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Short Communication

The utility of one-dimensional homonuclear Hartmann–Hahn spectroscopy (1D HOHAHA) for identifying the suprofen glucuronides fraction from an HPLC separation

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Introduction

Many drug substances that contain carboxylic acid moieties are eliminated from the body via an enzymatic pathway that results in their conjugation with glucuronic acid. Characterization of these acyl glucuronides is complicated because of the ease of acyl migration and hydrolysis. Acyl rearrangements have been well studied and this area has been reviewed by Faed [1]. Liquid chromatography (LC), mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are the standard analytical methods used for studying glucuronides [2–6]. NMR studies are very well suited for identification of positional isomers of acyl glucuronides because of the large chemical shift changes induced by acylation [7]. Additionally, differentiation of the α- and the β-anomers generally can be obtained from measurement of the proton–proton coupling constant of the anomeric proton [7]. What is needed is a NMR method that aids in the identification of the positional isomers of glucuronides within a mixture. Analysis of this type reduces the chance of hydrolysis or acyl migration as additional purification and separation steps are not required. Homonuclear Hartmann–Hahn (HOHAHA) NMR spectroscopy is well suited for the analysis of mixtures. The technique was first demonstrated by Davis and Bax [8] to assign the proton resonances from the ribose rings in a trinucleotide. They clearly showed the power of the method as a means for spectral editing and thus simplifying complicated spectra. It uses a selective 180° pulse as a means of accessing the spin network followed by a mixing period to label the protons within the coupling network. One-dimensional HOHAHA requires only one resolved proton resonance to be able to identify all the resonances within a sugar ring. For simple sugars this requirement is generally met using the anomeric proton. If needed the sequential assignment of protons can be had by varying the experimental mixing period (spin lock time) because the relay of magnetization occurs sequentially between coupled protons at a rate dependent on their coupling constant [8]. One-dimensional HOHAHA and in particular its 2D analogue [9, 10] has found wide application for structural studies of small proteins, nucleic acids and polysaccharides. For metabolite studies the 2D version may not be practical because of small sample size. Herein is demonstrated the utility of 1D HOHAHA spectroscopy for the identification of acyl glucuronides within a mixture.

The glucuronide fraction isolated from an LC column separating the components of human urine from four healthy male subjects given suprofen, (±)-α-methyl-4-(2-thienyl-

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carbonyl)benzene acetic acid, was used to illustrate the applicability of 1D HOHAHA for metabolism studies. Suprofen was a non-steroidal anti-inflammatory drug (NSAID) used for alleviation of mild to severe pain. Its metabolism has been characterized and many suprofen metabolites have been identified and are excreted predominantly via the urine [11–14]. Glucuronides are the major metabolites (>65%) isolated from human urine as determined by fast atom bombardment-mass spectrometry, FAB-MS; Glusulase® treatment (i.e. enzymatic treatment with β-glucuronidase and arylsulphatase); base hydrolysis and chromatographic methods (HPLC and TLC).

Experimental

Human studies

Four healthy male subjects were given racemic suprofen (200 mg per subject) labelled with trace levels of tritium. Urine samples were quantitatively collected for 2 days following the dose administration and a total of 85.6% of the dose was recovered. The work-up of the metabolites was performed under slightly acidic conditions.

Chromatography

LC analysis was performed at 254 nm using LiChrosorb RP-18 MPLC™ guard and analytical columns (130 × 4.6 mm i.d., 5 μm). A gradient elution method was used for each 50–100 μl sample injection. Solvent A was water containing 0.01% acetic acid, while solvent B was acetonitrile containing the same percentage of acetic acid. The solvent flow rate was maintained at 1 ml min^{-1}. The gradient program was carried out from (a) 0% B to 100% B in 20 min or (b) 0% B to 100% B in 30 min.

NMR analysis

All NMR spectra were acquired at room temperature using a Bruker AM-360 with an inverse detection upgrade. The NMR probe was a standard 5 mm proton–carbon dual probe. The spectral chemical shifts were referenced using TSP, sodium 3-(trimethylsilyl) propionate as an internal standard. The 1D HOHAHA pulse sequence reported by Subramanian and Bax [15] was used to generate purely absorptive 1D correlated spectra. A Dante pulse train [16] was used to generate the selective 180° pulse. The delay, τ_z was randomized over the range $(2\delta_{min})^{-1} \pm 100\%$ where δ_{min} is the frequency between the selectively inverted resonance and the nearest neighbour within its spin network.

Sample preparation

The enzymatic hydrolysis of the glucuronides was performed on the suprofen glucuronide mixture buffered with 1 M sodium acetate (pH 5.1) to which was added Glusulase®, a 1:4 (v/v) mixture of arylsulphatase and β-glucuronidase from *Helix pomatia* (Endo Laboratories, Inc., Wilmington, DE, USA) and incubated overnight at 37°C.

The glucuronide fraction that was isolated in an amorphous form from the urine pool by LC methods was dissolved in acetone-d_6 (MSD Isotopes) (approximately 2 mg in 0.5 ml). The material was sparingly soluble in acetone dried over sieves but upon the addition of a drop of D_2O to exchange the hydroxyl resonances the material was completely solubilized.

Results

The standard proton spectrum of the suprofen glucuronide mixture is shown in Fig. 1. The glucuronide ring protons were all located between 5.7 and 3.3 ppm. The hydroxyl protons were exchanged by the addition of D_2O (the large resonance at about 3.6 ppm). Because of the electron withdrawing effect of the two vicinal oxygens the anomeric proton was the most downfield of the sugar resonances [7] (the overlapping doublets near 5.6 ppm). The other resonances of the sugar ring were not so readily identified. When the overlapping doublets at approximately 5.6 ppm were selected out by the 1D HOHAHA method then the resulting difference spectra were obtained in Figs 2(b) and 3(b) (with and without D_2O, respectively). Only the regions of interest were plotted. These 1D HOHAHA difference spectra clearly represent a great spectral simplification compared to the normal proton spectrum. This spectral unravelling allows for more facile interpretation. Due to unfortuitous overlap of the resonances, the D_2O exchanged spectrum [Fig. 2(b)] was less useful for assignment purposes than the spectrum in Fig. 3(b) to which D_2O was not added. The resonance assignments in Fig. 3(b) were based on a comparison of experiments where the 1D HOHAHA spin lock time was varied and NOE difference data which showed NOEs from H_1

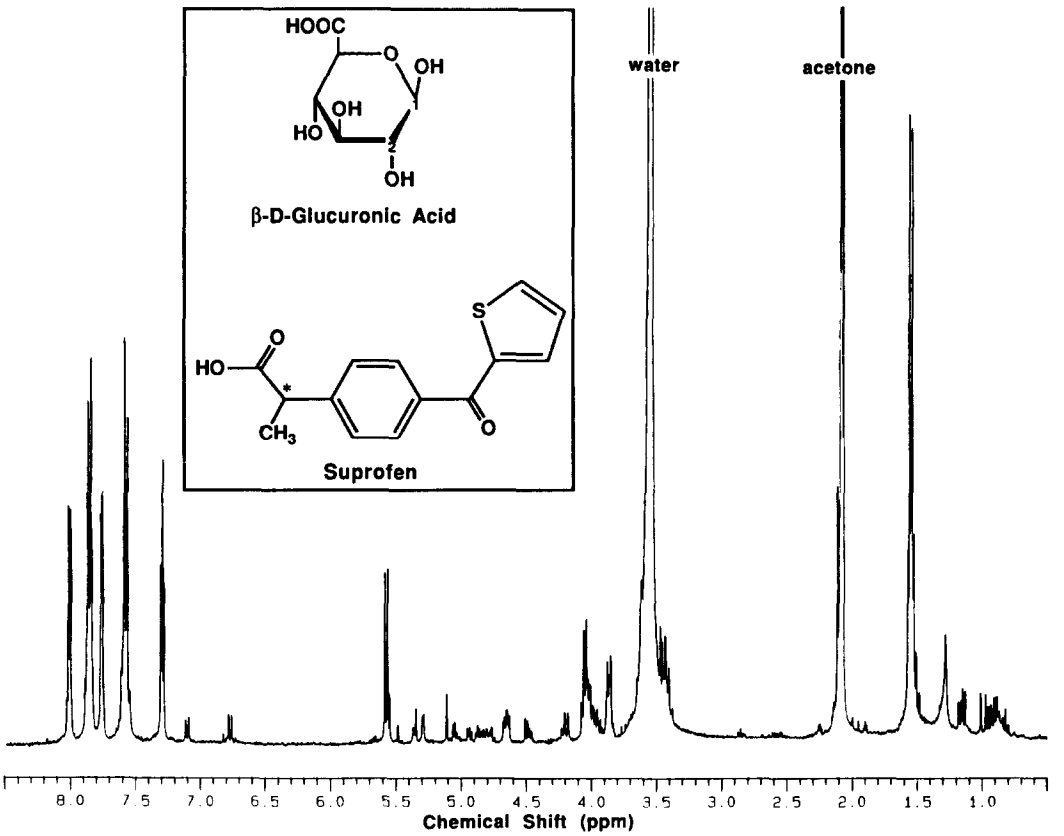

Figure 1
Standard ^1H NMR spectrum of the suprofen glucuronide fraction isolated by HPLC in acetone-d_6 plus a drop of D_2O. 7250 Hz sweep width, 32k complex data points, 2.26 s acquisition time, 1.0 s recycle delay, 30° pulse width, 1088 scans, 0.3 Hz exponential line-broadening. The insert shows the structures of suprofen and glucuronic acid.

to H_5 and H_3 (data not shown). The proton–proton couplings seen between all the proton pairs are 8–10 Hz [Fig. 3(b)]. The anomeric proton coupling value was consistent with values for β-anomers (7–10 Hz) but not α-anomers (2–4 Hz) [7, 17]. The chemical shifts and the couplings of the resonances were consistent with β-1-*O*-acyl-glucuronides. The minor component could not be selected independently of the major component because of overlap. Close inspection of the spectra (the HOHAHA difference spectrum and the normal proton spectrum) supported the assumption that the minor component has the same sugar configuration as the major component but because of overlap this could not be stated unambiguously. The overlap suggested the presence of diastereomers. Spectral simulation, although not pursued, might be used to get an approximation of the minor isomer proton–proton couplings but caution is recommended as the 1D HOHAHA difference spectra are not completely distortion free. The next ring network probed was the one associated with the doublet resonance at about 5.29 ppm (Fig. 2). The 1D HOHAHA difference spectra for this system are shown in Fig. 2(c). The difference spectrum in Fig. 3(c) was generated by selectively inverting the hydroxyl resonance. In Fig. 3(c) the hydroxyl resonance shows some broadening due to exchange whereas all other hydroxyls were flattened out by exchange broadening in the 4.5 ppm region. Since the hydroxyls underwent exchange there was a problem with magnetization being transferred to other sugar resonances not in the sugar ring selected. This was manifested in the negative peaks in Fig. 3(c) and (d) which were removed for the sake of clarity. This was not a problem when the hydroxyl protons were exchanged out with D_2O. (The dispersive peak which is cut off in the negative going direction [Fig. 2(c) and (d)] was due to incomplete subtraction of the very large water signal.) The hydroxyls selectively inverted in Fig. 3(c) and (d) were the HO-C_1 hydroxyis based on 1D

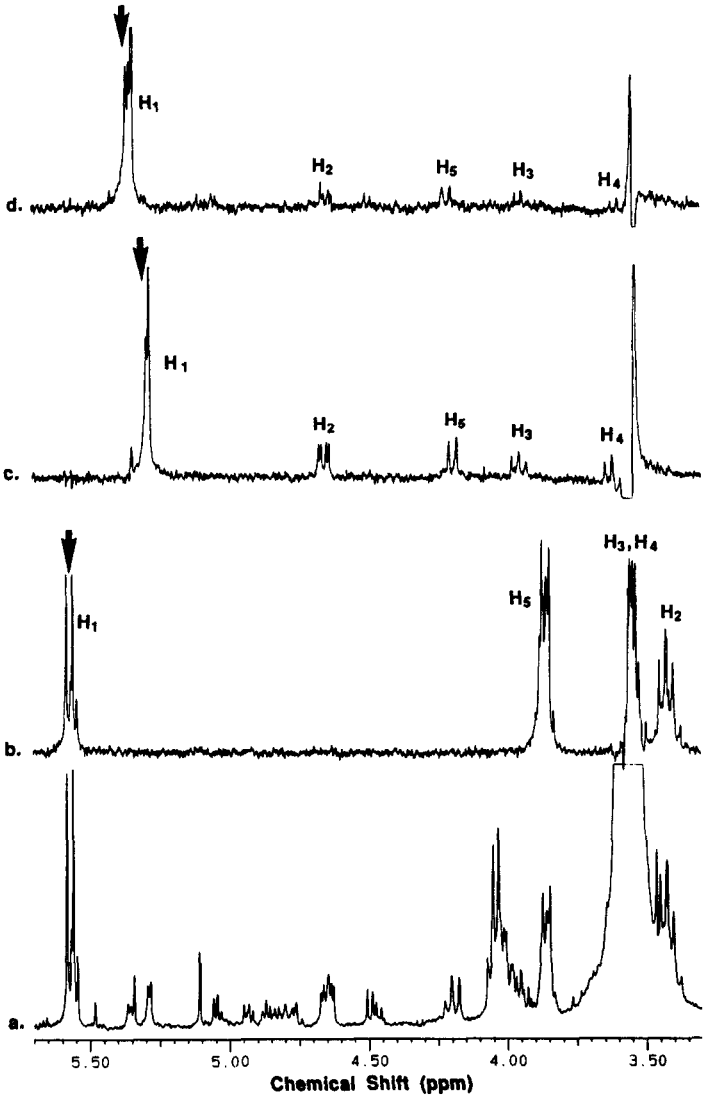

Figure 2
(a) An expansion of Fig. 1 showing the region from 3.3 to 5.7 ppm. (b–d) 1D HOHAHA difference spectra of the various spin systems selected. The arrows indicate the peaks which were selectively inverted by the Dante sequence. 4000 Hz sweep width, 32k complex data points, 4.1 s acquisition time, 0.5 s recycle delay, 7960 Hz spin lock field, 32 Hz Dante excitation bandwidth, 0.4 Hz exponential line broadening, 116 ms spin lock time (b), 161 ms spin lock time (c,d), 4096 scans (b), 16384 scans (c, d).

HOHAHA experiments using short mixing times where magnetization is transferred only to the nearest neighbour (data not shown). The large downfield shift of the H_2 proton was consistent with acylation at the C_2 position [7]. The coupling constants were consistent with an α-anomer, that is, all the couplings were large except for the coupling from the anomeric proton to the adjacent H_2 (3.4 Hz). Thus, this component of the mixture was consistent with an α-2-*O*-acyl-glucuronide. Another ring network was elucidated by examining the small doublet at approximately 5.36 ppm which was partially overlapped by an unknown singlet [Fig. 2(d)]. The subset spectra in Figs 2(d) and 3(d) showed the same coupling patterns and very similar chemical shifts to those seen in the spectra of Figs 2(c) and 3(c) suggesting diastereomeric α-2-*O*-acyl-glucuronides. The ring proton upfield of the H_5 resonance was barely observed above the noise in Figs 2(d) and 3(d); in Fig. 3(d) the partial loss of the signal was probably due to cancellation caused by (negative) transfer to the H_5 of the β-glucuronide. These four isomers (two β-1-*O*-acyl-glucuronides and two α-2-*O*-acyl-glucuronides)

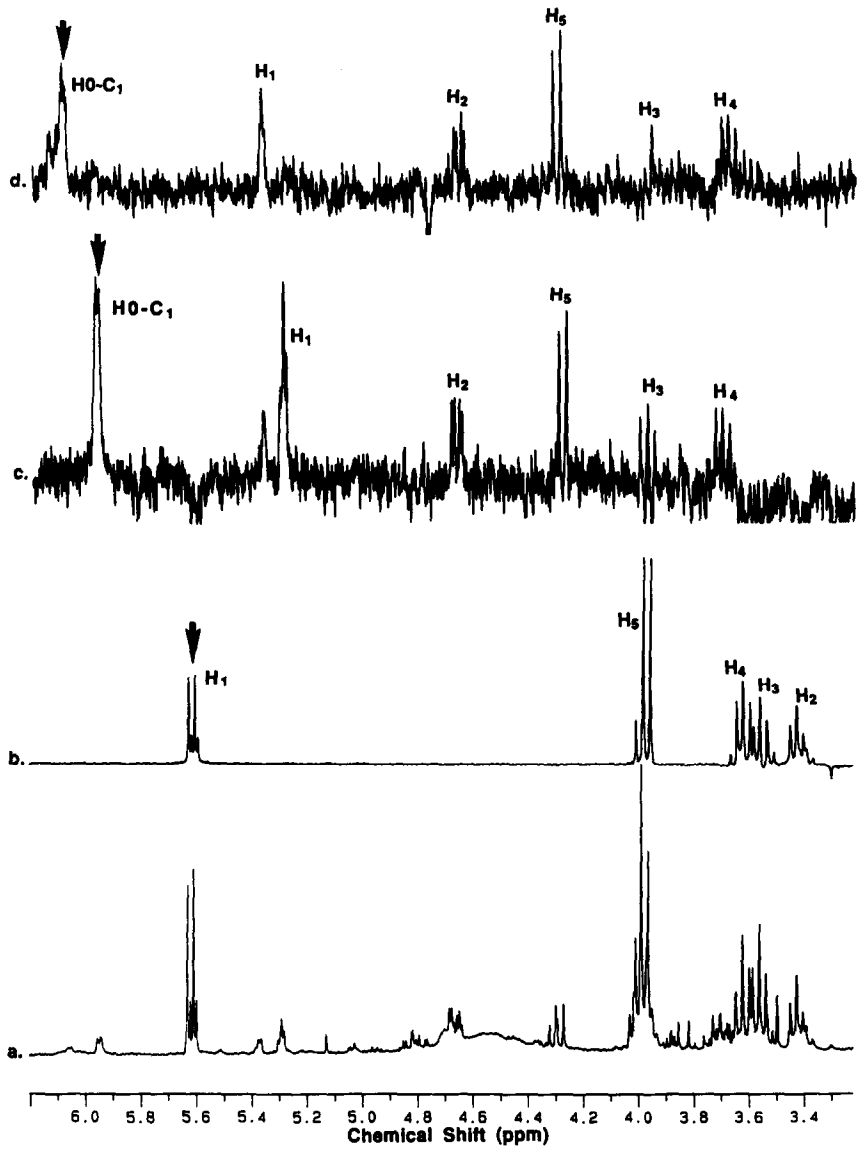

Figure 3
(a) The region from 3.3 to 6.1 ppm of a proton spectrum obtained from the sample when D_2O was not added and the acetone-d_6 was not placed over drying sieves. 7250 Hz sweep width, 32k complex data points, 2.26 s acquisition time, 45° pulse, 2.0 s recycle delay, 0.4 Hz exponential line broadening. (b–d) The 1D HOHAHA difference spectra. The arrows indicate the peaks which were selectively inverted by the Dante sequence. 2.0 s recycle delay, 2.26 s acquisition time, 90° pulse width = 21.5 us (also used for MLEV17 pulse train), 35 Hz Dante excitation bandwidth, 128, 175, 192 mixing time (b, c, d, respectively) at a field strength of 7960 Hz and 4096, 6400, 19200 scans (b, c, d, respectively).

accounted for most of the sugar resonances seen in the normal proton spectrum [Figs 2(a) and 3(a)]. The ratio of the β to the α forms was about 4:1. The difference in intensities between the two β (or α) forms was approximately 3:1. The smaller components observed in the sugar region were not characterized.

The LC profile of the sample used for the 1D HOHAHA experiments is shown in Fig. 4(a) and (b) (fraction VII). Some closely eluting peaks (the suprofen glucuronides) are observed along with some residual suprofen. After Glusulase® treatment of the sample three peaks were separated [Fig. 4(c)]. One peak eluted with the same retention time as suprofen and two small peaks had the same retention times as the original glucuronides. This was consistent with a mixture of glucuronides having both α and β linkages since Glusulase® treatment cleaves only β-1-*O* type linkages. Hydrolysis of both alpha and beta linkages by alkaline treatment resulted in only

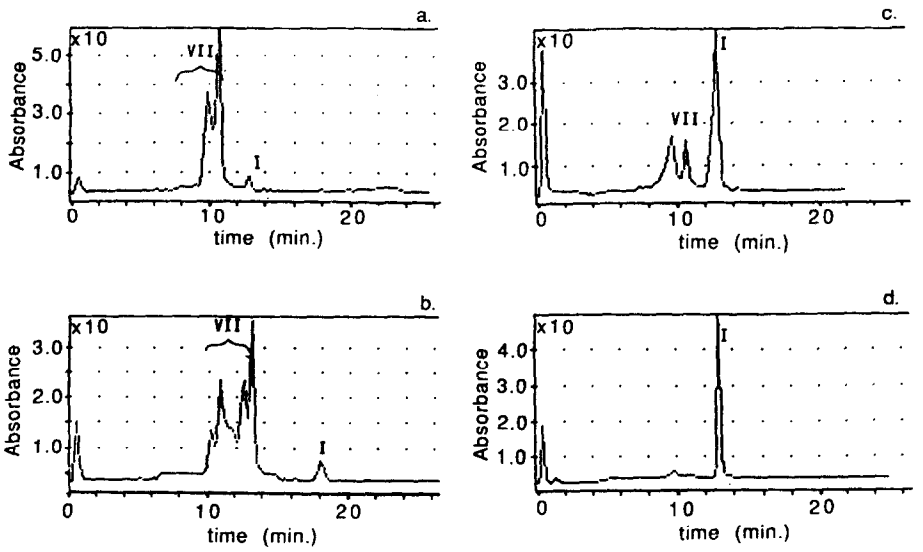

Figure 4
HPLC profiles of the suprofen glucuronide mixture before and after hydrolysis. (a) and (b) present a comparison of the HPLC profiles for the suprofen glucuronide mixture using different elution gradients (gradients A and B respectively, listed in the Experimental). The HPLC profiles (using gradient A) after Glusulase® treatment and alkaline hydrolysis of the sample are shown in (c) and (d), respectively.

one peak which co-eluted at the retention time of suprofen [Fig. 4(d)]. For fraction VII the FAB-MS results gave a molecular weight of 436 (data not shown).

Discussion

Using 1D HOHAHA four suprofen glucuronides were identified, two with β linkages and two with α linkages. These results were consistent with the HPLC and hydrolyses data. The NMR data did not show a 50:50 distribution of the diastereomers as noted by the integrated areas under the anomeric proton resonances. This unequal distribution might be explained by stereoselective metabolism. The stereoselective metabolism of 2-aryl propionates has been reviewed in the literature [18]. Selective racemization was reported *in vivo* for suprofen in plasma [19] but liver experiments *in vitro* showed that the elimination of racemic suprofen was not stereoselective and there was no inversion measured during (*R*)-suprofen perfusion [18]. The plasma studies showed that suprofen was stereospecifically inverted an average of 6.8% from the *R*-isomer to the *S*-isomer. There was no inversion measured in the opposite direction.

Conclusion

The use of 1D HOHAHA clearly showed that great spectral simplification could be obtained from a mixture of glucuronides and that reasonable conclusions could be drawn from the data in conjunction with other sources of information about the sample (e.g. hydrolysis experiments or mass spectral data). A limitation of the technique is that the molecule must contain a coupled network of protons. If the proton–proton coupling constants become very small then the magnetization transfer may get bottle-necked so that there may be difficulties observing all the protons within the spin network. Samples containing less than 1 mg per component are amenable for study by this technique using available high field spectrometers.

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References

[1] E.M. Faed, *Drug Metab. Rev.* **15**, 1213–1249 (1984).
[2] J. Hansen-Møller, C. Cornett, L. Dalgaard and S.H. Hansen, *J. Pharm. Biomed. Anal.* **6**, 229–240 (1988).
[3] I.A. Muhiudeen, T.A.W. Koerner, B. Samuelsson, Y. Hirabayashi, R. DeGasperi, S.-C. Li and Y.-T. Li, *J. Lipid Res.* **25**, 1117–1123 (1984).
[4] J. Hellou, J.H. Benoub and H.J. Hodder, *Chemosphere* **16**, 1381–1386 (1987).
[5] P. Gallice, J.P. Monti, A. Crevat, C. Durand and A. Murisasco, *Clin. Chem.* **31**, 30–34 (1985).
[6] P.C. Smith and L.Z. Benet, *Drug Metab. Disp.* **14**, 503–505 (1986).
[7] L.M. Jackman and S. Sternhell, in *Applications of Nuclear Magnetic Resonance Spectroscopy in Organic*

Chemistry, 2nd edn, p. 176. Pergamon Press, New York (1969).
[8] D.G. Davis and A. Bax, *J. Am. Chem. Soc.* **107**, 7197–7198 (1985).
[9] A. Bax and D.G. Davis, *J. Magn. Reson.* **65**, 355–360 (1985).
[10] L. Braunschweiler and R.R. Ernst, *J. Magn. Reson.* **53**, 521–528 (1983).
[11] Y. Mori, Y. Sakai, N. Kuroda, F. Yokoya, K. Toyoshi, M. Horie and S. Baba, *Drug Metab. Disp.* **12**, 767–771 (1984).
[12] Y. Sakai, Y. Mori, K. Toyoshi, M. Horie and S. Baba, *Drug Metab. Disp.* **12**, 795–797 (1984).
[13] Y. Mori, N. Kuroda, Y. Sakai, F. Yokoya, K. Toyoshi and S. Baba, *Drug Metab. Disp.* **13**, 239–245 (1985).
[14] Y. Mori, F. Yokoya, K. Toyoshi, S. Baba and Y. Sakai, *Drug Metab. Disp.* **11**, 387–391 (1983).
[15] S. Subramanian and A. Bax, *J. Magn. Reson.* **71**, 325–330 (1987).
[16] G.A. Morris and R. Freeman, *J. Magn. Reson.* **29**, 433–462 (1978).
[17] K. Yoshimoto, K. Tahara, S. Suzuki, K. Sasaki, Y. Nishikawa and Y. Tsuda, *Chem. Pharm. Bull.* **27**, 2661–2674 (1974).
[18] J.M. Mayer, *Acta Pharm. Nord.* **2**, 197–216 (1990).
[19] Y. Shinohara, M. Hiroshi and S. Baba, *J. Pharm. Sci.* **80**, 1075–1078 (1991).

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