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N^4 -alkyloxycarbonyl derivatives of cytosine: physicochemical characterisation, and cytosine regeneration rates and release from alginate acid gels

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Abstract

Nucleobase containing compounds might constitute a potential alternative to conventional antibiotics in the treatment of *Helicobacter pylori* infections. N^4 -alkyloxycarbonyl-cytosine derivatives were synthesized and subjected to basic physicochemical characterisation including assessment of hydrolytic stability in various matrices. pH-rate profiles of selected compounds (range 0–12) were constructed. Hydrolysis of the derivatives in slightly alkaline solution (60 °C) resulted in quantitative conversion to parent cytosine whereas at acidic pH (60 °C) liberation of cytosine was in most cases accompanied by the parallel formation of uracil. Interestingly the lipophilic N^4 -adamantylloxycarbonyl-cytosine prodrug exhibited a half-life of 41 min (pH 1.1 at 37 °C) with quantitative conversion to parent cytosine, the degradation rate being approximately 200 times faster than that of the non-cyclic aliphatic derivatives investigated. The presence of pig stomach homogenates, pepsin A and *H. pylori* did not have a noteworthy catalytic effect on the hydrolysis of the derivatives. The release of parent cytosine was markedly delayed from alginate acid gels loaded with the acid-labile and poorly soluble ADC prodrug as compared to gels loaded with parent cytosine. © 2004 Elsevier B.V. All rights reserved.

Keywords: Prodrug; Cytosine; N -alkyloxycarbonyl derivatives; Solubility; Lipophilicity; Hydrolysis; *Helicobacter pylori*; Ulcer; Release

1. Introduction

Helicobacter pylori, a spiral bacterium colonizing the gastric mucus, is recognized to be a major cause of gastric ulcer

Abbreviations: MC, N^4 -methyloxycarbonyl-cytosine; EC, N^4 -ethyl-oxycarbonyl-cytosine; NBC, N^4 -*n*-butyloxycarbonyl-cytosine; IBC, N^4 -isobutyloxycarbonyl-cytosine; NC, N^4 -neopentylloxycarbonyl-cytosine; HC, N^4 -hexyloxycarbonyl-cytosine; OC, N^4 -octyloxycarbonyl-cytosine; ADC, N^4 -adamantylloxycarbonyl-cytosine; BC, N^4 -benzyloxycarbonyl-cytosine

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(Hopkins et al., 1996). To eradicate *H. pylori* conventional antibiotics are generally used. In light of the fact that pathogenic bacteria may develop resistance towards conventional antibiotics (Perri et al., 2003), nucleobase containing compounds such as cytosine nucleosides (Isono, 1991) and peptide nucleic acids (Good and Nielsen, 1998) might constitute a potential therapeutic alternative.

Various derivatives of the exocyclic amino group (N^4) in cytosine containing compounds have been prepared and evaluated as prodrugs: (i) *N*-mannich bases (Koch and Sloan, 1987); (ii) amides (Miwa et al., 1990; Choe et al., 2002); (iii) alkyloxycarbonyl derivatives (Choe et al., 2002) and

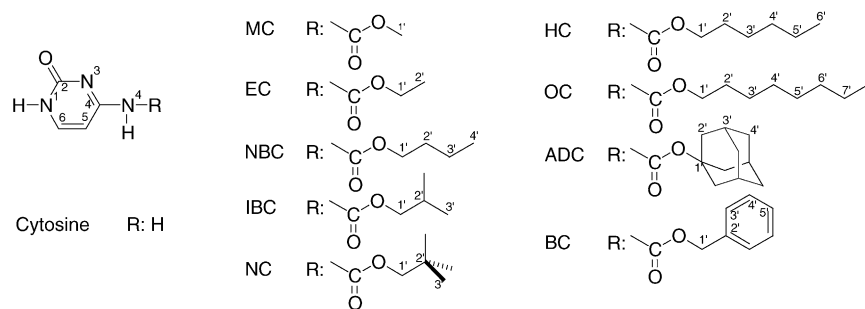


Fig. 1. Structure of cytosine and the N^4 -alkyloxycarbonyl-derivatives of cytosine.

(iv) dialkylamino-methylene derivatives (Kerr and Kalman, 1994). Capecitabine is a pentyloxycarbonyl prodrug derivative where drug release involves deacylation by a carboxylesterases (Shimma et al., 2000).

Antibiotics used to eradicate *H. pylori* are usually administered in the form of conventional oral dosage forms. The concentration of the antibiotics at the target site is thus mainly dependent on drug transport/diffusion from the blood into the gastric lumen (exsorption). In contrast, transport of an antibiotic from the gastric lumen into the mucus through the mucus layer has been suggested to be more effective in the management of such infections (Kimura et al., 1995). Thus, by employing a delivery system exhibiting prolonged retention and drug release in the gastric environment the therapeutic value of the antibiotics might be enhanced. Alginate gel formulations have been shown to provide gastric retention for up to 6 h (Whitehead et al., 1998). The prolonged gastric residence time of alginate eventually admixed with cationic polymers like chitosan has been ascribed to mucoadhesive properties of the polymer systems (Bernkop-Schnurch et al., 2001).

In the present study the nucleobase cytosine has been used as a model compound for antibiotics containing cytosine substructures. The aim of this study was to characterise synthesized N^4 -alkyloxycarbonyl derivatives of cytosine (Fig. 1) with regard to physicochemical properties, chemical stability, and stability in biological matrices. In addition, preliminary data of drug release from alginate gels formed in situ are reported.

2. Materials and methods

2.1. Materials

Cytosine and *N,N*-dimethylacetamide (DMA) were purchased from Bie & Berntsen, Copenhagen, Denmark. *n*-Octanol was obtained from VWR International, Copenhagen, Denmark. Alkyl haloformates and pyridine were purchased from Sigma–Aldrich, Copenhagen, Denmark. Sodium alginate (Protanal LF200DL) was a gift from FMC BioPolymer, Drammen, Norway. Chemicals for preparation of buffers and HPLC mobile phases were of analytical grade. Demineralised water was used throughout. The buffers used were acetate

(pH 4 and 5), phosphate (pH 3, 6 and 7.4), borate (pH 8.5 and 9.5) and carbonate (pH 11). At pH-values below 2 and above 12, hydrochloric acid and sodium hydroxide was used, respectively. A constant ionic strength of the buffer solutions ($\mu = 0.5$) was maintained by addition of calculated amounts of potassium chloride.

2.2. General procedure for synthesis of N^4 -alkyloxycarbonyl-cytosine derivatives

The N^4 -alkyloxycarbonyl derivatives were synthesized according to a procedure for synthesis of N^4 -benzyloxycarbonyl-cytosine (Dueholm et al., 1994) with modifications. The respective alkyl chloroformate (0.036 mol) was added over a period of 1 h to a suspension of cytosine (0.018 mol) in dry pyridine (100 ml) at -10 to 0 °C under argon. In case of the N^4 -adamantylloxycarbonyl derivative, the fluoroformate was used. The mixture was allowed to heat to room temperature and stirred overnight. The suspension was evaporated to dryness in vacuo. Water (20 ml) was added and pH was adjusted to 1 with 4 M hydrochloric acid. The resulting white precipitate was filtered off, washed with water, boiled with absolute ethanol (50–100 ml) for 10 min, cooled to 10 °C, filtered, washed with ether and dried in vacuo over phosphorous(V)-oxide.

The derivatives were subjected to elemental analysis, melting points (differential scanning calorimetry), mass spectrometry and NMR spectrometry. NMR spectra were obtained at 25 °C on a Bruker AMX 400 or Bruker AV 600 spectrometer (proton frequency 400.13 and 600.13 MHz) in DMSO- d_6 . Chemical shift values (δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS) used as an internal standard. Coupling constants (J) are given in Hz, and multiplicities are reported as apparent splittings abbreviated as s (singlet), d (doublet), t (triplet), q (quartet), p (pentet), n (nontet), m (multiplet), and br (br). Due to solubility limitations, ^{13}C NMR spectra were not recorded for all the derivatives.

N^4 -methyloxycarbonyl-cytosine (MC): 17% yield. Anal. Calcd. ($\text{C}_6\text{H}_7\text{N}_3\text{O}_3$): C, 42.61; H, 4.17; N, 24.84. Found: C, 42.57; H, 4.16; N, 24.63. Mp: 270.6 °C (275 – 280 °C (Nery, 1969)). MS: $m/z = 169.9$ (MH^+). ^1H NMR (400.13 MHz, DMSO- d_6): δ 3.67 (s, 3H, H-1'); 6.92 (d, $J = 7.1$ Hz, 1H,

H-5); 7.77 (d, $J = 7.1$ Hz, 1H, H-6); 10.66 (br, s, 1H, NH); 11.40 (br, s, 1H, NH). ^{13}C NMR (100.6 MHz, DMSO- d_6): δ 52.3 (C-1'); 93.3 (C-5); 146.6 (C-6); 155.7 (C-2); 163.5 (C-4) NCOO was not observed due to exchange broadening.

N^4 -ethylloxycarbonyl-cytosine (EC): 92% yield. Anal. Calcd. ($\text{C}_7\text{H}_9\text{N}_3\text{O}_3$): C, 45.90; H, 4.95; N, 22.94. Found: C, 45.73; H, 4.93; N, 22.78. Mp: 280.7 °C (302–308 °C (Nery, 1969); decompose above 290 °C (Vincze et al., 1973)). MS: $m/z = 183.9$ (MH⁺). ^1H NMR (400.13 MHz, DMSO- d_6): δ 1.22 (t, $J = 7.1$ Hz, 3H, H-2'); 4.13 (q, $J = 7.1$ Hz, 2H, H-1'); 6.91 (d, $J = 7.1$ Hz, 1H, H-5); 7.77 (d, $J = 7.1$ Hz, 1H, H-6); 10.50 (br, s, 1H, NH); 11.41 (br, s, 1H, NH).

N^4 -*n*-butyloxycarbonyl-cytosine (NBC): 41% yield. Anal. Calcd. ($\text{C}_9\text{H}_{13}\text{N}_3\text{O}_3$): C, 51.18; H, 6.20; N, 19.89. Found: C, 50.93; H, 6.16; N, 19.70. Mp: 297.6 °C. MS: $m/z = 211.9$ (MH⁺). ^1H NMR (400.13 MHz, DMSO- d_6): δ 0.90 (t, $J = 7.3$ Hz, 3H, H-4'); 1.35 (m, 2H, H-3'); 1.58 (m, 2H, H-2'); 4.09 (t, $J = 6.7$ Hz, 2H, H-1'); 6.89 (d, $J = 7.1$ Hz, 1H, H-5); 7.76 (d, $J = 7.1$ Hz, 1H, H-6); 10.48 (br, s, 1H, NH); 11.35 (br, s, 1H, NH).

N^4 -isobutyloxycarbonyl-cytosine (IBC): 63% yield. Anal. Calcd. ($\text{C}_9\text{H}_{13}\text{N}_3\text{O}_3$): C, 51.18; H, 6.20; N, 19.89. Found: C, 51.47; H, 6.27; N, 19.72. Mp: 304.6 °C. MS: $m/z = 211.9$ (MH⁺). ^1H NMR (400.13 MHz, DMSO- d_6): δ 0.91 (d, $J = 6.7$ Hz, 6H, H-3'); 1.90 (n, $J = 6.7$ Hz, 1H, H-2'); 3.88 (d, $J = 6.7$ Hz, 2H, H-1'); 6.89 (d, $J = 7.1$ Hz, 1H, H-5); 7.77 (d, $J = 7.1$ Hz, 1H, H-6); 10.95 (br, s, 2H, NH). ^{13}C NMR (100.6 MHz, DMSO- d_6): δ 18.6 (C-3'); 27.3 (C-2'); 70.7 (C-1'); 93.4 (C-5); 146.5 (C-6); 153.7 (NCOO); 155.7 (C-2); 163.6 (C-4).

N^4 -neopentylloxycarbonyl-cytosine (NC): 69% yield. Anal. Calcd. ($\text{C}_{10}\text{H}_{15}\text{N}_3\text{O}_3$): C, 53.32; H, 6.71; N, 18.66. Found: C, 53.29; H, 6.74; N, 18.56. Mp: 309.6 °C. MS: $m/z = 225.9$ (MH⁺). ^1H NMR (400.13 MHz, DMSO- d_6): δ 0.92 (s, 9H, H-3'); 3.79 (s, 2H, H-1'); 6.89 (d, $J = 7.1$ Hz, 1H, H-5); 7.77 (d, $J = 7.1$ Hz, 1H, H-6); 10.95 (br, s, 2H, NH). ^{13}C NMR (100.6 MHz, DMSO- d_6): δ 26.0 (C-3'); 31.2 (C-2'); 73.8 (C-1'); 93.4 (C-5); 146.6 (C-6); 153.7 (NCOO); 155.7 (C-2); 163.6 (C-4).

N^4 -hexyloxycarbonyl-cytosine (HC): 55% yield. Anal. Calcd. ($\text{C}_{11}\text{H}_{17}\text{N}_3\text{O}_3$): C, 55.22; H, 7.16; N, 17.56. Found: C, 55.28; H, 7.23; N, 17.43. Mp: 292.2 °C (285–288 °C (Nery, 1969)). MS: $m/z = 239.9$ (MH⁺). ^1H NMR (400.13 MHz, DMSO- d_6): δ 0.87 (t, $J = 7.0$ Hz, 3H, H-6'); 1.23–1.37 (m, 6H, H-3', H-4', H-5'); 1.59 (p, $J = 6.7$ Hz, 2H, H-2'); 4.08 (t, $J = 6.7$ Hz, 2H, H-1'); 6.89 (d, $J = 7.1$ Hz, 1H, H-5); 7.76 (d, $J = 7.1$ Hz, 1H, H-6); 10.48 (br, s, 1H, NH); 11.38 (br, s, 1H, NH).

N^4 -octyloxycarbonyl-cytosine (OC): 72% yield. Anal. Calcd. ($\text{C}_{13}\text{H}_{21}\text{N}_3\text{O}_3$): C, 58.41; H, 7.92; N, 15.72. Found: C, 58.34; H, 7.93; N, 15.62. Mp: 294.0 °C. MS: $m/z = 268.0$ (MH⁺). ^1H NMR (400.13 MHz, DMSO- d_6): δ 0.86 (t, $J = 6.9$ Hz, 3H, H-8'); 1.21–1.37 (m, 10H, H-3', H-4', H-5', H-6', H-7'); 1.59 (p, $J = 6.7$ Hz, 2H, H-2'); 3.79 (t, $J = 6.7$ Hz, 2H, H-1'); 6.87 (br, s, 1H, H-5); 7.76 (d, $J = 7.1$ Hz, 1H, H-6); 10.48 (br, s, 1H, NH); 11.37 (br, s, 1H, NH).

N^4 -adamantylloxycarbonyl-cytosine (ADC): 23% yield. Anal. Calcd. ($\text{C}_{15}\text{H}_{19}\text{N}_3\text{O}_3$): C, 62.27; H, 6.62; N, 14.52. Found: C, 61.95; H, 6.83; N, 14.20. Mp: 279.8 °C. MS: $m/z = 290.0$ (MH⁺). ^1H NMR (400.13 MHz, DMSO- d_6): δ 1.63 (m, br, 6H); 2.09 (m, br, 6H); 2.15 (m, br, 3H); 6.85 (d, $J = 7.1$ Hz, 1H, H-5); 7.76 (d, $J = 7.1$ Hz, 1H, H-6); 10.30 (br, s, 1H, NH); 11.44 (br, s, 1H, NH). ^{13}C NMR (100.6 MHz, DMSO- d_6): δ 30.1 (C-3'); 35.4 (C-4'); 40.8 (C-2'); 80.5 (C-1'); 93.4 (C-5); 146.5 (C-6); 155.3 (C-2); 163.4 (C-4) NCOO was not observed due to exchange broadening.

N^4 -benzyloxycarbonyl-cytosine (BC): 43% yield. Anal. Calcd. ($\text{C}_{12}\text{H}_{11}\text{N}_3\text{O}_3$): C, 58.77; H, 4.52; N, 17.13. Found: C, 58.61; H, 4.49; N, 17.05. Mp: 292.1 °C (>250 °C (Dueholm et al., 1994; Thomson et al., 1995)). MS: $m/z = 245.9$ (MH⁺). ^1H NMR (600.13 MHz, DMSO- d_6): δ 5.17 (s, 2H, H-1'); 6.92 (d, $J = 7.1$ Hz, 1H, H-5); 7.34 (m, 1H, H-5'); 7.40 (m, 4H, H-3' and H-4'); 7.79 (d, $J = 7.1$ Hz, 1H, H-6); 10.81 (br, s, 1H, NH); 11.33 (br, s, 1H, NH). ^{13}C NMR (150.9 MHz, DMSO- d_6): δ 66.4 (C-1'); 93.5 (C-5); 127.8 (C-3'); 128.1 (C-5'); 128.4 (C-4'); 136.0 (C-2'); 146.6 (C-6); 153.4 (NCOO); 155.7 (C-2); 163.6 (C-4). The spectral data are consistent with those reported earlier (Thomson et al., 1995).

2.3. Determination of aqueous solubility and partition coefficient

Excess amounts of the test compounds were suspended in buffer solution (5–10 ml) in screw-capped test tubes. The tubes were sonicated (30 min) followed by agitation at 37 ± 0.5 °C in an incubator hood until equilibrium was attained (4–24 h). The supernatant was filtered through a 0.45 μm membrane filter discarding the first 3–5 ml. The filtrate was diluted prior to HPLC analysis. The filtration process was carried out in the incubator hood and the equipments used were all preheated to 37 °C. The partition of the test compounds between *n*-octanol and buffer solutions was determined at 37 ± 0.5 °C. The phases were mutually saturated before use. The partition coefficient was calculated from the concentration of the test compound in the aqueous phase before and after attained equilibrium (6–24 h) measured by HPLC. Solubilities and partition coefficients were calculated from experiments done in triplicate.

2.4. Stability measurements in aqueous solutions

The reactions were initiated by adding 100–500 μl of a stock solution of the test compounds in methanol to 10.0 ml of preheated buffer solution in screw-capped test tubes resulting in a final concentration of 0.6–10 μM . The reaction solutions were kept at constant temperature in a water bath and at appropriate time intervals samples were taken and analysed by HPLC. In order to, respectively, stop fast proceeding reactions and neutralise acidic samples prior to product analyses, these samples were mixed with an equal volume of 1 M phosphate buffer pH 7.4.

2.5. Stability measurements in biological media

A stock homogenate (50% (w/v)) of pig stomach mucus was prepared in ice cold 0.02 M phosphate buffer pH 7.40. A stock solution of pepsin A (7500 U/ml) was prepared in 0.02 M acetate buffer pH 4.4 (Rajagopalan et al., 1966). The final homogenates (10% (w/v)) and enzyme solutions (750 U/ml) were prepared by dilution with 0.01 M hydrochloric acid. Pepsin A was stable in the final solutions for 24 h based on studies using haemoglobin as reference substrate (Anson, 1938). *H. pylori* ATCC 700392 were dispersed in 0.9% (w/v) NaCl at a concentration of 10^8 cells/ml (estimated using OD measurement at 600 nm). A part of this suspension was sonicated for 10 min to disturb cell membranes and release intracellular enzymes. The preheated media were spiked with the individual test compound (2–10 μ M) and kept at 37 ± 0.5 °C. At appropriate time intervals two aliquots were withdrawn and added to one aliquot of a 6% (w/v) perchloric acid solution in order to deproteinize the samples. In case of ADC one aliquot were added to two aliquots of 97% (v/v) methanol in 1 M aqueous sodium hydroxide. After mixing and centrifugation for 15 min at 13000 rpm, the supernatant was analysed by HPLC.

2.6. Preparation of alginate formulations

Sodium alginate was suspended in DMA solutions of ADC and cytosine, respectively. By addition of water under stirring, dissolution of sodium alginate was accompanied by precipitation of the test compound in case of ADC, whereas cytosine remained dissolved. The precipitated ADC prodrug was uniformly distributed throughout the viscous suspension. Trapped air was removed prior to use. The formulations consisted of 1% (w/v) aqueous sodium alginate solution containing 20% (v/v) DMA.

2.7. In vitro release from alginate formulations

Release of the compounds from the alginate formulations was examined using the paddle method (Eur. Ph. 4th dissolution test apparatus) at 37 ± 0.5 °C according to previous studies (Katayama et al., 1999) with modifications. The release medium (500 ml; 0.1 M HCl pH 1.1) was placed in the vessel and preheated to 37 ± 0.5 °C. The liquid formulation (36 ml comprising 18 μ mol of the test compound) was placed in a Petri dish (6.9 cm \times 1.2 cm). A piece of woven gauze made from stainless steel wire 0.30 mm in diameter and having mesh apertures of 1.00 mm was placed on top of the Petri dish. The Petri dish was then located at the base of the dissolution vessel and stirring of the dissolution medium was started (50 rpm). At appropriate time intervals samples were withdrawn, mixed with an equal volume of 1 M phosphate buffer pH 7.4 and analysed by HPLC. The samples were replaced with fresh release medium. Release experiments were performed in triplicate.

2.8. HPLC analysis

HPLC analyses were performed with a Shimadzu LC-6A, Merck Hitachi L-6000 or Merck Hitachi L-7100 pump and a Merck Hitachi L-7480, Jasco 821-FP or Merck Hitachi F1000 FL-detector operating at Ex 288 nm/Em 350 nm when detecting the derivatives and a Merck Hitachi D-4000 UV-detector operating at 266 nm for detection of degradation products. Reversed phase chromatography was carried out using a Phenomenex Aqua C₁₈ column (150 mm \times 4.6 mm i.d.; 5 μ m particles) equipped with a C₁₈ precolumn (4 mm \times 3.0 mm i.d.) (Supware, Copenhagen, Denmark). The flow rate was set at 1 ml min⁻¹. Mobile phase systems of 10–80% (v/v) methanol in 0.1% (v/v) phosphoric acid were used with the methanol content adjusted for each compound to provide retention times in the range of 3–8 min. To detect degradation products and cytosine 0.02 M phosphate buffer pH 7.4 was used as mobile phase.

3. Results and discussion

3.1. Physicochemical characterisation

The pK_a-values of parent cytosine are pK_{a1} 4.6 and pK_{a2} 12.2 (Shugar and Fox, 1952) for the protonation at N³ and deprotonation at N¹, respectively. Thus cytosine is positively charged, neutral and negatively charged under acidic, neutral and basic conditions, respectively. The pH-solubility profile of EC (Fig. 2) reveals enhanced solubility at low and high pH, respectively. The pK_{a1}-value for EC was calculated (Table 1) according to the method previously described for a weak base (Yalkowsky, 1999).

$$C_s = C_0(1 + 10^{pK_a - pH}) \quad (1)$$

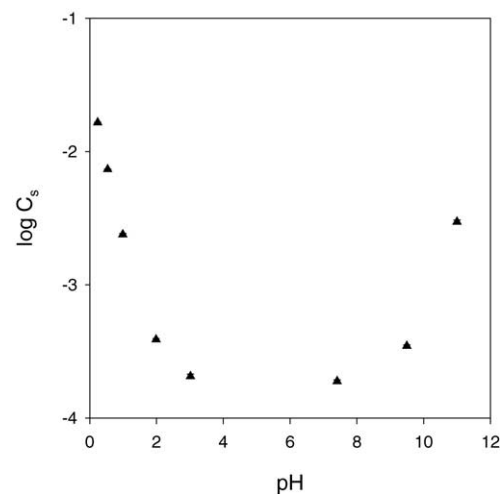


Fig. 2. The pH-solubility profile for EC at 37 ± 0.5 °C and $\mu = 0.5$. C_s refers to the total pH-dependent solubility (M). Each value is the mean \pm S.E.M.

Table 1
The values of $\log P$, solubility and pK_a for cytosine and the N^4 -alkyloxycarbonyl-cytosine derivatives at $37 \pm 0.5^\circ\text{C}$

Compound	$\log P$	Solubility (μM) (RSD (%))		
		$C_{s,pH 1.1}$	$C_{s,pH 7.4}$	pK_a
Cytosine	-1.5 (3)	n.d.	102624 (4) ^a	4.6 ^b
MC	-0.55 (2)	n.d.	1843 (0.3)	n.d.
EC	-0.085 (2)	2377 (3) ^c	199 (9)	1.9 ^d
NBC	1.1 (2)	983 (3)	147 (2)	1.9
IBC	1.1 (1)	1670 (3)	235 (2)	1.9
NC	1.5 (1)	886 (3)	122 (2)	1.9
HC	2.3 (4)	34 (4)	4 (2)	1.9
OC	3.3 (10)	0.7 (11)	0.3 (16)	1.9
ADC	2.9 (1)	n.d.	19 (9)	n.d.
BC	1.3 (2)	n.d.	51 (2)	n.d.

Experiments performed in triplicate; n.d. = not determined.

^a In H_2O .

^b From Shugar and Fox, 1952.

^c At pH 1.0.

^d From solubility and partition experiments.

where C_0 is the intrinsic solubility determined at least two pH-units above pK_{a1} and C_s is the total pH dependent solubility. Similarly, pK_{a1} -values for the other non-cyclic derivatives were estimated using the solubility at pH 1.1 and pH 7.4 (Table 1). The pK_{a1} -value is lowered approximately 2.7 units by the introduction of the alkyloxycarbonyl group whereas it seems to be unaffected by the chemical structure of the alkyl substituent (Table 1). These observations are in favourable agreement with the behaviour of N -alkyloxycarbonyl derivatives of imidazole-containing compounds (Buur and Bundgaard, 1991; Nielsen et al., 1994) and N^6 -alkyloxycarbonyl derivatives of adenine (Giner-Sorolla and Bendich, 1958). Compared to cytosine, EC possesses an additional ionisation constant (pK_{a2}) for the leaving of the proton at N^4 which can be calculated to 9.8 using solubility data and the equation for a divalent acid (Yalkowsky, 1999). Similar observations have been reported for N^6 -alkyloxycarbonyl derivatives of adenine (Giner-Sorolla and Bendich, 1958). The lowered intrinsic solubility of the derivatives relative to parent cytosine (50- to 10^5 -fold) can most likely be ascribed to an increasing crystal lattice energy, achieved by enhanced intermolecular attractive interactions by introduction of the alkyloxycarbonyl-groups. Similarly N -alkyloxycarbonyl derivatives of mitomycin C (Mukai et al., 1985) exhibited decreased solubility relative to the parent drug.

The partition of the test compounds between n -octanol and 0.02 M phosphate buffer pH 7.4 were determined at $37 \pm 0.5^\circ\text{C}$. The hydrophobic substituent constant ($\pi = 0.56$) determined from the slope of the straight line correlation ($R^2 = 0.996$) between $\log P$ and the number of C-atoms in the alkyl chain for the non-cyclic aliphatic derivatives is in excellent agreement with earlier findings (Hansch and Leo, 1979). The $\log P$ -values for ADC and BC are given in Table 1. Using the expression previously given (Larsen et al., 2000) the pK_{a1} -value for EC was calculated from the distribution coefficient

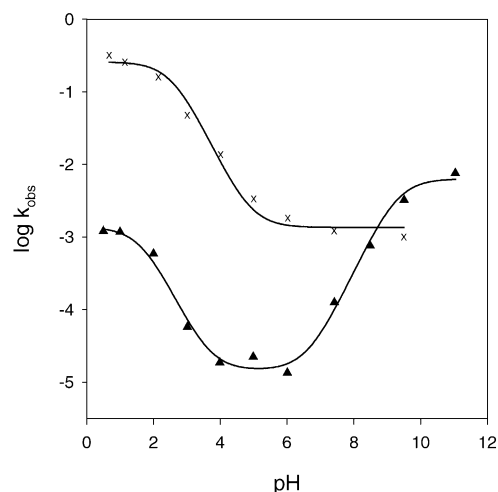


Fig. 3. The pH-rate profile for the hydrolysis of EC (▲) and ADC prodrug (X) at $60 \pm 0.5^\circ\text{C}$ and $\mu = 0.5$. The solid lines have been calculated from Eqs. (2) and (3), respectively. The value, k_{obs} refers to the observed pseudo-first-order rate constant (min^{-1}).

determined at pH 1.5 and the partition coefficient determined at pH 7.4 ($37 \pm 0.5^\circ\text{C}$) and agreed well with those obtained from solubility data (Table 1).

3.2. Stability measurements in aqueous solutions

To investigate the kinetics of hydrolysis the pH-rate profiles of EC and ADC ($60 \pm 0.5^\circ\text{C}$) were established (Fig. 3). Under constant pH, ionic strength and temperature the degradation reactions displayed first-order kinetics for several half-lives. Pseudo-first-order rate constants were determined from the slopes of linear plots of the logarithm of intact N^4 -alkyloxycarbonyl-cytosine against time. From kinetic runs with EC employing buffer concentrations in the range 0.02–0.1 M ($\mu = 0.5$) it was observed that the decomposition rates were not subject to significant general acid–base catalysis. The shape of the pH-rate profile for EC suggests that the overall hydrolysis can be accounted for in terms of spontaneous degradation of the cationic, neutral and anionic species as described by:

$$k_{\text{obs}} = k_{01} \frac{[a_{\text{H}^+}]^2}{[a_{\text{H}^+}]^2 + [a_{\text{H}^+}]K_{a1} + K_{a1}K_{a2}} + k_{02} \frac{[a_{\text{H}^+}]K_{a1}}{[a_{\text{H}^+}]^2 + [a_{\text{H}^+}]K_{a1} + K_{a1}K_{a2}} + k_{03} \frac{K_{a1}K_{a2}}{[a_{\text{H}^+}]^2 + [a_{\text{H}^+}]K_{a1} + K_{a1}K_{a2}} \quad (2)$$

where $[a_{\text{H}^+}]$ is the hydrogen ion activity, and K_{a1} and K_{a2} are the ionisation constants for the equilibrium between the cationic and neutral, and neutral and anionic species,

Table 2

Half-lives, ratio of degradation products and pseudo-first-order rate constants associated the formation of degradation products by hydrolysis of the *N*⁴-alkyloxycarbonyl-cytosine derivatives at 60 ± 0.5 °C and μ = 0.5

Compound	Half-life (min) (RSD (%))		C/U ^a	Rate constants (× 10 ³ min ⁻¹) at pH 1.2	
	pH 1.2	pH 9.5		<i>k</i> _C	<i>k</i> _U
MC	689 (1)	210 (0.4)	39/64	0.39	0.64
EC	589 (2) ^b	216 (1) ^b	20/80	0.24 ^b	0.94 ^b
EC ^c	6480 (1) ^b	n.d.	20/80	0.02 ^b	0.09 ^b
NBC	624 (2)	388 (0.3)	13/92	0.14	1.02
IBC	619 (1)	412 (2)	14/88	0.16	0.99
NC	617 (1)	492 (5)	78/24	0.88	0.27
HC	590 (5)	423 (1)	n.d.	n.d.	n.d.
OC	625 (2)	510 (2)	n.d.	n.d.	n.d.
ADC	3 (7)	475 (0.3)	96/0	238	0
ADC ^c	41 (3)	n.d.	100/0	16.7	0
BC	350 (2)	211 (0.5)	55/47	1.09	0.93

Experiments performed in triplicate; n.d. = not determined.

^a %cytosine/%uracil formed after 8 half-lives at pH 1.2.

^b At pH 1.0.

^c At 37 °C.

respectively. Similarly, the hydrolysis of ADC can be described by:

$$k_{\text{obs}} = k_{01} \frac{[a_{\text{H}^+}]}{[a_{\text{H}^+}] + K_{a1}} + k_{02} \frac{K_{a1}}{[a_{\text{H}^+}] + K_{a1}} \quad (3)$$

In Fig. 3 the solid curves drawn were constructed from Eqs. (2) and (3), respectively. From computer fitting magnitudes of the pseudo-first-order rate constants *k*₀₁, *k*₀₂ and *k*₀₃ for EC of 1.37, 0.0145 and 6.33 × 10⁻³ min⁻¹, respectively, were calculated and p*K*_{a1} and p*K*_{a2} estimated to 1.7 and 9.3, respectively. The corresponding *k*₀₁ and *k*₀₂ values for ADC were 259 and 1.36 × 10⁻³ min⁻¹, respectively and p*K*_{a1} were 2.6. The observed agreement between the fitted curve and experimental data points indicates that Eqs. (2) and (3) adequately describe the degradation kinetics for EC and ADC, respectively. The shape of the pH-rate profile of EC is comparable to those previously reported for *N*-alkyloxycarbonyl derivatives of imidazoles (Buur and Bundgaard, 1991) and benzimidazoles (Nielsen et al., 1994) and benzamide (Kahns and Bundgaard, 1991).

To extend the study of the hydrolysis, the degradation of all the derivatives was studied under acidic and basic conditions (pH 1.2 and 9.5, respectively). At pH 9.5 the decompositions resulted in quantitative conversion to parent cytosine whereas at pH 1.2 liberation of cytosine was in most cases accompanied by the formation of uracil. After 8 half-lives the sum of the two degradation products were close to those of the initial concentrations of test compound (±5%) (Table 2). Identification of the degradation products formed in acidic and basic media were done by HPLC. The findings under acidic conditions were supported by experiments done in 0.1 M deuterium chloride (60 ± 0.5 °C) determined by ¹H NMR for ADC and EC. Comparable cytosine to uracil ratios was found after 2 and 8 half-lives for MC (pH 1.2) suggesting that the two products were formed by parallel reactions. Both cytosine and uracil were stable under the conditions

used in agreement with earlier findings (Garrett and Tsau, 1972). The pseudo-first-order rate constants, *k*_C and *k*_U for the release of cytosine and uracil from the derivatives at pH 1.2 were calculated from:

$$k_{\text{C}} = \frac{[C]_{\infty} k_{\text{obs,pH1.2}}}{[RC]_0} \quad (4)$$

$$k_{\text{U}} = \frac{[U]_{\infty} k_{\text{obs,pH1.2}}}{[RC]_0} \quad (5)$$

where [RC]₀ is the initial concentration of the derivative investigated and [C]_∞ and [U]_∞ are the concentrations of cytosine and uracil after 8 half-lives, respectively. Although exhibiting fairly identical overall chemical stability at pH 1.2 (60 ± 0.5 °C) the non-cyclic aliphatic derivatives degrade to cytosine and uracil in different ratios dependent on the alkyl chain structure (Table 2). To this end the hydrolysis of *N*-alkyloxycarbonyl benzamides at pH > 7 resulted in the formation of benzoic acid and benzamide favouring the formation of benzoic acid the longer the alkyl chain (Kahns and Bundgaard, 1991). The slow deamination of cytosine to uracil has been reported to involve H₃O⁺ attack at C⁴ followed by leaving of NH₄⁺ (pH 1.1; 70 °C) (Garrett and Tsau, 1972). Under the assumption that the degradation of the *N*⁴-alkyloxycarbonyl derivatives to uracil follows an analogous pathway with the leaving of NH₃⁺COOR, it is noted that NH₃⁺COOR is a better leaving group than NH₄⁺, which can be ascribed to the electron withdrawing properties of -COOR. Similar electronic effects have been reported for *N*⁴-methylsubstituted cytidines (Kusmieriek et al., 1989). The influence of the temperature (range 37–60 °C) on the degradation rate of EC was studied at pH 1.0 and the activation energy (*E*_a) from Arrhenius plot was 90 kJ mol⁻¹ allowing the half-life of EC at 37 ± 0.5 °C (pH 1.0) to be estimated to 108 h. Interestingly, hydrolysis of ADC resulted in quantitative release of cytosine under acidic conditions at 60 ± 0.5 °C

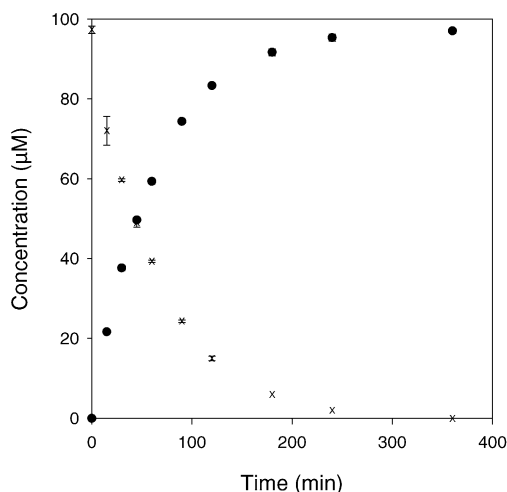


Fig. 4. Time course for the degradation of ADC prodrug (X) and formation of cytosine (●) at pH 1.1 at 37 ± 0.5 °C and $\mu = 0.5$. Each value is the mean \pm S.E.M. of three determinations.

(Table 2) and 37 ± 0.5 °C (Fig. 4) exhibiting half-lives of 3 and 41 min, respectively. Thus, under acidic conditions ADC is approximately 200 times more hydrolytic labile than the non-cyclic aliphatic derivatives. Although strain often decelerates hydrolysis of bridgehead systems, hyperconjugation often has the opposite effect. Thus a carbocation at C₁ in adamantyl will be stabilised by hyperconjugative electron donation from C₃ and the other identical carbons (Lowry and Richardson, 1987). Thus, the hydrolysis of ADC may be expected to proceed via an unimolecular reaction (E1) involving the formation of the 1-adamantyl carbocation intermediate, in contrast to the bimolecular reaction (A_{AC}2) involving the tetrahedral intermediate formed from a nucleophile attack by a water molecule in case of the non-cyclic aliphatic derivatives (March, 1992).

3.3. Stability measurements in biological media

Estimated half-lives from experiments run for 24 h (37 ± 0.5 °C) in the presence of pepsin A (pH 2.2) and mucus homogenate (pH 3.3) for NBC were 19 and 30 h, respectively. The correspondent stabilities of IBC were 50 and 69 h, respectively, indicating that NBC and IBC are subject to enzymatic hydrolysis by pepsin A. The degradation of the other derivatives was not significantly affected in the presence of these media. The presence of *H. pylori* did not affect the stability of any of the derivatives, even though the alkyloxycarbonyl bond in pentyloxycarbonyl-5'-deoxy-5-fluorocytidine has been shown to be cleaved by carboxylesterases (Miwa et al., 1998), enzymes that also are expressed in *H. pylori* (Mendz et al., 1993). The data may suggest that the N⁴-alkyloxycarbonyl derivatives, except ADC, are stable in the milieu residing in the stomach and that regeneration of the parent drug has to rely on cleavage of the alkyloxycarbonyl bond effected by intracellular bacterial enzymes.

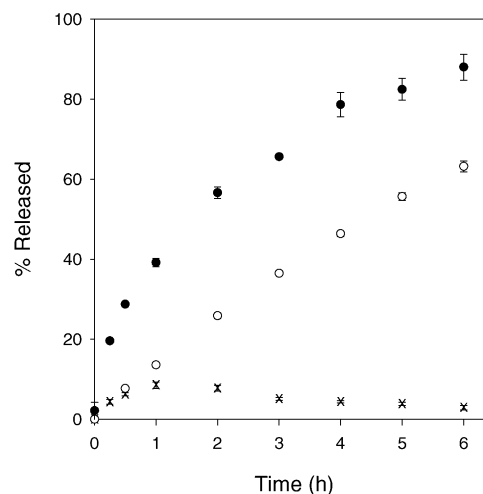


Fig. 5. Release of ADC (X) and cytosine formed by hydrolysis of ADC (○) from alginate acid gels loaded with the acid-labile and poorly water-soluble ADC prodrug derivative. For comparison the release of cytosine from similar gels loaded with equimolar amounts of parent cytosine (●). Each value is the mean \pm S.E.M.

3.4. In vitro release from alginate formulations

The obtained stability data indicates that ADC may be of interest in prodrug design of cytosine containing antibacterial compounds in relation to stomach specific delivery. In order to prolong gastric retention of ADC the prodrug was incorporated into an alginate formulation. Preliminary studies of the release from alginate formulations containing cytosine and ADC prodrug, respectively, were conducted in 0.1 M HCl at 37 ± 0.5 °C. Immersing the alginate formulation into the acidic release medium resulted in the formation of alginate acid gel structures. In Fig. 5 the release profile of cytosine and ADC from gels loaded with ADC prodrug are shown. It is seen that parent cytosine is the main compound released while only minute amounts of ADC appears in the release media. For comparison the release of cytosine from a similar alginate formulation loaded with equimolar amounts of parent cytosine is also depicted in Fig. 5. It is apparent that release of parent cytosine is markedly delayed by employment of the acid-labile and poorly-soluble adamantyl prodrug derivative with the time of 50% released being 1.5 h and 4.5 h from cytosine and ADC loaded gels, respectively. The minute amounts of ADC present in the release medium show that the formation of cytosine by hydrolysis of ADC predominantly takes place inside the gel. The retarded release of cytosine from ADC loaded gels may be ascribed to the low solubility of the ADC prodrug and subsequently minor concentration gradient from the gels to release media as compared to cytosine loaded gels. Since parent compound is released from the formulation achievement of a therapeutic effect requires that the parent cytosine compound is capable of reaching the intended site of action.

4. Conclusion

The synthesized N^4 -alkyloxycarbonyl-cytosine derivatives exhibited increased lipophilicity, in a predictive manner ($\pi = 0.56$), and lowered intrinsic aqueous solubility, relative to parent cytosine. The degree of ionisation in acidic media is markedly suppressed due to the decrease in pK_{a1} of the derivatives. The hydrolysis under alkaline conditions resulted in quantitative conversion to parent cytosine whereas at acidic pH the liberation of cytosine was in most cases accompanied by parallel formation of uracil. The lipophilic and poorly water-soluble ADC prodrug exhibited a hydrolytic half-life of 41 min at pH 1.1 (37 °C) with quantitative conversion to parent cytosine, the degradation rate being approximately 200 times faster than that of the non-cyclic aliphatic derivatives. The time of 50% cytosine released from gels loaded with ADC prodrug at acidic pH was 4.5 h compared to that of 1.5 h from gels loaded with parent cytosine. A study of various combined formulations based on N^4 -alkyloxycarbonyl derivatives of cytosine and alginate based in situ gelling systems are to be published elsewhere.

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