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Ester prodrugs of a potent analgesic, morphine-6-sulfate: syntheses, spectroscopic and physicochemical properties

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Abstract

The aim of this work is to develop 3-acyl prodrugs of the potent analgesic morphine-6-sulfate (M6S). These are expected to have higher potency and/or exhibit longer duration of analgesic action than the parent compound. M6S and the prodrugs were synthesized, then purified either by recrystallization or by semi-preparative HPLC and the structures confirmed by mass spectrometry, IR spectrophotometry and by detailed 1- and 2-D NMR studies. The lipophilicities of the compounds were assessed by a combination of shake-flask, group contribution and HPLC retention methods. The octanol–buffer partition coefficient could only be obtained directly for 3-heptanoylmorphine-6-sulfate, using the shake-flask method. The partition coefficients (P) for the remaining prodrugs were estimated from known methylene group contributions. A good linear relationship between log P and the HPLC log capacity factors was demonstrated. Hydrolysis of the 3-acetyl prodrug, as a representative of the group, was found to occur relatively slowly in buffers (pH range 6.15–8.01), with a small buffer catalysis contribution. The rates of enzymatic hydrolysis of the 3-acyl group in 10% rat blood and in 10% rat brain homogenate were investigated. The prodrugs followed apparent first order hydrolysis kinetics, with a significantly faster hydrolysis rate found in 10% rat brain homogenate than in 10% rat blood for all compounds.

Keywords: Morphine; Morphine-6-sulfate; 3-Acyl prodrugs; Nuclear magnetic resonance; Lipophilicity; HPLC capacity factors; Hydrolysis kinetics; Aqueous solubility; Oil solubility

1. Introduction

Morphine is the recommended drug of choice by the World Health Organization for the management of severe pain, especially in terminally ill patients. However, it has a short plasma elimina-

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tion half life (Osborne et al., 1990). Although sustained release morphine tablets are available for twice daily doses, they are not useful for patients unable to swallow. Repeated injections may cause compliance problems from venepuncture pain, patient-controlled drug infusion pumps are costly and nasogastric tubes are inconvenient. Morphine-3-glucuronide (M3G), a major metabolite of morphine, has no analgesic action, and has been found to possess anti-analgesic activity (Smith et al., 1990). Conversely, the minor metabolite morphine-6-glucuronide (M6G) has been shown to be a much more potent antinociceptive than morphine itself (Shimomura et al., 1971). This finding contradicts general belief that a polar compound or metabolite is quickly excreted from the body, and should not cross the blood-brain-barrier (BBB) to interact with receptors.

A recent study (Carrupt et al., 1991) has suggested that the 3- or 6-glucuronide metabolites are not as polar as expected. The lower than expected polarity was claimed to result from intramolecular folding of the metabolites in such a way that the more hydrophilic regions of each molecule could be shielded, while the more hydrophobic portion was exposed to interact with the lipid environment of the BBB and the CNS.

Mori et al. (1972) found that peripherally administered morphine-6-sulfate (M6S) in mice had a potency and duration of antinociceptive action similar to that of M6G, which was several times longer than equivalent doses of morphine. A later study showed that M6S had 28-fold greater potency than morphine following intracerebroventricular (i.c.v.) administration in mice (Brown et al., 1985). A more recent study suggested that M6S also had highly potent antinociceptive properties after i.c.v. injection in rats (Houdi et al., 1992, 1996). Thus, the major objective of this work is to enhance potency and/or duration of analgesic action by developing 3-acyl ester prodrugs of M6S.

The overall objective of this work was to evaluate effects on the potency and/or duration of antinociceptive action from peripheral administration of a limited range of 3-acyl ester prodrugs of M6S. The 3-acyl group is expected to be susceptible to in vivo hydrolysis by plasma esterases, thus providing active M6S, as occurs with heroin (3,6-diacetylmorphine) and 3-acetylmorphine (Hite, 1989). The prodrugs themselves are expected to be more lipophilic and should cross the BBB more readily than M6S itself, where they may then undergo hydrolysis to M6S in the CNS by brain esterases. Thus, the prodrugs may deliver additional M6S to the brain. Furthermore, release of the more polar M6S inside the BBB may result in trapping some of it in the CNS, resulting in a longer duration of action.

This paper presents synthetic, structure confirmation, partitioning, hydrolysis kinetics and solubility data. The physicochemical data are important factors which affect the in vitro and in vivo properties of the parent and its prodrugs, which will be the subject of a following publication (Preechagoon et al., 1997).

2. Materials and methods

2.1. Materials

Morphine HCl (BP grade; Macfarlan Smith Ltd.) was purchased from Royal Brisbane Hospital Pharmacy, Brisbane. Repackaged acetic anhydride and chlorosulphonic acid (both L.R. grade) were bought from the Chemistry Department, University of Queensland. Propionic anhydride was obtained from Ajax (Ajax Chemical, Brisbane). Butyric anhydride, heptanoic anhydride and anhydrous pyridine were purchased from Aldrich (Sigma-Aldrich, Sydney). 1-Octanol (spectroscopic grade) was purchased from FSE (Fisons Scientific Equipment, Brisbane). d_5-Pyridine (Merck; 99 atom% D) was used for NMR spectra. All other chemicals were either AR or HPLC grade. pH values were measured with a Corning pH meter (Model 220) after calibration with pH standard solutions (Ajax Chemical, Brisbane; pH 4.0 and 7.0).

2.2. HPLC

The semi-preparative HPLC system consisted of a solvent delivery pump (Model LC-10AS,

Shimadzu, Japan), a rotary injector (Model 7125, Rheodyne, Cotati, CA, USA) equipped with a 2 ml loop, a fixed wavelength UV detector at 254 nm (Model 440, Millipore-Waters, Brisbane, Australia) and an integrator (Model C-R5A Chromatopac, Shimadzu, Japan). A 250×10 mm C18 reversed-phase (RP) semi-preparative column (Waters 25×10) and a C18 precolumn were used. The mobile phase was filtered (0.45 μm millipore) 11% v/v aqueous acetonitrile (ACN) at a flow rate of 9.9 ml/min. The retention time observed for 3BuM6S was about 10 min.

Analytical HPLC used a Nucleosil 4.6×50 mm, 5 μm, RP C8 column (Keystone Scientific, Bellefonte, PA, USA) and a 20-μl injector loop with the HPLC system described above. The flow rate was 1.0 ml/min and the analytical wavelength was 214 nm (Waters Model 440 UV Detector with extended wavelength module) for the hydrolysis studies. Aqueous solubility measurements used a Waters Model 464 dual electrode electrochemical detector with a pre-oxidation voltage of 0.5 V and an analytical oxidation voltage of 1.1 V.

2.3. Mass spectrometry

Mass spectra were acquired on a PESciex API 111 triple quadrupole mass spectrometer (PE-Sciex, Ontario, Canada), equipped with an ion-spray atmospheric pressure ionization source (Centre for Drug Design and Development, University of Queensland). Samples (5 μl) in 0.5% trifluoroacetic acid were injected into a moving solvent (30 μl/min; 50:50 ACN/0.5% trifluoroacetic acid), coupled directly to the ionization source via a fused silica capillary interface (50 μm I.D. $\times$ 50 cm long). Sample droplets were ionized at a positive potential of 5 kV and entered the analyser through an interface plate and subsequently through an orifice (100–120 μm diam.) at a potential of 60 and 100 V. Full scan spectra were acquired over the mass range 200–800 daltons (scan step size of 0.1 daltons) in 3 s.

2.4. Syntheses

2.4.1. 3-Acetylmorphine-6-sulfate (3AcM6S)

Morphine HCl (1 g; 3.108 mmol) and aqueous sodium bicarbonate (100 ml; 10% w/v) were mixed. Acetic anhydride (4.44 g; 43.5 mmol, in three portions) was added dropwise at 5 min intervals, then ice-cold H_2O (100 ml) was added and stirred for 5 min. 3-Acetylmorphine (3AcM) was extracted from the mixture with $CHCl_3$ (4×20 ml), washed with cold H_2O, and dried over anhydrous sodium sulfate. The extract was rotary evaporated, leaving an oil (95% yield by weight). Chlorosulfonic acid (redistilled; 0.43 ml; 2 equiv) was placed in a 50 ml 3-neck RB flask containing hot (70–80°C) dry pyridine (4A molecular sieve; 2.58 ml; 12 equiv). The mixture was stirred and heated for 10 min. The crude 3AcM was transferred to the reaction flask with 1 ml dry pyridine, mixed without heating for 30 min, then left overnight at RT. Aqueous MeOH (3 ml of 50%) was triturated with the mixture to obtain a fine precipitate. This was washed with small amounts (1–2 ml) of ice-cold 50% MeOH. Two recrystallizations from 20% methanol (2×50 ml) gave white crystals.

2.4.2. Morphine-6-sulfate (M6S)

M6S was obtained by hydrolyzing 3AcM6S (1271 mg) in 60 ml hot H_2O. The solution was heated until boiling and filtered using a warmed sintered glass funnel. The filtrate was left at RT to deposit white needles.

2.4.3. 3-Propionylmorphine-6-sulfate (3PrM6S)

Synthesis of 3PrM6S followed that of 3AcM6S, using propionic anhydride (2.5 equiv). The sodium bicarbonate solution was replaced by sodium carbonate (1% w/v), while the sulfonation reaction used chlorosulfonic acid (1.3 equiv) and pyridine (1.5 equiv). MeOH (12 ml) was added to the reaction mixture, which on standing overnight at RT deposited large yellowish crystals. These were washed with ice-cold MeOH prior to three recrystallizations from 10% aqueous MeOH (3×50 ml) to give white crystals.

2.4.4. 3-Butanoylmorphine-6-sulfate (3BuM6S)

The synthesis of 3BuM6S mainly followed that of 3PrM6S, except that semi-preparative HPLC was used for purification. Butyric anhydride (3.4 equiv), chlorosulfonic acid (1.5 equiv) and pyridine (1.6 equiv) were used in the synthesis steps. The reaction mixture was evaporated after addition of ACN (3×10 ml). The impure 3BuM6S residue was triturated with 50 ml cold water, the pH of the aqueous phase was adjusted to 7.0 with concentrated ammonia, then extracted with $CHCl_3$ (3×20 ml). The collected aqueous phase was evaporated to a white residue. One hundred mg of the residue was dissolved in 1.5 ml of the mobile phase and injected (0.45 μm in-line filter) into the HPLC system (Section 2.2). The eluted peak corresponding to 3BuM6S was evaporated to dryness under vacuum with the addition of small portions of ACN.

2.4.5. 3-Heptanoylmorphine-6-sulfate (3HpM6S)

The synthesis of 3HpM6S mainly followed that of 3BuM6S. In the acylation reaction, sodium carbonate 10% w/v in 90% aqueous methanol was used with heptanoic anhydride (3.4 equiv). 3HpM was extracted with 2×25 ml CCl_4. The sulfonation reaction was as for 3BuM6S, except that the impure compound was extracted with 25 ml of CCl_4, then the aqueous layer was evaporated. Semi-preparative HPLC was used as described above, except that the mobile phase was 32% v/v aqueous ACN, which gave a retention time for 3HpM6S of about 15 min.

2.5. Partition coefficients

2.5.1. Shake-flask method

3HpM6S was distributed between buffer-saturated 1-octanol and phosphate buffered saline (PBS, pH 7.4), at different volume ratios (1:4, 1:3, 1:2, 1:1 and 2:1). 3HpM6S was dissolved in PBS (50 μg/ml), then the appropriate volume of solution transferred to a 10 ml vial. The calculated volume of buffer-saturated 1-octanol was added to the vial, which was sealed and placed in a water-jacketted beaker at 37.0 ± 0.1°C. The PBS phase was stirred continuously with a small magnetic bar. Aliquots (100 μl) of the buffer layer were taken hourly for 12 h and assayed immediately for the remaining 3HpM6S by HPLC ($\lambda_{anal} = 254$ nm). The mobile phase for the HPLC system was ACN/acetate buffer (0.02 M, pH 4.75, 31:69 v/v) which gave a retention time of 3.2 min. Plots were constructed of % remaining in the aqueous phase versus time. The hydrolysis of 3HpM6S at 37.0°C in PBS alone was also investigated. The P value was calculated as follows:

$$P = \frac{(C_0 - C)(V_b)}{C(V_o)} \quad (1)$$

where C_0 is the initial solute concentration and C is the equilibrium solute concentration. V_b and V_o are the buffer and octanol volumes, respectively.

2.5.2. HPLC capacity factors

These were determined with the analytical HPLC system described above and a mobile phase of 0.01 M acetate buffer with varying percentages (3–25% v/v) of ACN. The aqueous component of each mobile phase was varied in pH as follows: 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.4. The column temperature was controlled to 37.0 ± 0.1°C. Aliquots (20 μl) of each compound were injected into the system. Sodium nitrate was used as a non-retained marker for the system void volume (Jinno and Kawasaki, 1984). Retention times were used to calculate capacity factors (k') as follows:

$$\log k' = \log \frac{(R_s - R_o)}{R_o} \quad (2)$$

where k' is the capacity factor, R_s is the retention time of the compound and R_o the retention time of sodium nitrate.

Log k'_o was obtained by extrapolating plots of log k' versus % ACN to 0% ACN by fitting the non-linear log k' versus mobile phase composition data to an empirical quadratic equation with Microsoft Excel®. The log k'_o values were then plotted versus apparent pH.

2.6. Hydrolysis kinetics in buffers and in biological matrices

2.6.1. HPLC analyses

RP HPLC analyses of reaction solutions for prodrug concentrations used the same system as for the HPLC capacity factors. Mobile phases consisted of varying percentages of ACN in 0.05

M acetate buffer, pH 4.75. The concentration of ACN was adjusted to obtain a retention time in the range 3–12 min for each prodrug. The flow rate was maintained at 1 ml/min and the prodrugs were detected at 214 nm (3AcM6S in buffers) or 254 nm (all 3AM6S compounds in biological matrices). The peak area for the remaining prodrug was recorded.

2.6.2. Hydrolysis kinetics for 3AcM6S in buffers

Twenty one reaction solutions (25 ml) were prepared containing 2×10^{-4} M 3AcM6S at the following pH values 6.15, 7.13, 7.32, 7.70 and 8.01. pH values were measured for each final solution. Each solution was prepared by weighing 2.00 ± 0.1 mg of the prodrug (Sartorius M5P Microbalance) into a 25 ml volumetric flask by difference, then dissolution with the solvent (pre-equilibrated at 37.0 ± 0.1°C) during the 30 s prior to time zero. The solvent for each solution was either 25 ml PBS or 10 ml PBS plus 15 ml isotonic saline. All solutions were prepared in duplicate, except for pH 6.13 in 25 ml PBS, which was prepared in triplicate. Reaction solutions were sampled periodically by direct injection of an aliquot into the HPLC system described above. All solutions were checked for isotonicity with a Fiske Model 110 freezing-point osmometer (Selby Scientific, Brisbane, Australia) and were found to have an osmotic effect equivalent to 280–300 mOsm/kg (expected, 300 mOsm/kg). Pseudo-first order rate constants (k_{cat}) were estimated by linear regression of the ln (peak area) as a function of time. All plots had correlation coefficients $r >$ 0.96. The buffer-dependent pseudo-first order rate constants were then plotted as a function of total phosphate concentration. The buffer-independent rate constants (k_{uncat}) were taken as the intercepts of the (assumed) linear plots.

2.6.3. Hydrolysis of the prodrugs in 10% rat brain homogenate

A male Sprague-Dawley rat (Medical School Animal House, University of Queensland, Brisbane, Australia; UQ AEEC approval Pharm/575/95/PhD) was euthanased with 100% carbon dioxide and decapitated. The brain was removed, washed several times with ice-cold 0.9% normal saline (NS) solution, then weighed. It was then immediately homogenised (Glas-col, Extech Equipment, Wantirna, Victoria) with four times its weight of ice-cold PBS, pH 7.4, for 1 min. One ml aliquots of the homogenate were transferred to glass reaction tubes, which were then equilibrated at 37.0 ± 0.1°C. A 1.0 ml aliquot of the prodrug (1000 μg/ml in PBS, pH 7.4), was added to the tube, which was vortexed for 1 min. A 200 μl aliquot of the sample was transferred to a 1.5 ml Eppendorf tube at time zero and periodically for up to 2 h. Prior to taking each aliquot, the reaction tube was vortexed for 10 s. After the aliquot was placed in the Eppendorf tube, the reaction tube was immediately returned to the water bath and mixed. ACN (300 μl) was added to each Eppendorf tube. The mixture was vortexed for 1.5 min at high speed, then the precipitated protein centrifuged off for 2.5 min ($11\,600 \times g$, Micro Centaur, MSE Scientific, Sussex). Duplicate 50 μl aliquots of the supernatant were assayed by HPLC for the % remaining prodrug. Triplicate hydrolysis runs were performed for each prodrug, each run using fresh brain homogenate from a different rat.

2.6.4. Hydrolysis of the prodrugs in 10% rat blood

The hydrolysis rate for each prodrug was determined in triplicate following the method described for rat brain homogenates. Rat blood was taken by cardiac puncture and kept in a heparinized tube immersed in an ice bath prior to the experiment. The blood was diluted with four times its weight of PBS and the experiments performed as described above.

2.7. Solubility studies

2.7.1. Solubilities in soybean oil

Soybean oil (Sigma-Aldrich, Sydney, Australia) was chosen as a potential long acting formulation vehicle for subsequent pharmacodynamic studies. Duplicate 1.00 mg samples of M6S, 3AcM6S and 3PrM6S were weighed (Model M5P, Sartorius AG, Göttingen, Germany), transferred into 20 ml test tubes and capped. One ml of the soybean oil was added to each tube, vortexed for 10 s, then

Table 1
Physicochemical properties of M6S and its prodrugs

Compounds	MW	R_f value[a]	% Yield	MP[b] (°C)	HPLC[c] R_t	IR bands (cm^{-1})[d]		MS[e] (M+1)
						C=O	S–OH	
M6S	365.38	0.38	58	289–292	2.2	—	1230, 1030	366
3AcM6S	407.42	0.43	67	262–265	3.4	1749	1230, 1038	408
3PrM6S	421.44	0.44	55	248–251	5.7	1752	1250, 1035	422
3BuM6S	435.47	0.45	60	239–242	11.6	1770	1265, 1052	436
3HpM6S	477.54	0.58	55	224–226	–	1765	1250, 1045	478

[a] TLC used 10% ACN/MeOH as eluent. TLC analysis used aluminium-backed silica gel 60 F254 plates (5 × 10 cm; 0.2 mm thickness; E. Merck).
[b] With decomposition (lit. MP of M6S and 3AcM6S are 288–290°C and 278–281°C, respectively) (Mori et al., 1972).
[c] 17% ACN/acetate buffer.
[d] IR spectra were obtained for KBr discs with a Perkin-Elmer 599 infrared spectrophotometer.
[e] Positive ion mass spectra for the fully protonated species.

sonicated for 30 min. Additional 1.0 ml aliquots of soybean oil were added half-hourly if undissolved particles were seen visually. To check for complete dissolution, samples were examined microscopically (× 400, Olympus BH-2 Microscope, Tokyo, Japan). Blank soybean oil was also examined microscopically.

2.7.2. Solubility in buffer

The aqueous solubilities of M6S, 3AcM6S and 3PrM6S at 37.0 ± 0.1°C were obtained by equilibrating 2–4-fold excess amounts of the compounds with PBS (pH 7.4; with sodium ascorbate (2 mg/ml) as antioxidant). One ml of the buffer solution was pipetted into a 5 ml test tube containing the compound. The suspension of each compound was sonicated for 5 min at 40–42°C and then transferred to a jacketted beaker connected to a constant-temperature water bath. Each sample was stirred magnetically. After 6 h equilibration, 100 μl of the suspension was pipetted into an Eppendorf tube and centrifuged at high speed for 10 min (Hettich Mikroliter, HD Scientific, Australia). Fifty microliters of the clear supernatant were diluted with mobile phase and assayed by HPLC according to a method modified from the literature (Wright et al., 1994). Sampling was performed every 60 min until no change in concentration of each compound was observed.

3. Results and discussion

3.1. Syntheses and structure confirmation of M6S and derivatives

The synthesis of 3AcM6S was modified from the literature (Mori et al., 1972). It was noted that the molar ratio of morphine to acid anhydride influenced the success of the syntheses, especially for the longer chain esters. Pre-purification was essential to remove contaminants such as unreacted 3-acylmorphine and polar reagents prior to semi-preparative HPLC separation. Analytical and spectroscopic properties of the compounds are reported in Table 1. IR spectra (Table 1), mass spectra (Table 1) and NMR spectra (Section 3.2) of all compounds were consistent with the assigned structures. The overall yield of each compound was not less than 55% by weight with high purity (99%) from HPLC. The high melting points with decomposition and low TLC R_f values suggest that these compounds are very polar. There is a clear trend to lower melting point with increasing ester chain length, suggesting progressive disruption of the M6S crystal lattice for the longer acyl groups. The solid compounds are highly likely to be zwitterionic. This has been confirmed for the closely related 3-*O*-acetyl-7,8-dihydromorphine-6-*O*-sulfate by X-ray crystallography (Brock et al., 1996), in which the zwitterions are linked into chains by hydrogen bonds. Such

Table 2
^{1}H chemical shifts (ppm) for morphine, morphine-6-sulfate and its derivatives

	Compounds					
Proton(s)	Morphine	M6S	3AcM6S	3PrM6S	3BuM6S	3HpM6S
H-1	6.73	6.73	6.74	6.74	6.75	6.77
H-2	7.13	7.15	7.11	7.12	7.14	7.18
H-5	5.21	5.57	5.57	5.57	5.56	5.54
H-6	4.60	5.61	5.62	5.64	5.63	5.60
H-7	6.11	6.52	6.54	6.53	6.54	6.57
H-8	5.40	5.54	5.46	5.42	5.43	5.47
H-9	4.14	4.55	4.21	3.99	4.09	4.36
H-10α	2.85	2.99	2.83	2.71	2.75	2.91
H-10β	3.35	3.34	3.30	3.24	3.27	3.34
H-14	3.83	3.44	3.24	3.12	3.16	3.30
H-15eq	1.90	1.95	1.78	1.74	1.75	1.78
H-15ax	2.79	2.61	2.36	2.25	2.28	2.40
H-16eq	3.21	3.41	3.11	2.94	3.02	3.21
H-16ax	2.97	3.08	2.79	2.68	2.73	2.86
N-CH_3	2.95	3.16	2.91	2.78	2.84	3.00
Acyl-CH_3			2.10	1.11	0.96	0.89
$CH_2(\alpha)$				2.45	2.44	2.50
$CH_2(\beta)$					1.69	1.70
$CH_2(\gamma)$						1.33
$CH_2(\delta)$						1.23
$CH_2(\epsilon)$						1.28

Chemical shifts were measured from the pyridine resonance at 8.80 ppm and are relative to TMS.

chains would be hard to disrupt, thus contributing to their high melting points.

3.2. NMR studies

The ^{1}H and ^{13}C NMR were obtained with a 500 MHz Bruker AMX FT-Spectrometer at a sample temperature of 303 K, using standard pulse sequences (Center for Magnetic Resonance, The University of Queensland). For 2D experiments, typically, 64 scans were acquired for each of 512 increments using a spectral width of 10 ppm in both dimensions. ^{1}H and ^{13}C chemical shifts for morphine, M6S and its 3-acyl derivatives are summarized in Tables 2 and 3.

The NMR studies are reported here in some detail, as the literature on assignment of signals to morphine derivatives is not complete. In this work, the complete assignment of the ^{1}H resonances of morphine, M6S and the 3AM6S esters was achieved by 1D and 2D (DQF-COSY and heteronuclear multiple-quantum coherence (HMQC)) NMR experiments and by comparison with published spectra in other solvents (Brown et al., 1983; Eliel and Morris-Natschke, 1984; Brown et al., 1985; Krowech et al., 1986; Sy et al., 1986; Neville, 1987). Assignment of chemical shifts was obtained for complex multiplets from examination of cross peaks in the DQF-COSY spectra. The ^{1}H NMR spectra of morphine, M6S and the M6S derivatives displayed the H-1 and H-2 resonances at a downfield chemical shift typical of aromatic protons. The DQF-COSY experiment distinguished H-1 from H-2 by the coupling of H-10α and H-10β with H-1, but not with H-2. For H-5, H-6 and H-7, the resonances of M6S and all its derivatives were deshielded to 5.5, 5.6 and 6.5 ppm, respectively, compared with 5.2, 4.6 and 6.1 ppm in morphine. The lower field chemical shifts of these signals presumably resulted from an electron-withdrawing effect of the sulfate group. COSY experiments showed very strong coupling between the vinylic protons H-7 and H-8, and a strong correlation was also observed

Table 3
^{13}C chemical shifts (ppm) for morphine, morphine-6-sulfate and its derivatives

	Compounds					
Carbon(s)	Morphine	M6S	3AcM6S	3PrM6S	3BuM6S	3HpM6S
C-1	119.90	120.14	119.41	119.45	119.20	119.55
C-2	118.24	118.85	123.03	123.05	123.65	123.27
C-3	—	141.48	–	–	–	–
C-4	–	147.82	–	–	–	–
C-5	92.06	90.34	91.73	91.99	91.63	91.56
C-6	67.16	73.03	72.74	72.69	72.60	72.69
C-7	135.53	132.39	132.41	132.51	132.43	132.54
C-8	126.05	126.54	126.81	127.13	126.62	126.54
C-9	60.31	60.82	59.91	59.74	59.85	60.08
C-10	22.26	22.19	22.12	21.92	21.86	22.19
C-11	–	121.72	–	–	–	–
C-12	–	129.23	–	–	–	–
C-13	–	43.17	–	–	–	–
C-14	38.60	39.48	39.71	40.13	39.74	39.70
C-15	33.64	33.45	33.50	33.93	33.11	33.33
C-16	46.76	47.36	46.68	46.55	46.35	46.68
N-CH_3	41.25	41.47	41.73	42.02	41.55	41.53
Acyl-CH_3			20.24	9.02	13.25	14.53
$CH_2(\alpha)$				27.26	35.35	33.91
$CH_2(\beta)$					18.30	25.07
$CH_2(\gamma)$						22.54
$CH_2(\delta)$						31.45
$CH_2(\epsilon)$						22.25

Chemical shifts were measured from the d_5-pyridine carbon at 150.00 ppm and are relative to TMS.

between H-8 and H-14, which clarified their chemical shift assignments. The presence of the sulfate group resulted in small but significant shifts for protons H-9, H-15 and H-16, whereas H-10 protons remained relatively constant. In general, acylation at the 3-position produced relatively minor changes in 1H chemical shifts of the morphine moiety. Assignment of acyl chain proton resonances was confirmed from DQF COSY spectra.

On comparing the spectra with published data (Brown et al., 1983; Eliel and Morris-Natschke, 1984; Brown et al., 1985; Krowech et al., 1986; Sy et al., 1986; Neville, 1987), it is also clear that the chemical shifts of most protons are solvent dependent. For example, in D_2O, the H-1 proton of morphine was assigned to lower field than H-2 (Krowech et al., 1986). However, in CD_3OD, $CDCl_3$ (Neville, 1987) and in d_5-pyridine (this study), the H-2 proton is assigned to lower field resonances than those of H-1. The H-1 and H-2 resonances of morphine and the 3-acylmorphine-6-sulfates in pyridine were shifted downfield compared to those for morphine in $CDCl_3$ and CD_3OD. In this study, the chemical shifts for the H-5, H-6, H-7 and H-8 resonances of morphine in d_5-pyridine were found to be in the same relative order as in other solvents ($CDCl_3$, D_2O, CD_3OD). However, for M6S and derivatives, the H-5 and H-6 resonances were shifted downfield to 5.5–5.6 ppm, with the H-8 resonance moved to higher field. In general, most resonances were shifted to lower field in d_5-pyridine, compared to CD_3OD and $CDCl_3$.

The assignment of carbon-13 resonances was achieved with the aid of HMQC and heteronuclear multiple-bond correlation (HMBC) 2-D experiments. The HMQC experiment allows assignment of carbon-13 resonances by correlation with the resonance of directly attached pro-

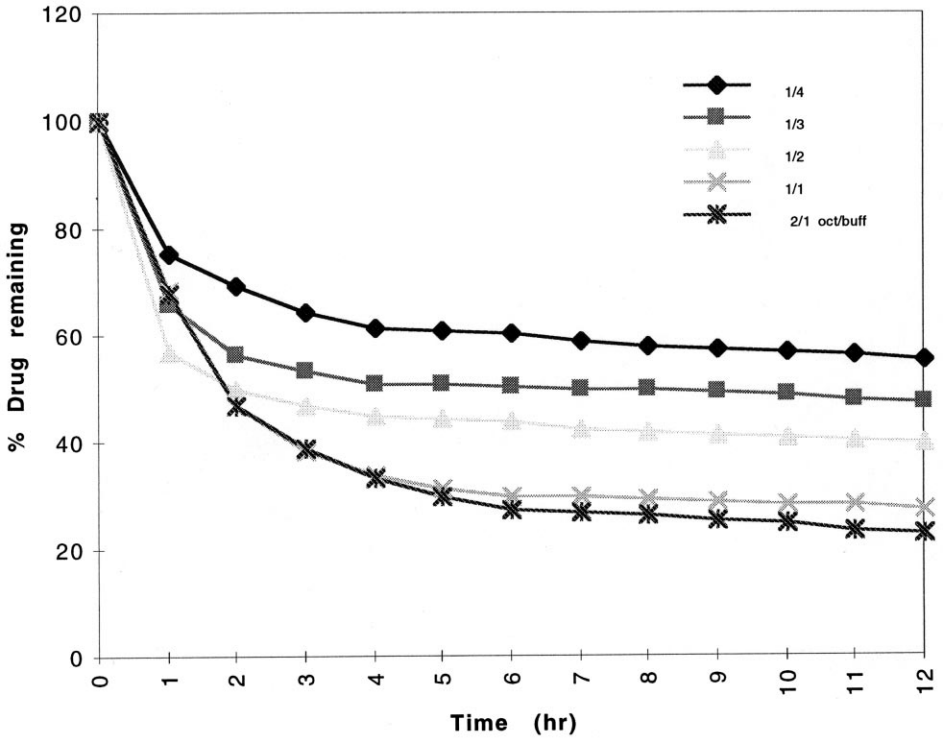

Fig. 1. Plot of [3HpM6S] in the buffer phase versus time at various volume ratios for the shake-flask partition coefficient determination.

tons. Quaternary carbons were assigned using the HMBC experiment, which shows correlations between proton resonances and carbons which are three (or two) bonds removed. Changing the acyl group had little effect on the carbon-13 chemical shifts of the skeletal carbons. As expected, the effect of the sulfate group was most marked for C-6, compared to morphine, being shifted down field by 5 ppm to 72 ppm. The 3-acyl methyl group demonstrated the well-known gamma effect on carbon shifts.

3.3. Partition coefficient determinations

3.3.1. Shake-flask method

Partition coefficient determination using the shake-flask method was successful only for 3HpM6S (Fig. 1), as the other prodrugs were too hydrophilic for distribution from the aqueous phase to be measured with quantitative accuracy. Interfering peaks prevented HPLC analysis of the prodrugs in the 1-octanol phase, thus the concentration of 3HpM6S in this phase was calculated from the aqueous phase concentration by difference for replicated experiments. The prodrug was rapidly lost from the aqueous phase during the first 2 h after mixing, due to redistribution to the octanol phase, then there was a slow loss from 4 to 8 h in all volume ratios, due to hydrolysis. The concentration-time profile of the compound in buffer alone was closely similar to that in the partitioning experiment after equilibration of 3HpM6S between the two layers was achieved. The mean partition coefficient of 3HpM6S from all measurements was found to be $P = 2.30 \pm 0.18$, i.e. $\log P = 0.36 \pm 0.03$.

3.3.2. Methylene group contributions

The reported methylene group contribution to octanol–water partitioning, derived from numerous homologous series, ranges from 0.52 (Davis et al., 1974) to 0.54 (Prankerd and McKeown, 1992).

Taking this value to be 0.53 ± 0.01 allows estimation of $\log P_{(cal)}$ for each prodrug from the $\log P$ value of 3HpM6S ($\log P = 0.36 \pm 0.03$). Therefore, the $\log P_{(cal)}$ values for 3AcM6S, 3PrM6S and 3BuM6S are -2.23 ± 0.04, -1.71 ± 0.04 and -1.19 ± 0.04, respectively.

3.3.3. Capacity factors

Except for 3HpM6S, the prodrug lipophilicities could not measured by the shake-flask method. Instead, they were estimated by difference from the value for HpM6S, using known methylene group contributions for each compound. HPLC retention time data has also been widely used to assess the lipophilicity of many compounds (Hammers et al., 1982; Hafkenscheid and Tomlinson, 1983, 1984; Bechalany et al., 1989). The log capacity factor (Eq. (2)), is often found to correlate with conventional $\log P$ values.

$\text{Log } k'$ was obtained from the retention times, using Eq. (2). $\text{Log } k'_o$, the capacity factor for a purely aqueous mobile phase is of interest, as most conventional $\log P$ values are determined using an aqueous phase which contains only buffers and no organic co-solvents. Nonetheless, it is not always readily accessible, as organic compounds may have lengthy retention times. Thus, $\log k'_o$ was estimated by extrapolating plots of $\log k'$ versus %ACN to 0%.

The relationships between %ACN and $\log k'$ for all compounds were non-linear. It is likely that the non-linear relationship results from the use of low concentrations of ACN in the mobile phase, rather than higher concentrations of MeOH, as used elsewhere (Hafkenscheid and Tomlinson, 1984). The data for all compounds in this study were modelled well by an empirical fit to a series of quadratic equations, with r^2 values > 0.991. In the worst case, the overall Fisher F-ratio for significance of the quadratic fit was 268, with three and five degrees of freedom. This corresponded to $p < 0.001$. Most F-ratios were > 1000. For all compounds, Student's t-test on the standard error for the quadratic term (five degrees of freedom) was significant at $p < 0.001$, except for morphine itself, where the results were significant with $p < 0.01$. The coefficients of variance for all estimated $\log k'_o$ values were not more than 3%.

The extrapolated $\log k'_o$ values gave a shallow sigmoidal relationship with apparent mobile phase pH. A substantially more exaggerated sigmoidal plot was observed for morphine. This is probably related to a significant overall change in polarity for morphine on changing the extent of ionization of the 3° nitrogen. Fig. 2 shows the plots of $\log k'$ versus pH for morphine, M6S and three of its prodrugs. For M6S and its esters, the overall change in polarity on ionization of the 3° nitrogen is probably less than for morphine, as the 6-sulfate group remains fully ionised at all pH values studied. This maintains a high overall polarity for the M6S compounds which is relatively pH-independent. This is also different to the reported changes in hydrophilicity for the 3- and 6-glucuronides (Carrupt et al., 1991), for which rather marked sigmoidal curves were observed over a pH range which included the measured pK_a value of the glucuronide carboxyl group. The change in ionisation of the carboxyl group should contribute significantly to an overall change in polarity, and thus a marked sigmoid should result.

At pH 7.4, it was found that the differences in $\log k'_o$ values between 3AcM6S and 3PrM6S, and between 3PrM6S and 3BuM6S, were 0.38 and 0.43, respectively. These estimates for the methylene group contribution to hydrophobicity are less than those usually found by the 1-octanol–water shake flask method, for which the $\log P$ difference is 0.52–0.54 (Davis et al., 1974; Prankerd and McKeown, 1992). This indicates that although the $\log k'_o$ values on the C8 RP HPLC column may be linearly related to the $\log P$ values (Hafkenscheid and Tomlinson, 1983; Prankerd, 1985), the relationship has a slope which is < 1.

The relationship between $\log k'_o$ and $\log P_{(cal)}$ for the three shorter 3-acylmorphine-6-sulfate prodrugs was examined. An apparently good linear relationship was found ($r^2 = 0.999$). The relationship between the $\log P_{(cal)}$ values and the $\log k'_o$ values is given by:

$$\log k'_o = 0.775 * \log P_{(cal)} + 3.441 \quad (3)$$

The standard errors for the slope and intercept were 0.022 and 0.039, respectively, while the over-

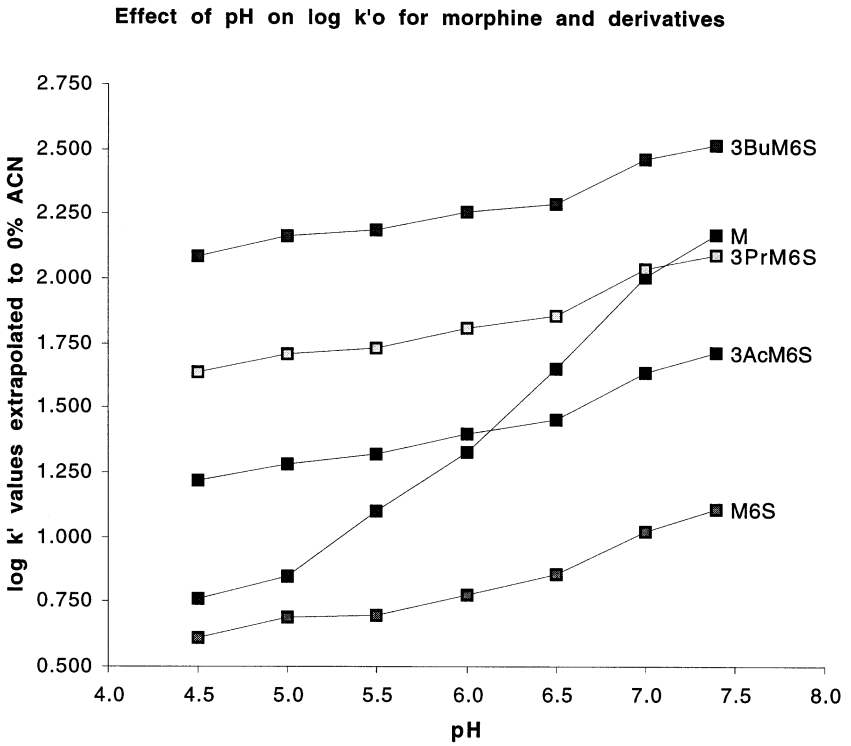

Fig. 2. Plots of $\log k'_o$ for morphine (M), M6S and three prodrugs as a function of pH.

all Fisher F-ratio was 1218. This was significant at $p < 0.025$, even though there were only three points in the correlation. From this relationship, a $\log P_{(cal)}$ value of about -3.1 could be estimated for M6S.

3.3.4. Chemical hydrolysis studies

The prodrug 3AcM6S was shown by these studies to be relatively stable in PBS over the pH range 6.15–8.01 at 37.0°C. The half-lives ranged from 2030 min (pH 8.01) to 32 600 min (pH 6.15). Under physiological conditions, the pseudo-first order hydrolysis rate constant is about 1.4×10^{-4} min^{-1}. The other 3AM6S prodrugs are expected to be less susceptible to chemical hydrolysis, due to their longer alkyl sidechains. The slopes for the plots of k_{cat} (s^{-1}) versus [total phosphate] ranged from 2.8×10^{-6} to 1.8×10^{-5} $l \cdot mol^{-1} \cdot s^{-1}$. Fig. 3 shows the buffer-independent rate constants (k_{uncat}) (s^{-1}) as a function of the experimental pH values. This partial pH-rate profile shows hypothetical lines of slope $+1$, which would be expected for base-catalyzed ester hydrolysis in this pH region.

The data for the partial pH-rate profile has slope < 1 in the pH range expected for change in the state of ionization of the 3° nitrogen. The pK_a values for M6S or its 3-acyl derivatives have not been measured. The reduced slope is due to different susceptibilities expected for base-catalyzed hydrolysis of the species with either a protonated or a deprotonated 3° nitrogen in the morphine nucleus. Similar changes in slope are found in pH-rate profiles for the hydrolyses of esters containing a 3° nitrogen, such as cocaine (Connors et al., 1986).

3.3.5. Enzymatic hydrolysis studies

These studies show that enzymes in blood and brain homogenate, presumably esterases, catalyse hydrolysis of the prodrugs very considerably (Table 4). Some variability in the measured hy-

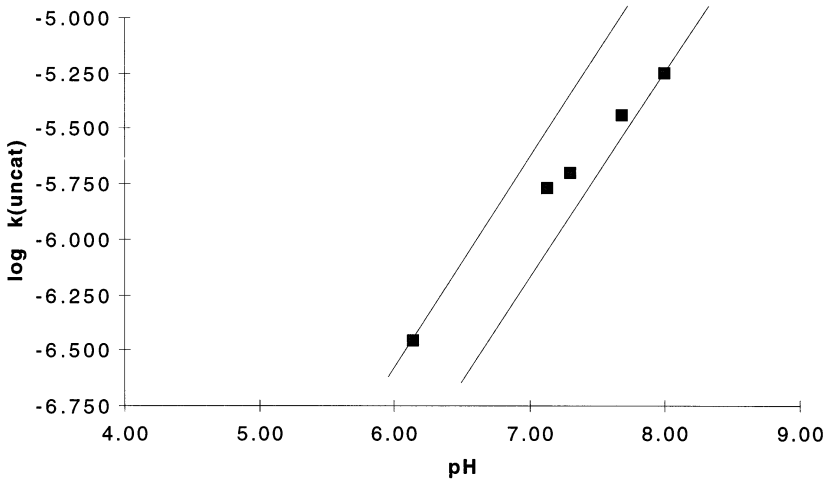

Fig. 3. Partial pH-rate profile (buffer-corrected) for hydrolysis of 3AcM6S (2×10^{-4} M) at 37°C.

drolysis rates for each compound was expected, as the blood or brain homogenate used for the replicate experiments on each prodrug was taken from a different animal. From the time-courses for % remaining of 3AcM6S, 3PrM6S and 3BuM6S in 10% rat blood and 10% rat brain homogenate, pseudo-first order reaction kinetics were observed with good linear relationships between log % prodrug remaining and time ($r^2 > 0.97$). The dilution is performed to ensure that the pH of each system does not change significantly from physiological pH, which would occur when blood or tissues are separated from the buffering action of the carbon dioxide tension maintained in vivo by the respiratory system (Flear et al., 1987). The hydrolysis rate for each compound was substantially faster in 10% brain homogenate than in 10% blood. Assuming that the rate of hydrolysis in whole blood or brain is about ten-fold faster than in the present study, the likelihood of the prodrugs surviving intact (estimated half-lives, 7–11 min) in the blood to be absorbed across the BBB is high. After hydrolysis of the esters in the brain (estimated half-lives, 2.5–3.6 min), the higher polarity of the resulting M6S is expected to help its retention in the CNS.

The difference in hydrolytic rate constants for the 3-acetyl and 3-butanoyl groups in 10% brain homogenate was significant at $p < 0.05$, while that for the 3-propionyl group appeared to be intermediate. Similar behaviour was seen for the hydrolyses in 10% rat blood. The standard deviations used to assess the statistical significance were large, as no comparisons were made of the hydrolytic data for all three esters in blood or brain samples from the same rat. The hydrolysis kinetics of 3HpM6S were not studied, due to its low aqueous solubility. The anticipated relationship between chain length and hydrolysis rate may have several causes, including: (i) steric constraints of fitting the substrate into the esterase reaction site, particularly in rat plasma esterases; (ii) electron release from the alkyl side-chain to increase electron density at the labile acyl oxygen bond ($+$I effect); (iii) steric hindrance at the reactive carbonyl group caused by the side-chain.

It has been suggested from receptor binding and intracerebroventricular administration studies that 3AcM6S is a centrally acting analgesic (Houdi et al., 1996). The estimated rates of hydrolysis by whole rat brain (half-lives of 2.5–3.6 min, based on the rates in 10% brain homogenate) for the ester prodrugs from the present study indicates that there is only a relatively short time for the intact esters to produce any pharmacological effect. It should be noted that the rate of hydrolysis by brain homogenate may not reflect in vivo disposition and dynamics. Furthermore, 3-*O*-substituted morphine derivatives (e.g. codeine) generally do not bind well to mu opioid receptors.

Table 4
Rate constants[a] for the hydrolysis of three M6S prodrugs in 10% rat blood and 10% rat brain at pH 7.4 and 37°C

Compounds	Rate constant (min^{-1}) ± S.D.		Half-life (min) ± S.D.		No. of half-lives followed	
	Blood	Brain	Blood	Brain	Blood	Brain
3AcM6S	0.0146 ± 0.0021	0.0403 ± 0.0044	68 ± 12	24.8 ± 3.1	2.2	3.7
3PrM6S	0.0111 ± 0.0020	0.0350 ± 0.0035	90 ± 20	28.6 ± 3.2	2.3	3.2
3BuM6S	0.0087 ± 0.0022	0.0278 ± 0.0037	114 ± 38	36.0 ± 5.5	2.0	3.4

[a] Rate constants were determined in triplicate using blood or brain material from three separate rats with six time points in each kinetic run.

It is more likely that the 3-*O*-acyl group is simply acting as a carrier moiety to improve transport to the brain.

3.4. Solubility studies

Due to the high polarities of M6S, 3AcM6S and 3PrM6S, dissolution in soybean oil was not complete even after 20 ml of the oil had been added to 1 mg of each compound. Cloudy suspensions of the samples were observed visually and microscopically, compared to the oil blank. Thus, it was concluded that the solubility of each compound in soybean oil was less than 50 μg/ml. Attempts to determine visual solubilities at lower concentrations were not made, due to difficulties in seeing the suspended crystals.

The aqueous solubilities of M6S, 3AcM6S and 3PrM6S in PBS (pH 7.4) at 37.0 ± 0.1°C were found to be 3.83 ± 0.03 (1.05×10^{-5} M), 3.33 ± 0.01 (8.17×10^{-6} M) and 9.50 ± 0.04 (2.25×10^{-5} M) mg/ml, respectively. These solubilities were achieved after 6 h and were constant for an additional 3 h. The aqueous solubilities for the three compounds were not ranked by either their melting points or their partition coefficients. This indicates that neither crystal lattice energies nor lipophilicities dominate the aqueous solubilities, but both factors are of comparable magnitude.

4. Conclusions

The prodrugs of M6S have a range of octanol–water partition coefficients ($\log P = -2.23$ to $+0.36$). The increased lipophilicity on lengthening the 3-*O*-acyl chain of the M6S prodrugs was clearly demonstrated by HPLC capacity factor measurements. There was a parabolic relationship between the HPLC capacity factors and the percentage of mobile phase organic modifier in this study. A linear relationship was found between the log capacity factors extrapolated to zero modifier concentration and octanol–water partition coefficients calculated from methylene group contributions. 3AcM6S was found to hydrolyse relatively slowly in PBS over the pH range 6.15–8.0. The other prodrugs are expected to hydrolyse at similar or slower rates. The longer side-chain prodrug was hydrolysed at a slower rate in both of the enzymatic systems than shorter chain prodrugs for the compounds examined. The observed slower hydrolysis kinetics for the prodrugs in blood, in addition to their increased lipophilicity compared to M6S, may lead to improved in vivo transport of the prodrugs across the blood-brain barrier. These properties may increase the in vivo duration of analgesic action of M6S. Pharmacodynamic properties of these compounds are the subject of a following communication.

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