J. Pharm. Pharmacol. 1993, 45: 559–562
Received June 1, 1992

Application of the WATR Technique for Water Suppression in ^{1}H NMR Spectroscopy in Determination of The Kinetics of Hydrolysis of Neostigmine Bromide in Aqueous Solution

ABU J. FERDOUS* AND ROGER D. WAIGH†

Department of Pharmacy, University of Manchester, Manchester M13 9PL, UK

Abstract—Both ammonium chloride and guanidinium chloride were used to secure water suppression in ^{1}H NMR spectra using the 'Water Attenuation by T_2 Relaxation' (WATR) technique. The effect of phosphate buffer in the suppression was investigated over a range of pH values at 80 MHz. The spin–spin relaxation time of water protons at 80 MHz was found to reach a minimum at pH 7·3 in the presence of 0·1 M phosphate buffer and 1 M guanidinium chloride; these conditions were therefore chosen for subsequent use of the WATR technique in a study of the kinetics of hydrolysis of neostigmine bromide. The method was found to be very convenient for studies of the hydrolysis of this representative amide.

Nuclear magnetic resonance (NMR) spectroscopy has been widely used in the study of drug degradation rates (Mitsumori et al 1977; Baltzer et al 1979; Degelaen et al 1979; Nishikawa et al 1987). As an analytical tool it offers various advantages compared with other methods of analysis (Ferdous 1989). Drugs undergoing hydrolytic degradation in aqueous solution are not amenable to direct ^{1}H NMR spectroscopic analysis; the high concentration of water protons (ca. 110 M) causes dynamic range problems in recording the NMR spectra and the huge water peak obscures or distorts all nearby weak solute peaks. The problem of removing the water peak from the spectra of aqueous solutions has been successfully overcome by using the 'Water Attenuation by T_2 Relaxation' (WATR) method (Rabenstein et al 1985). Using this method, peaks in the region of δ 4–5 ppm can be easily observed (Rabenstein & Fan 1986; Dickinson et al 1987). Various relaxation agents have been used to suppress the water peak. In the present study the relaxation effect of ammonium chloride and guanidine hydrochloride and the effect of pH on water suppression are reported. As an extension of the use of the technique in studies of ester hydrolysis (Ferdous et al 1991), the method has been used to study the hydrolysis rate of a representative amide, neostigmine bromide.

Materials and Methods

Ammonium chloride (BDH, Poole, Dorset), guanidine hydrochloride (Aldrich, Gillingham), acetamide (BDH), potassium dihydrogen phosphate and sodium hydroxide were of analytical grade. Deuterium oxide (99·88%D) was obtained from Fluorochem (Glossop). Neostigmine bromide (Aldrich) was used without further purification. Distilled water was used in all experiments.

The spin–spin relaxation time (T_2) was measured using the CPMG pulse sequence (Carr & Purcell 1954; Meiboom & Gill 1958). The sample solution contained 5% (v/v) D_2O and a measured amount of relaxation agent (ammonium chloride or guanidine hydrochloride). The solution contained buffer salts to stabilize pH. About 0·5 mL solution was used in a 5-mm diameter NMR tube. The CPMG data were obtained as a stack plot. Digital data printouts were used to calculate T_2 by least square regression analysis.

The water suppressed ^{1}H NMR spectra of neostigmine bromide samples in aqueous solution were recorded using the WATR method (Ferdous et al 1991). The experiments were carried out on a Bruker WP80 NMR spectrometer operating at 80 MHz at 20°C.

The degradation of neostigmine bromide was studied at four different temperatures at pH 9·3. Water (50 mL) was adjusted to the desired pH. A flask containing 45 mL of this solution was placed in the water bath and kept there until it reached the required temperature. Neostigmine bromide (1% w/v) was measured and dissolved in the remaining 5 mL solution and the pH was adjusted. The two solutions were mixed and transferred to a jacketed vessel through which hot water was circulated to maintain the desired temperature. The jacketed vessel was placed in a pH-stat and the pH was constantly monitored. As the degradation continued the pH of the solution was kept constant by adding sodium hydroxide solution (0·7–1·4 M) automatically. Samples were collected at the beginning and at timed intervals. Each sample was placed in a screw-cap tube, instantly frozen in liquid nitrogen and kept at −6°C until the spectrum was recorded. Before obtaining the spectrum, the sample was thawed and a solution was added containing acetamide (0·075% w/v) as internal standard, 0·1 M phosphate buffer, 5% (v/v) D_2O for an NMR lock signal and 1 M guanidine hydrochloride as relaxation agent. The final pH of the solution was adjusted to 7·3. A total of 32 scans was accumulated for each spectrum with a total delay of 0·8 s.

After acquisition of NMR spectra, digital printouts were obtained containing peak area (integral) for each chemical shift. The integral of the two singlets at δ 3·1 ppm was normalized with reference to the integral of the internal standard acetamide peak at δ1·9 ppm. Three spectra were recorded for each sample. The integrals for the peak from

* Present address: Department of Pharmacy, University of Dhaka, Dhaka 1000, Bangladesh.

† Present address and correspondence: R. D. Waigh, Department of Pharmaceutical Sciences, University of Strathclyde, Glasgow G1 1XW, UK.

each spectrum after normalization were added together and the average value was taken. The ln integral values were plotted against time for each degradation experiment. The slope of the line of best fit was computed which gave the first order rate constant for that degradation experiment. All experiments were performed in triplicate and the average value was recorded.

Results and Discussion

The spin–spin relaxation time (T_2) of water protons was determined in varying concentrations of phosphate buffer and at two different pH values. The change in T_2 of water protons in the presence of 0·25 M ammonium chloride as relaxation agent and increasing concentration of phosphate buffer at pH 5·5 and 6·0 is shown in Fig. 1. The change in T_2 of water protons with increasing concentration of phosphate buffer in the presence of 1 M ammonium chloride at pH 5·5 is shown in Fig. 2. For Fig. 1, two pH values were chosen because the relaxation effect of ammonium chloride was maximum around the pH range 5·5–6·0 (Dickinson et al 1987). The T_2 of water protons increased with increasing phosphate buffer up to a maximum (0·35–0·45 M; Figs 1, 2); T_2 then slightly decreased with further increase of phosphate buffer. The spin–spin relaxation times are slightly longer at pH 6·0 than at pH 5·5 in the presence of the same

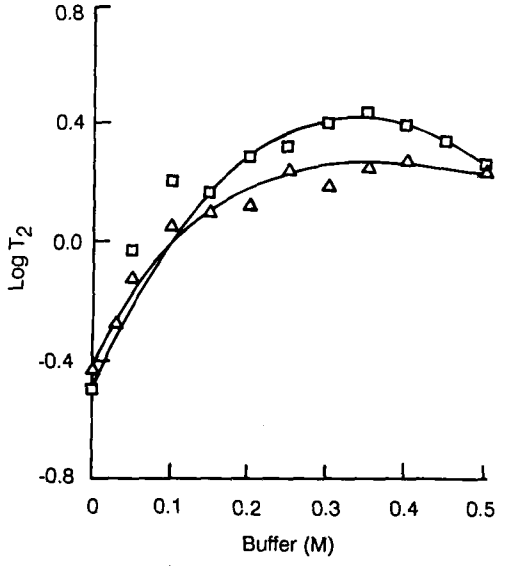

FIG. 1. Ln T_2 of water protons (in presence of 0·25 M ammonium chloride at pH 5·5 (△) and 6·0 (□)) plotted as a function of phosphate-buffer concentration.

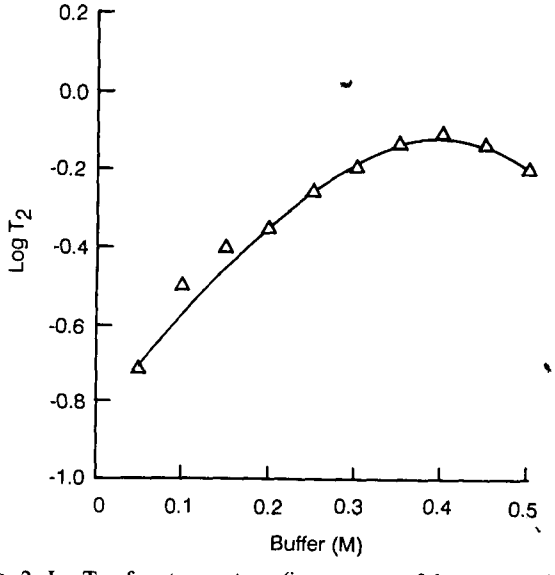

FIG. 2. Ln T_2 of water protons (in presence of 1 M ammonium chloride at pH 5·5) plotted as a function of phosphate-buffer concentration.

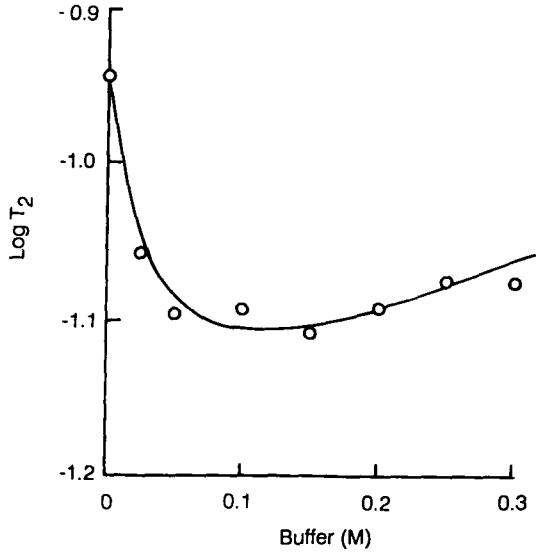

FIG. 3. Ln of spin–spin relaxation time (T_2) of water protons (in presence of 1 M guanidine hydrochloride at pH 7·2) plotted as a function of phosphate-buffer concentration.

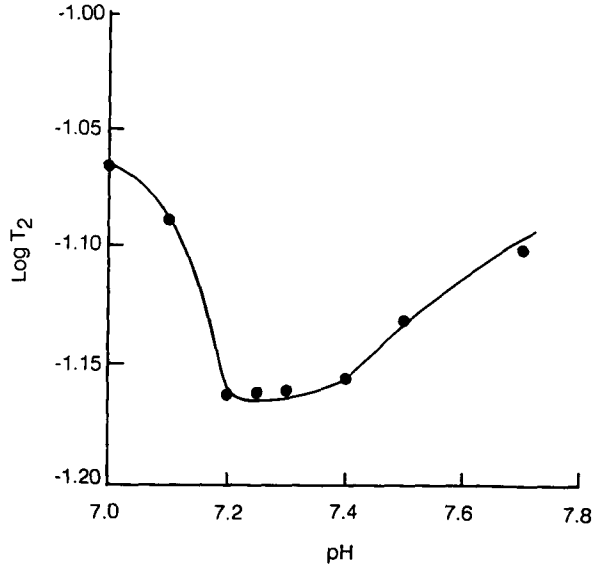

FIG. 4. pH dependence of Log T_2 of water protons in the presence of 1 M guanidine hydrochloride and 0·1 M phosphate buffer.

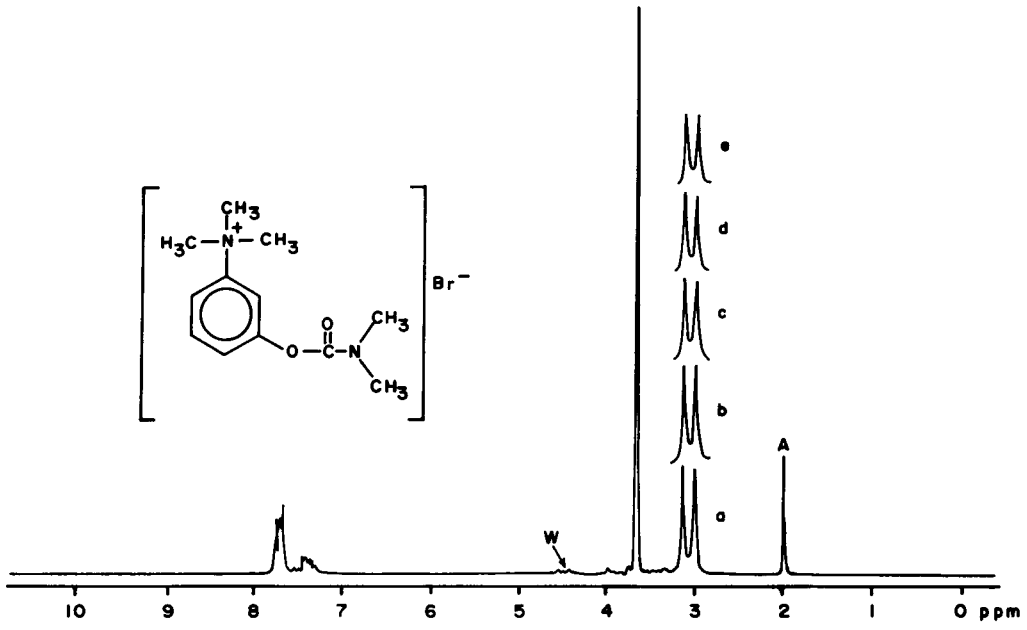

FIG. 5. Water suppressed ^{1}H NMR spectrum of neostigmine bromide before hydrolysis and (inserts) the double singlets at δ 3·1 ppm after (a) 0, (b) 30, (c) 60, (d) 120 and (e) 180 min. Samples were degraded at 70°C. W marks the suppressed water peak, and A acetamide peak. The structure of neostigmine bromide is given in the insert.

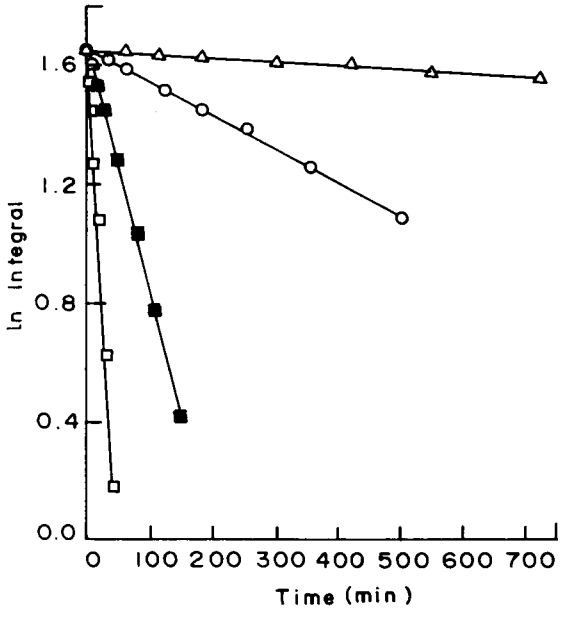

FIG. 6. Plot of ln integral of double singlet at δ 3·1 ppm against time for neostigmine bromide degradation at four different temperatures and at pH 9·3. △ 60, ○ 70, ■ 80 and □ 88°C.

Table 1. First order rate constants for hydrolysis of neostigmine bromide at pH 9·3.

Temperature (°C)	K (min^{-1})	Log K	s.d.
60	$1{\cdot}38947 \times 10^{-4}$	−3·857144	0·01134
70	$1{\cdot}13133 \times 10^{-3}$	−2·946410	0·01215
80	$8{\cdot}39509 \times 10^{-3}$	−2·075974	0·01956
88	$3{\cdot}83084 \times 10^{-2}$	−1·416706	0·01057

Table 2. Activation energy for hydrolysis of neostigmine bromide.

Temperature (°C)	pH	Activation energy (kJ mol^{-1})	Source
70–90	7·6	101·1	Porst & Kny (1985)
10–45	13·0	14·0*	Christenson (1964)
60–88	9·3	43·9	Present study

* Calculated from Christenson (1964).

concentration of phosphate buffer and ammonium chloride. Increasing the ammonium chloride concentration from 0·25 to 1 M significantly decreased the relaxation time (Fig. 2) which is in accordance with previous findings (Dickinson et al 1987). Phosphate buffer at pH 5·5 is slightly below its recommended buffering range. Phosphate buffers at or above pH 5·5 can be used in obtaining the WATR spectra, but a higher concentration of ammonium chloride would be necessary at higher pH values for complete suppression of the water signal.

The effect of phosphate buffers on T_2 of water protons in the presence of 1 M guanidine hydrochloride is shown in Fig. 3. T_2 decreased with increase of phosphate buffer up to 0·05 M. In the range of 0·05–0·2 M phosphate buffer, T_2 was almost constant. Above that concentration, there was a slight increase in the T_2 of the water protons. Therefore, subsequently the WATR spectra of all compounds were measured in 0·1 M phosphate buffer. The relaxation effect of guanidine hydrochloride was greater than that of ammonium chloride (Dickinson et al 1987). Guanidine hydrochloride was also found to be less susceptible to interference from phosphate

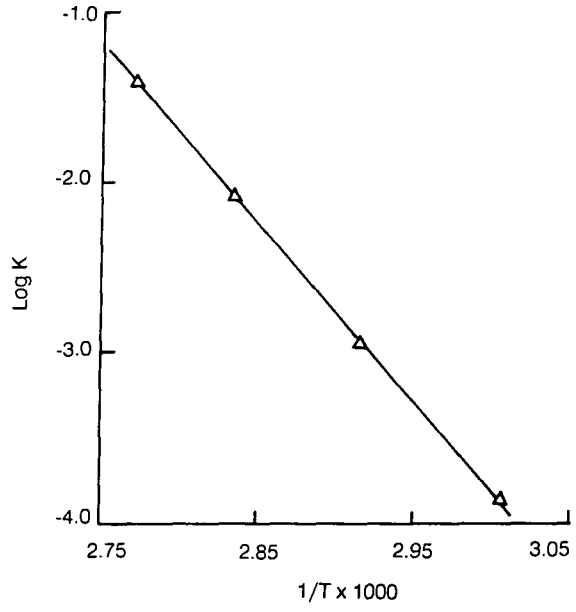

FIG. 7. Arrhenius plot of ln K (min^{-1}) against reciprocal of absolute temperature of neostigmine hydrolysis degradation.

buffers (Fig. 3), so guanidine hydrochloride was used as the relaxation agent to suppress the water peak in all subsequent experiments.

The T_2 of water protons in 0·1 M phosphate buffer and 1 M guanidine hydrochloride in the pH range 7·0–7·7 was determined: the results are presented in Fig. 4. In this pH range in unbuffered solution Dickinson et al (1987) found T_2 to be at a minimum. In phosphate buffer, the T_2 was found to be at a minimum in the pH range 7·2–7·4. Above and below the pH range, T_2 increased significantly. Therefore, pH 7·3 was chosen as the optimum pH for obtaining WATR spectra in the presence of 0·1 M phosphate buffer and 1 M guanidine hydrochloride as the relaxation agent.

The hydrolysis rate of neostigmine bromide was determined at 60, 70, 80 and 88°C at pH 9·3. The WATR spectrum was recorded for each sample obtained during each degradation experiment. Since spectra were obtained at pH 7·3, at which pH hydrolysis is likely to be slow (compare data in Table 2) and at ambient temperature, ca. 20°C (compare data in Fig. 6), it can be assumed that hydrolysis during acquisition of the WATR spectra would be negligible. A typical spectrum of neostigmine bromide at the beginning of degradation is shown in Fig. 5. The integral of the two methyl singlets at δ 3·1 ppm thus obtained was normalized with the integral of the peak of the internal standard acetamide. The amide methyl peaks at δ 3·1 ppm were chosen because they were well separated from the peak for dimethylamine at ca. δ 2·7, present after hydrolysis. The ln integral (after normalization) was then plotted against time (Fig. 6). The slope of each line gave the rate constant (K) for hydrolysis (Table 1). The Arrhenius plot (Fig. 7) was obtained by plotting the rate constant for hydrolysis against the reciprocal of absolute temperature. From the slope of the line, the activation energy was calculated. The results thus obtained can be compared (Table 2) with those reported by Christensen (1964) and Porst & Kny (1985). The differences in Table 2 are due to temperature, pH, buffer salts and drug concentration. At low pH, more energy is required for hydrolysis, so the activation energy is high; at higher pH values the activation energy is lower.

The WATR technique has the advantage over chromatographic procedures in that there is little method development, and separation of degraded products from the parent compound is not necessary. The method is rapid and straightforward and both undegraded and degraded products can be analysed and quantified easily. In this experiment an NMR spectrometer of relatively low sensitivity and resolving power was used. If an instrument of higher field strength were used then greater spectral resolution and better signal-to-noise ratio would be obtained which would further improve the scope of this technique.

Acknowledgements

We thank the Commonwealth Scholarship Commission for financial support of A. J. Ferdous.

References

Baltzer, B., Lund, F., Rustrup-Anderson, N. (1979) Degradation of mecillinam in aqueous solution. J. Pharm. Sci. 68: 1207–1215

Carr, H. Y., Purcell, E. M. (1954) Effects of diffusion on free precession in nuclear magnetic resonance experiments. Phys. Rev. 94: 630–639

Christenson, I. (1964) Alkaline hydrolysis of some carbamic acid esters. Acta Chem. Scand. 18: 904–922

Degelaen, J. P., Loukas, S. L., Feeney, J., Roberts, G. C. K., Burgen, A. S. V. (1979) A nuclear magnetic resonance study of degradation of Penicillin-G in acidic solution. J. Chem. Soc. Perkin Trans. II: 86–90

Dickinson, N. A., Lythgoe, R. E., Waigh, R. D. (1987) Some observations on the WATR method for water suppression at 80 MHz. Magn. Reson. Chem. 25: 996–997

Ferdous, A. J. (1989) NMR Spectroscopy in the Study of Drug Degradation Rates. Ph D Thesis, University of Manchester, UK

Ferdous, A. J., Dickinson, N. A., Waigh, R. D. (1991) ^{1}H NMR as an analytical tool for the investigation of hydrolysis rates: a method for rapid evaluation of the shelf-life of aqueous solutions of drugs with ester groups. J. Pharm. Pharmacol. 43: 860–862

Meiboom, S., Gill, D. (1958) Modified spin-echo method for measuring nuclear relaxation times. Rev. Sci. Instrum. 29: 688–691

Mitsumori, F., Arata, Y., Fujiwara, S., Muranaka, M. (1977) NMR study of spontaneous degradation of penicillin G in aqueous solution. Bull. Chem. Soc. Japan 50: 3164–3166

Nishikawa, J., Watanabe, F., Shudou, M., Terui, Y., Narisada, M. (1987) ^{1}H NMR study of degradation mechanisms of oxacepham derivatives with various 3′-substituents in alkaline solution J. Med. Chem. 30: 523–527

Porst, H., Kny, L. (1985) Zur kinetic des neostigmineabbaus in wasringer losung. Pharmazie 40: 713–717

Rabenstein, D. L., Fan, S. (1986) Proton nuclear magnetic resonance spectroscopy of aqueous solutions: complete elimination of the water resonance by spin-spin relaxation. Anal. Chem. 58: 3178–3184

Rabenstein, D. L., Fan, S., Nakashima, T. T. (1985) Attenuation of the water resonance in Fourier transform ^{1}H NMR spectra of aqueous solutions by spin–spin relaxation. J. Magn. Reson. 64: 541–546