MAGNETIC RESONANCE IN CHEMISTRY, VOL. 35, 2–8 (1997)

^{1}H NMR Characterization of Methacrylamide Polymer Conjugates with the Anti-Cancer Drug Doxorubicin

V. Pinciroli, V. Rizzo,* F. Angelucci, M. Tatò and A. Vigevani
Pharmacia and Upjohn, Pharmaceutical Research and Development, via Pasteur, I-20014 Nerviano, Italy

Two poly-2-hydroxypropylmethacrylamide conjugates with the anti-cancer drug doxorubicin were investigated by ^{1}H NMR spectroscopy. In both polymers (FCE 28068 and FCE 28069) the drug is connected to a polymethacrylamide backbone via a tetrapeptidyl spacer (Gly-L,D-Phe–L-Leu–Gly) and, in addition, FCE 28069 contains similarly bound D-galactosamine molecules as liver-targeting moieties. Signals of the drug and galactosamine protons were fully assigned in the spectra of the polymers by means of NOESY and TOCSY experiments and by comparison with the spectra of reference compounds. The integrity of doxorubicin and the connectivity between the drug and peptidyl chain was successfully confirmed in spite of the comparatively low abundance (< 5%) of the corresponding monomeric units and of signal spreading due to the diastereomeric mixture of peptidyl chains. Polymer-bound galactosamine molecules were found to be distributed among four isomeric forms (α- and β-anomers of pyranose and furanose forms, with predominance of the α-pyranose form). Diagnostic signals for possible alternative peptidyl chain endings (2-hydroxypropylamide or free carboxyl groups) were identified with the help of model polymers and the first, but not the second, were detected in the spectra of the two FCEs. In addition to the above results, high-field NMR is a rapid and sensitive method for the qualitative identification of low molecular weight contaminants in production batches of polymer–drug conjugates.

Magn. Reson. Chem. **35**, 2–8 (1997) No. of Figures: 7 No. of Tables: 3 No. of References: 11

Keywords: NMR; ^{1}H NMR; doxorubicin; drug targeting; D-galactosamine

Received 12 December 1995; revised 1 July 1996; accepted 5 August 1996

INTRODUCTION

Conjugates of synthetic polymers with drugs are actively investigated particularly for anti-cancer therapy.[1] The expected benefits of this approach derive from two main grounds: (i) an appropriate choice of the polymer and linker structure leads to pharmacological improvements with respect to the free drug and (ii) the polymeric backbone offers a convenient scaffold for the preparation of chemically connected structures containing both cytotoxic drugs and organ-targeting agents. The characterization of these conjugates is generally confined to chemical or enzymatic hydrolysis experiments aimed at determining the amount of bound drug and its release, whereas molecular structure is inferred on the basis of the synthetic route.[2] The strict requirements posed by regulatory authorities on identity verification of new drugs undergoing clinical evaluation suggest that new methods should be sought for the structural characterization of such conjugates and NMR appears to be the most suitable for this purpose.

* Correspondence to: V. Rizzo.

The present study was focused on two analogous polymers, FCE 28068 and FCE 28069, the former currently undergoing clinical evaluation for the assessment of the maximum tolerated dose.[3] Both compounds contain the anti-cancer drug doxorubicin[4] linked to a polymeric backbone based on the strongly hydrophilic poly-*N*-(2-hydroxypropyl)methacrylamide structure (Fig. 1). The tetrapeptide sequence connecting the drug with the polymer backbone has been selected to favor intracellular drug release[5] and contains a racemic mixture at the phenylalanine residue as a consequence of the original synthetic procedure,[6] which was not based on racemization free methods. In addition to doxorubicin, FCE 28069 contains, as liver-targeting moieties,[7,8] D-galactosamine residues covalently linked to some of the peptidyl chains.

Chemical characterization has been carried out independently on the two polymers and the amount of coupled drug and galactosamine has been established with procedures based on acid hydrolysis and high-performance liquid chromatographic quantitation of the corresponding products.[9] The typical drug loading is about 8% (w/w) for FCE 28068 and 7% (w/w) for FCE 28069. The galactosamine content is *ca.* 2% (w/w) in the examined batches of FCE 28069. In spite of these

CCC 0749–1581/97/010002–07

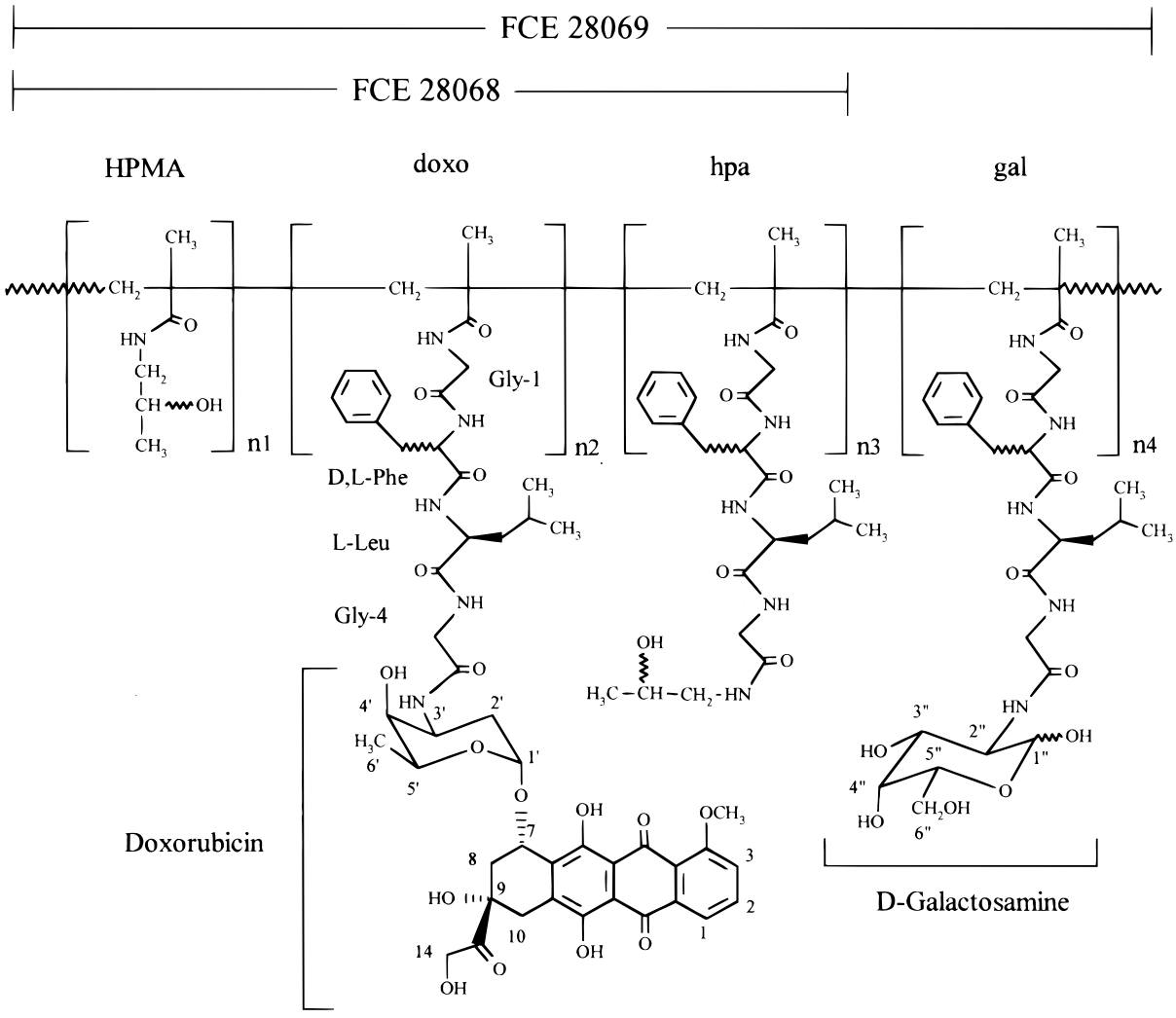

Figure 1. Structures of FCE 28068 and FCE 28069. The two compounds are copolymers based on the strongly hydrophilic poly-*N*-(2-hydroxypropyl)methacrylamide (poly-HPMA). Both contain HPMA as the most abundant repeating unit, whereas other monomers carry pendant peptide chains terminating with the anti-cancer drug doxorubicin (doxo) or 2-hydroxypropylamide (hpa) in both compounds, and also with D-galactosamine (Gal) in the case of FCE 28069.

low levels of drug and galactosamine substitution, preliminary ^{1}H NMR experiments with a high-field instrument (600 MHz) showed several characteristic signals of the attached molecules and thus encouraged an extensive study aimed at the assignment of all proton resonances and at the verification of molecular connectivity with standard 2D methods. The major difficulty in this study arose from the presence of many different pendant chains (Fig. 1), which was a cause of signal crowding and sensitivity loss, especially in the NOESY spectra. Assignments were facilitated by a comparison with the spectra of two low molecular weight model compounds (**1**, Ac–Gly-L-Phe–L-Leu–Gly–doxorubicin and **2**, trityl–Gly-L-Phe–L-Leu–Gly–D-galactosamine) and with those of a series of model polymers (**3–9**; see Table 1) each containing at most two types of pendant chain.

Table 1. Summary of structures of FCEs and model polymers

Polymer	Tetrapeptide	Chain ends
FCE 28068	Gly–L,D-Phe–L-Leu–Gly	hpa, doxo
FCE 28069	Gly–L,D-Phe–L-Leu–Gly	hpa, doxo, gal
3	Gly–L-Phe–L-Leu–Gly	hpa
4	Gly–D-Phe–L-Leu–Gly	hpa
5	Gly–L-Phe–L-Leu–Gly	doxo, hpa
6	Gly–D-Phe–L-Leu–Gly	doxo, hpa
7	Gly–L-Phe–L-Leu–Gly	hpa, gal
8	Gly–D-Phe–L-Leu–Gly	hpa, gal
9	Gly–L-Phe–L-Leu–Gly	OH

EXPERIMENTAL

Model compound **1** (Ac–Gly–L-Phe–L-Leu–Gly–doxorubicin) and **2** (trityl–L-Phe–L-Leu–Gly–D-galactosamine) were prepared by conventional stepwise condensation in solution with racemization-free methods, and their structures were confirmed by ^{1}H NMR and mass spectrometry. Batches of FCE 28068 and FCE 28069 were provided by Pharmacia R&D/Pharmaceutical Development (Nerviano, Italy). Model polymers **3–9** were prepared by a modification of the general scheme used for FCE 28068 and 28069, which included racemization-free methods in the preparation

of the tetrapeptides. A summary of structural characteristics of each model polymer is given in Table 1.

NMR experiments were performed on a Varian Unity-600 spectrometer operating at 599.919 MHz with DMSO-d_6 solutions of the polymers (about 10 mg ml^{-1}) at 28 °C. Two-dimensional NMR spectra were recorded in the phase-sensitive mode with quadrature detection in both dimensions using the hypercomplex method: 2048 data points were collected in t_2, with 400–500 FIDs in t_1. A total of eight transients were signal-averaged for each point in t_1. TOCSY, NOESY, DQFCOSY and HMQC were acquired by standard methods as supplied with the Varian software (VnmrS 4.1). The TOCSY experiments employed an average spin lock field of 10 kHz for 80 or 120 ms, consisting of a 2 ms trim pulse followed by an MLEV-17 composite pulse sequence. NOESY data were acquired with a mixing time of 150 ms, which was found to be a good compromise between NOE sensitivity and the onset of spin diffusion. HMQC data were acquired with a delay corresponding to $^1J(^{13}C, ^1H) = 140$ Hz. The first complex points in the t_2 dimension of TOCSY and NOESY spectra were scaled to eliminate ridge artifacts. All spectra were transformed into a 2048 × 2048 matrix by zero filling, with both time domains in each data set multiplied by shifted squared sine-bell functions before Fourier transformation. Sequence-specific resonance assignments of peptidyl chains were made by the standard method.[10] In general, cross peaks were classified according to the spin system with the through-bond experiments TOCSY or DQFCOSY. Sequential connectivities between spin systems were then made using the NOESY data.

RESULTS

The 1D ^1H NMR spectra at 600 MHz of FCE 28068 and 28069 are compared in Fig. 2, where the position of doxorubicin and galactosamine (main isomeric form) resonances is indicated schematically. These assignments correspond closely to those obtained with greater precision from the spectra of model compounds **1** and **2**, which are summarized in Tables 2 and 3. The 1D spectra are dominated by the intense, broad resonances of polymer backbone and of HPMA side-chains (Fig. 1), which constitute about 95% of the side-chains in FCE 28068 and about 90% in the case of FCE 28069. Nevertheless, several comparatively sharp signals deriving from the bound drug or galactosamine molecules are clearly observed in the two spectra and these provide a good starting point for an assignment strategy, which is generally obtained with 2D spectra. A comparison with spectra of model polymers **3–9** proved fundamental in this assignment strategy and verified the following additivity rule: for each given peptide chain ending (doxo, gal, hpa) and stereochemistry (L-Phe–L-Leu or D-Phe–L-Leu), signals are found at approximately the same frequencies in different polymers. This rule notably simplifies the spectral assignment in complex molecules

Table 2. Resonance assignments and coupling constants (Hz)[a] of doxorubicin protons in model compound 1[b]

Atom	δ (ppm), J (Hz)[c]	Atom	δ (ppm), J (Hz)[c]
H1′	5.22 (3.3)	H1, 2	7.91, m
H2′eq	1.44, m	H3	7.65, m
H2′ax	1.83, m	H7	4.95 (3.8, 5.8)
H3′	3.97, m	H8eq	2.20 (14.4, 3.8)
NH3′	7.44 (8.1)	H8ax	2.12 (14.4, 5.8)
H4′	3.39, m	OH9	5.45, s
OH4′	4.75 (6.1)	H10	2.97, m
H5′	4.16 (6.4)	H14	4.56 (6.0)
H6′	1.12 (6.4)	OH14	4.81 (6.0)

[a] s = Singlet; m = multiplet.
[b] Resonances of doxorubicin residues in FCE 28068 and 28069 and in model polymers agree with these assignments to within 0.05 ppm.
[c] *J* values in parentheses.

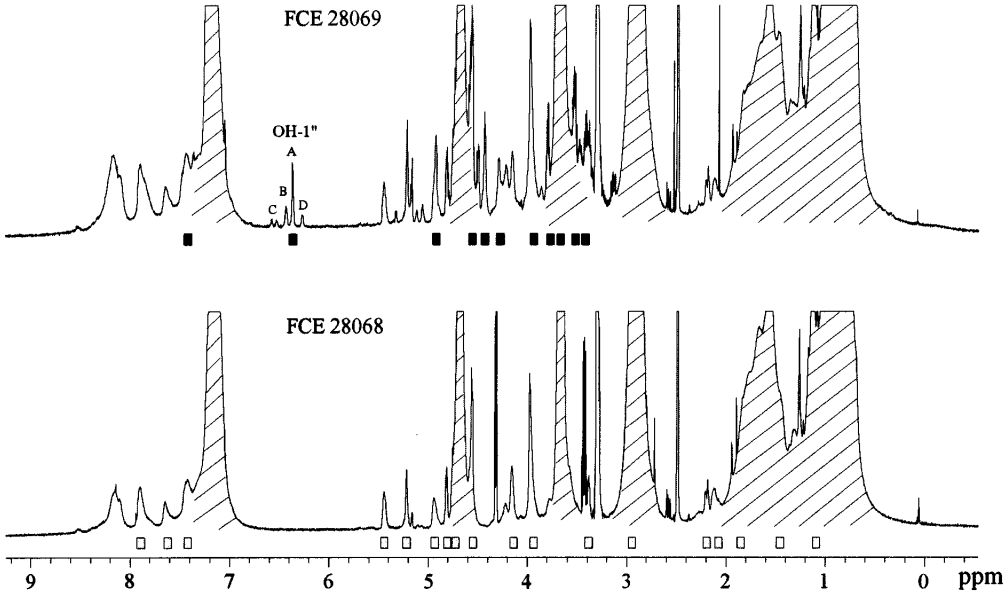

Figure 2. ^1H NMR spectra of FCE 28068 and FCE 28069. Shaded peaks denote the intense, broad resonances deriving from the polymeric backbone and from the side-chain of the most abundant repeating unit (HPMA). The position of resonances of the drug, doxorubicin, is indicated with small bars under the spectrum of FCE 28068. Resonances of the main isomeric form of galactosamine are similarly indicated in the spectrum of FCE 28069.

Table 3. Chemical shifts (ppm) and coupling constants (Hz)[a] of the galactosamine resonances in model compound 2[b]

	Pyranose forms		Furanose forms	
Atom	A = α	B = β	C = α	D = β
H1	4.92 (4.0, 3.2)	4.38 (6.4, 8.0)	5.04 (5.3, 4.4)	4.89 (5.5, 3.4)
H2	3.94 (3.2, 8.6, 11.5)	3.64 (nd)	3.96 (nd)	3.94 (nd)
H3	3.67 (nd)	3.42 (nd)	4.11 (m)	3.90 (nd)
H4	3.71 (nd)	3.61 (nd)	3.76 (nd)	3.87 (7.0, 2.6)
H5	3.82 (6.2, 6.2)	3.26 (nd)	3.44 (nd)	3.50 (nd)
H6, H6′	3.54, 3.42 (nd)	3.54, 3.48 (nd)	3.36 (nd)	3.38 (nd)
OH1	6.33 (4.0)	6.38 (6.4)	6.58 (5.3)	6.28 (5.5)
NH2	7.41 (8.6)	7.59 (8.6)	7.72 (nd)	7.74 (nd)
OH3	4.27 (6.5)	4.40 (6.2)	5.13 (6.3)	5.13 (5.1)
OH4	4.45 (nd)	4.45 (nd)	—	—
OH5	—	—	4.20 (7.1)	4.47 (6.4)
OH6	4.54 (5.6, 5.6)	4.61 (5.6, 5.6)	4.58 (5.7, 5.7)	4.54 (nd)

[a] J values in parentheses; nd = not determined; m = multiplet.
[b] Resonances of galactosamine residues in FCE 28069 and in model polymers agree with these assignments to within 0.05 ppm.

such as FCE 28069, which contains at least six different types of pendant peptidyl chains. Consistently, the assignment of doxorubicin bound chains is discussed only for FCE 28068, since identical results are obtained for the other polymer.

Resonances attributed to low molecular weight contaminants (solvents, free 2-propanolamine, free drug, free galactosamine) are detected in the NMR spectra of the two polymers with great sensitivity (detection limits below 0.1% are possible in some cases). Typically, these resonances are recognized through their narrow linewidth and identified from their characteristic chemical shifts and scalar connectivity maps, as developed from TOCSY spectra.

Connectivity of doxorubicin-bearing chains

Complete molecular connectivity of these chains is obtained by sequential cross-peak assignment in NOESY/TOCSY spectra (see Fig. 3 for an example). In particular, the cross peak between doxorubicin 3′NH and Gly-4 CαH (marked b in Fig. 3 and appearing as a double peak in correspondence with the diastereotopic proton pair of Gly), which is apparent in the NOESY spectrum, provides a convenient pivot for the tracing of both drug and peptide structural integrity and bonding. Splitting of cross peaks into two well distinguishable patterns is a consequence of the diastereoisomerism of

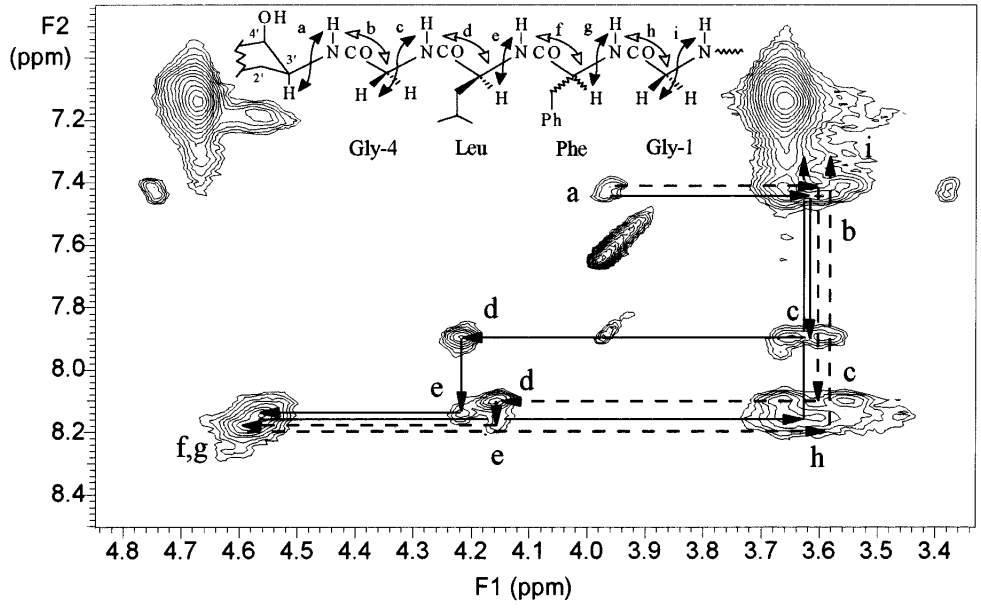

Figure 3. Portion of NOESY spectrum of FCE 28068 including cross resonances of CαH and amide NH of peptidyl chains. Sequential assignment is indicated with arrows for the two diastereomeric tetrapeptidyl chains connected to doxorubicin: solid lines relate to the Gly–L-Phe–L-Leu–Gly chain and dashed lines to the Gly–D-Phe–L-Leu–Gly chain. In some cases cross peaks are not clearly delimited by contour lines, because of signal crowding. The position of these cross peaks is deduced by comparison with the NOESY spectra of the two model polymers **5** and **6**, both of which contain one diastereomeric peptidyl chain. The inset shows in detail cross-peak assignment and presence in both NOESY and TOCSY (solid arrows) or just in NOESY (open arrows).

the tetrapeptidyl chain, and in particular the cross peaks involving the Gly-4 NH resonance are most affected in the portion of NOESY reported in Fig. 3. Other peaks which split significantly arise from the protons of the Leu side-chain (not shown). Notably this effect is much weaker for protons of the Phe residue, which is present in two enantiomeric configurations.

Progressive peak broadening is observed with increasing proximity of the corresponding chemical group to the polymer main chain. For this reason, the cross peak between Gly-1 NH and CαH resonances (labelled i in Fig. 3) is nearly undetectable in both the NOESY or TOCSY spectra of all polymers examined and is assigned on a best guess basis. The presence of an amide bond at the amino end of Gly-1 is nevertheless inferred from the chemical shift of the Cα protons (3.5–3.7 ppm). Some arrows in Fig. 3 point to crowded areas where cross peaks are difficult to locate. In those cases, the assignments are based on a comparison with the spectra of model polymers **5** and **6**, each containing only one configuration of the Phe residue. Correspondingly, the assignments of the Gly–L-Phe–L-Leu–Gly chain are in good agreement with those obtained from the spectrum of model compound **1**.

Isomeric equilibrium of galactosamine units

Multiple signals are observed for each proton of the galactosamine units in the spectra of FCE 28069; see, for instance, the signals corresponding to the anomeric 1″OH in the 6.2–6.6 ppm interval (Fig. 2). These four sets of signals, labeled A, B, C and D, respectively, correspond to the four isomeric cyclic forms of the sugar (Fig. 4) and appear with relative intensity 9:5:1:1. The same situation is more clearly observed in the spectrum of reference compound **2** (not shown), where assignment of the anomeric proton resonances to each form was obtained after identification of the corresponding ^{13}C resonances in a heterocorrelated 2D experiment (HMQC; Fig. 5). The assignment of ^{13}C resonances derives from standard literature values for D-galactose in D_2O[11] after correcting with a constant decrement ($\Delta\delta \approx -3$ ppm), which is partly attributed to the inductive shift difference of the amide nitrogen with respect to the sugar hydroxyl group, and partly to a solvent effect. Assignment of the ^{1}H resonances is

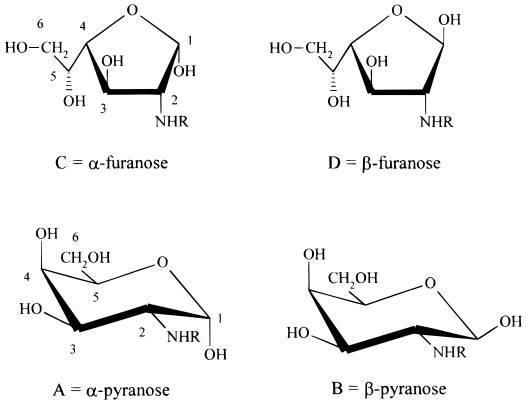

Figure 4. Structures of the four isomeric forms of the galactosamine unit in FCE 28069.

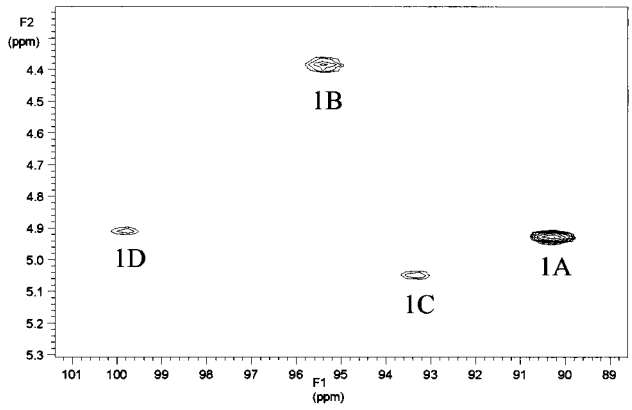

Figure 5. Portion of the HMQC spectrum showing cross peaks between the anomeric sugar protons and directly bound carbon atoms of model compound **2**.

derived from that of the corresponding anomeric proton through inspection of coupling constant correlation pattern in DQF-COSY and TOCSY experiments. For the galactosamine moiety, a complete listing of proton resonance assignment is reported in Table 3. The observed ^{1}H–^{1}H coupling constants (also listed in Table 3) confirm the α and β stereochemical assignments of pyranose forms. Assignments of the galactosamine resonances in the spectra of model polymers **7** and **8** and also in that of FCE 28069 agree with the above within 0.05 ppm.

Connectivity of galactosamine-bearing chains

As in the above case, molecular connectivity is established with sequential cross-peak assignment starting from the 2″CH–2″NH cross peak in the NOESY of FCE 28069 (Fig. 6). In the spectra of model polymers **7** and **8**, isomerism of the sugar structure produces a well defined splitting of the 2″CH–2″NH and of the 2″NH–C_αHGly-4 cross peaks, but is not detectable in the correlations arising from the other amino acid residues. Only signals of the major A-form are detectable in the NOESY spectrum of FCE 28069, but cross peaks arising from forms B, C and D are revealed by the more sensitive TOCSY experiment (Fig. 7). As already observed for the doxorubicin-bearing chain in FCE 28068, stereoisomerism of the peptidyl chain affects at most the position of the cross peak involving Gly-4 NH.

Diagnostic signals for alternative chain ending

Analytical data on the amino acid content of FCE 28068 indicate that the peptide chains are not all bound to doxorubicin. Within synthesis, a treatment with 2-hydroxypropylamine after doxorubicin loading is carried out for the transformation of unreacted active ester groups into the corresponding 2-hydroxypropylamide endings (hpa). Characteristic cross peaks of this group (NHhpa–C_αHGly-4, CH_2hpa–NHhpa) may be located in the NOESY

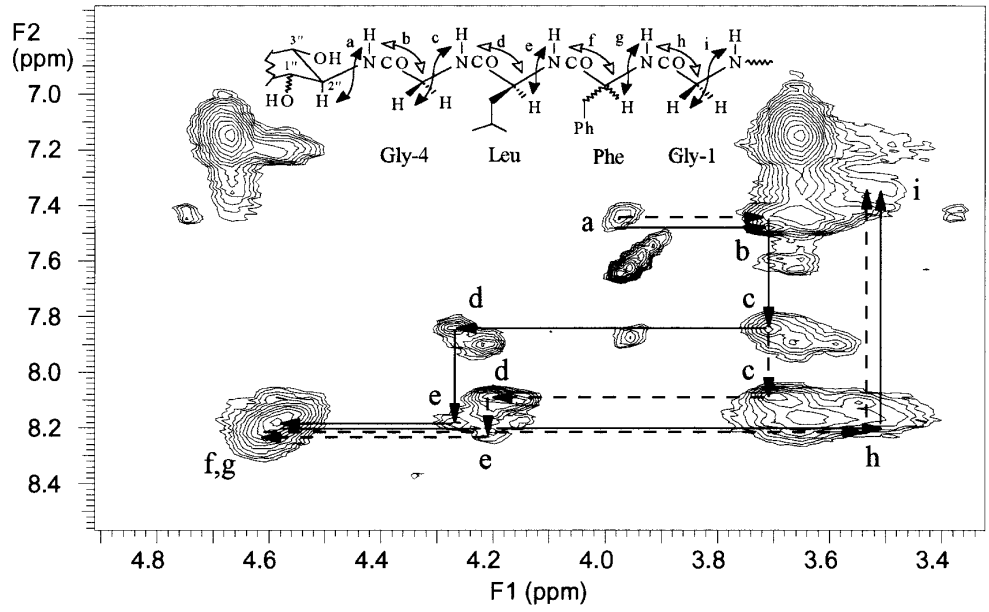

Figure 6. Portion of NOESY spectrum of FCE 28069 including cross resonances of CαH and amide NH of peptidyl chains. Sequential assignment is indicated with arrows for the two diastereomeric tetrapeptidyl chains connected to the galactosamine moiety (see Fig. 3 for details). In some cases, the position of cross peaks is deduced by comparison with NOESY spectra of model polymers **7** and **8**, both of which contain only one diastereomeric peptidyl chain. Cross resonances of chains carrying doxorubicin are easily identified by comparison with Fig. 3.

spectra of model polymers **3** and **4**. NOESY spectra of FCE 28068 batches contain at least some of these cross peaks, as low-intensity spots, close to background noise. Among other possible alternative chain endings, free carboxyl groups have been postulated and a suitable model polymer (**9**) has been prepared and tested for the identification of diagnostic cross peaks. In this case, the cross peak (NHGly-4–C_{α}HLeu-3), which is well distinguished from the corresponding peak of doxorubicin-containing chains, appears the most suitable. These signals are not revealed in the spectra of FCE's polymer within current sensitivity limits.

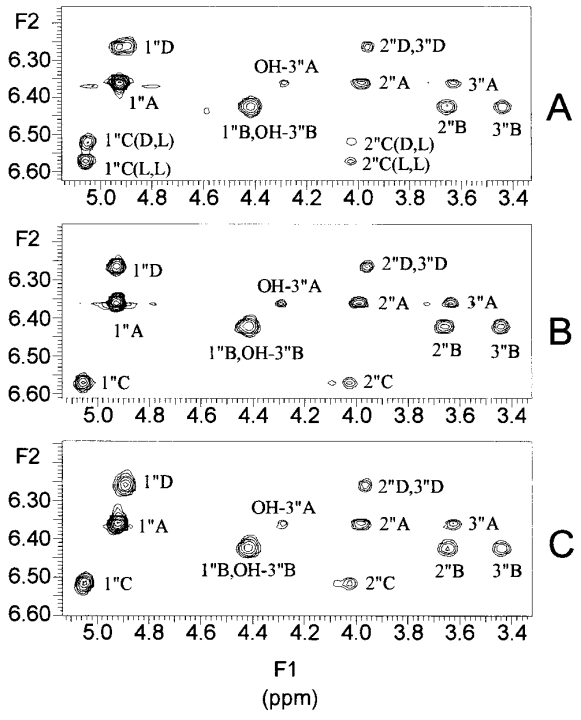

Figure 7. Portion of TOCSY spectrum showing the correlation of OH1″ signal with other sugar resonances: (A) FCE 28069; (B) model polymer **7**; (C) model polymer **8**. Cross peaks belonging to all four isomeric forms of the galactosamine residue are detected in this experiment. The spectrum of FCE 28069 clearly corresponds to the sum of the two model spectra. Sugar molecules bound to diastereomeric peptides produce distinguishable cross-peak patterns only for the α-furanose form.

CONCLUSIONS

To the best of our knowledge, past NMR structural studies of polymer–drug conjugates have focused on the characterization of the polymeric backbone. The present study demonstrates that complete structural analysis of the polymer–drug or polymer–carrier linkage is feasible with present-day NMR techniques, and that much useful information becomes accessible in this way. The low abundance of monomeric units carrying the drug or the targeting moiety ($< 5\%$ in the present case) is compensated by the different bandwith of signals arising from polymer backbone or pendant chains. Thus, the intense but broad resonances of the polymer backbone do not obscure sharper signals of drug (or carrier) containing pendant chains.

In practice, 2D methods on a 600 MHz instrument provide sufficient sensitivity and signal resolution to distinguish among polymer pendant chains with different terminations and also between chains with diastereomeric sequences. Accordingly, a full inventory of the polymer pendants may be generated. In favourable cases, as for galactosamine, even slowly interconverting isomers may be distinguished and approximately quantitated. NMR is also a quick and convenient way to

detecting low molecular weight contaminants with remarkable sensitivity (0.1% or better). In conclusion, it appears that full exploitation of NMR methods in the field of polymeric drugs could lead to considerable improvements in the characterization of these compounds and, in some cases, to structural and conformational information of great utility for the understanding of their organ- or cell-targeting properties.

REFERENCES

1. R. Duncan, *Anti-Cancer Drugs* **3**, 175 (1992).
2. K. D. Widder and R. Green (Eds), *Drugs and Enzyme Targeting. Part A. Methods in Enzymology*, vol. 112, Section IV. Academic Press, Orlando (1985).
3. P. A. Vasey, R. Duncan, S. B. Kaye and J. Cassidy, in *Abstracts of the 8th European Conference on Clinical Oncology, Paris*, 1995, Abstract 929.
4. F. Arcamone *Doxorubicin. Anticancer Antibiotics*. Academic Press, New York (1981).
5. R. Duncan, H. C. Cable, J. B. Lloyd, P. Rejmanova and J. Kopecek, *Makromol. Chem.* **184**, 1997 (1984).
6. R. Duncan, J. C. Hume, P. Kopecekova, K. Ulbrich, J. Strohlam and J. Kopacek, *J. Controlled Release* **10**, 51 (1989).
7. R. Duncan, L. C. W. Seymour, L. Scarlett, J. B. Lloyd, P. Rejmanova and J. Kopecek, *Biochim. Biophys. Acta* **880**, 62 (1986).
8. M. V. Pimm, A. C. Perkins, R. Duncan and K. Ulbrich, *J. Drug Targeting* **1**, 125 (1993).
9. E. Configliacchi, G. Razzano, V. Rizzo and A. Vigevani, *J. Pharm. Biomed. Anal.* **15**, 123 (1996).
10. K. Wüthrich, *NMR of Proteins and Nucleic Acids*. Wiley, New York (1986).
11. E. Breitmeier and W. Voelter, *Carbon-13 NMR Spectroscopy*. VCH, Weinheim (1987).