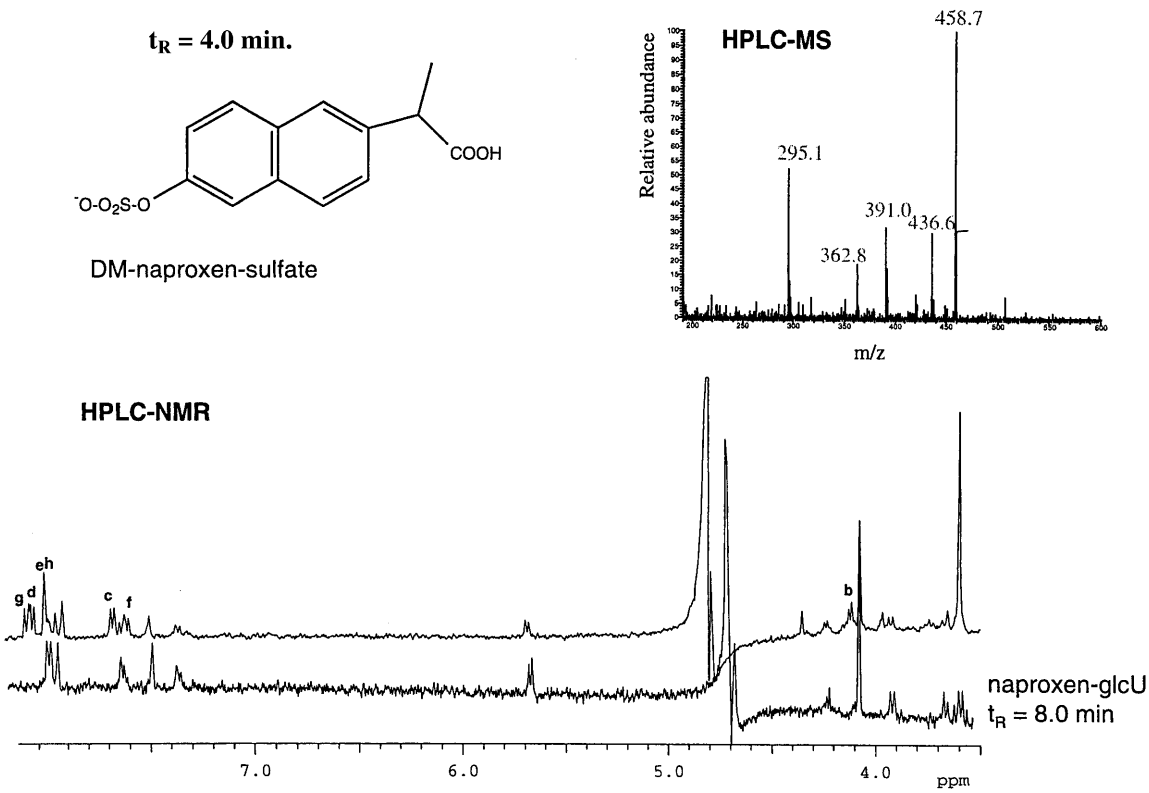

Fig. 5. The combined ^{1}H nuclear magnetic resonance (NMR) and mass spectrometry (MS) data obtained by the stop-flow HPLC-NMR and HPLC-MS experiments of the sulphate ester. The top NMR spectrum corresponds to the sulphate ester, the bottom NMR spectrum is the spectrum of the ester glucuronide of naproxen (6-methoxy-α-methyl-2-naphthyl acetic acid).

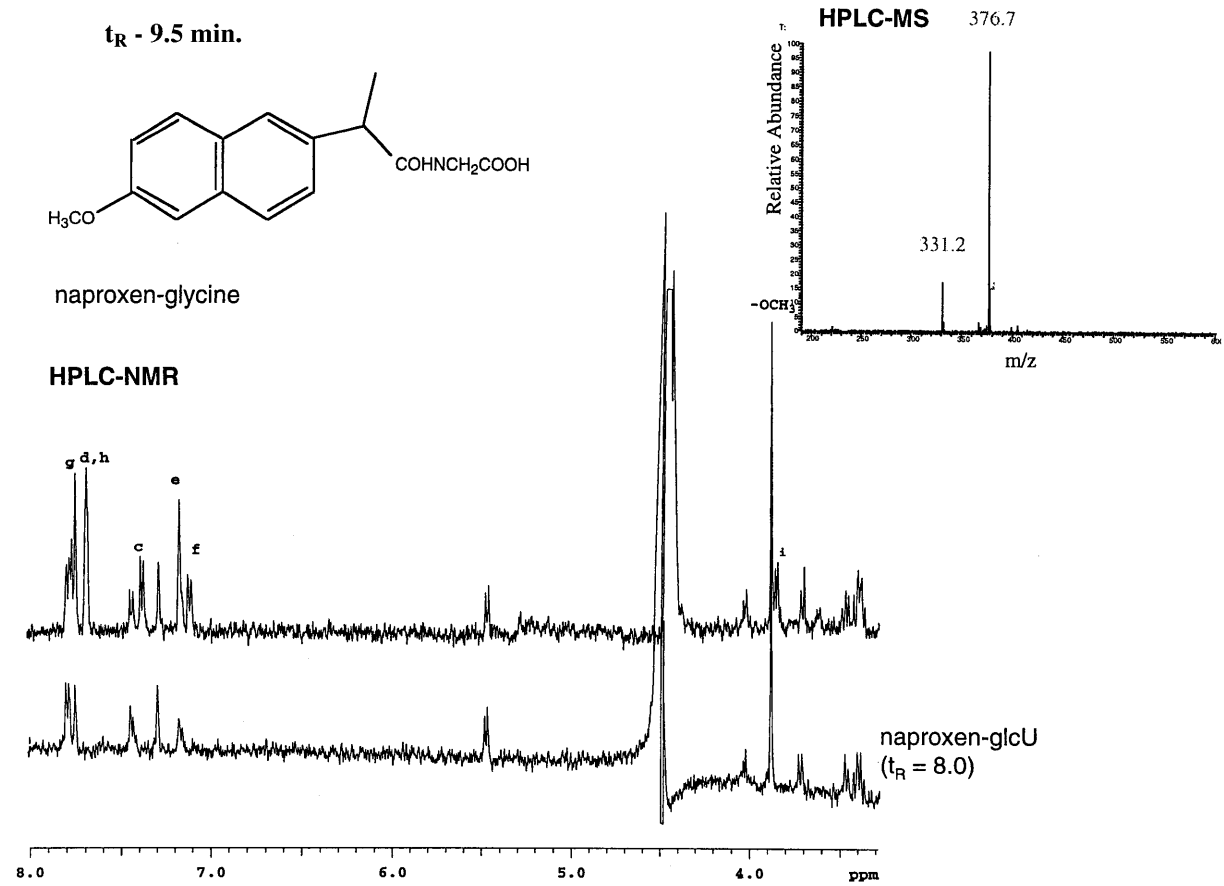

Fig. 6. The combined ^{1}H nuclear magnetic resonance (NMR) and mass spectrometry (MS) data obtained by the stop-flow HPLC-NMR and HPLC-MS experiments of the glycine conjugate of naproxen. The top NMR spectrum corresponds to the glycine conjugate and the bottom NMR spectrum is the spectrum of the ester glucuronide of naproxen (6-methoxy-α-methyl-2-naphthyl acetic acid).

because the detection limit of the continuous-flow experiment is higher or because the sulfate ester is not present in the earlier collected sample.

HPLC-NMR analysis is an efficient means of rapidly obtaining information on the structure of drug metabolites in biofluids. The experiments described here show the applicability of the HPLC-NMR technique within the fields of pharmaceutical and biological chemistry. Large amounts of structural information can be obtained very rapidly, because of the saving in analysis time compared to off-line analysis, where the individual metabolites would have to be separately isolated and purified, before they could be identified by NMR analysis. Also the study shows that even though the chromatographic separations during the stop-flow HPLC-NMR experiment were not optimal it was still possible to identify all the metabolites of naproxen present in the urine samples. It was proved that ammonium formate buffer was well-suited when both directly-coupled HPLC-NMR and HPLC-MS is applied with the same chromatographic system. The advantage of such a system is clear from the present investigations where the sulfate ester could not have been identified with NMR alone and where the MS analysis immediately aids the identifica-

tion of the glycine conjugate. On the other hand the MS analysis provides no information on the type of glucuronides formed. As mentioned earlier both the ether and the ester glucuronide of DM-naproxen has been proposed (see Fig. 1). In both the continuous-flow and the stop-flow HPLC-NMR experiments described here only the ester glucuronide of DM-naproxen was observed. This is valuable information as ester glucuronides are reactive metabolites that may give rise to allergic reactions in man [14–16].

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