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Identification and synthesis of norhydromorphone, and determination of antinociceptive activities in the rat formalin test

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Abstract

The main objective of this paper is to report the identification and synthesis of norhydromorphone, a novel metabolite of hydromorphone, and its antinociceptive activities when tested in the formalin test as compared to other known analgesics. In addition, we are reporting for the first time the lack of antinociceptive activities of hydromorphone-3-glucuronide, dihydromorphine-3-glucuronide and dihydroisomorphine-3-glucuronide in the rat formalin test. Norhydromorphone was isolated and identified as a metabolite of hydromorphone in a cancer patient's urine. An authentic standard of norhydromorphone was synthesized. The identity of norhydromorphone in the urine sample was confirmed by comparing the LC retention time and MS ion fragmentation with the synthetic standard using a liquid chromatographic-mass spectrometric-mass spectrometric (LC-MS-MS) assay. Norhydromorphone was found to be a minor metabolite of hydromorphone in the urine. Additionally, the antinociceptive activities of norhydromorphone, hydromorphone, morphine, dihydromorphine, dihydroisomorphine, hydromorphone-3-glucuronide, dihydromorphine-3-glucuronide and dihydroisomorphine-3-glucuronide were determined in the rat formalin test following intraperitoneal (i.p.) administration. Only limited antinociception was observed and no significant increase in antinociception was detected at the three doses tested. The increased polarity of norhydromorphone as compared to hydromorphone due to the primary piperidine nitrogen may make it less favorable to cross the blood-brain-barrier (BBB), which may be partly responsible. In addition, lower intrinsic antinociceptive activity, which remains to be determined, could also contribute to the low antinociception. Our results also show that

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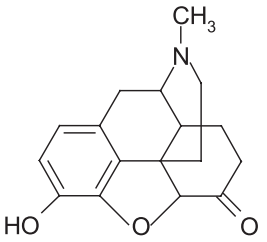
hydromorphone was five times as potent as morphine in the formalin test, while dihydromorphine and dihydroisomorphine were equipotent to and 36% as potent as morphine, respectively. Hydromorphone-3-glucuronide, dihydromorphine-3-glucuronide and dihydroisomorphine-3-glucuronide did not exhibit any antinociceptive effect at the doses tested. The results further underscore the importance of a free C_3-OH to the analgesic effect of morphine alkaloids.

Keywords: Norhydromorphone; Hydromorphone; LC-MS-MS; Metabolite identification; Formalin test; Antinociception

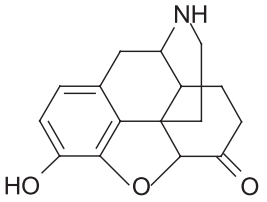
Introduction

Like morphine, hydromorphone is also a μ-opioid analgesic, and it is used for the relief of moderate to severe pain (Sarhill et al., 2001). Hydromorphone is about 8 times as potent as morphine in relieving postoperative pain (Mahler and Forrest Jr, 1975) and chronic cancer pain (Houde, 1986), although a 5:1 potency ratio has been reported after intravenous bolus administration in healthy volunteers undergone repeated electrical tooth pulp stimulation (Coda et al., 1997). At equianalgesic doses, hydromorphone and morphine have similar peak effects and duration of action (Mahler and Forrest Jr, 1975; Houde, 1986).

Hydromorphone has been reported to be metabolized and form dihydromorphine, dihydroisomorphine, dihydromorphine-3-glucuronide and dihydroisomorphine-3-glucuronide in humans (Cone et al., 1977; Zheng et al., 2002a). Dihydromorphine and dihydroisomorphine have been reported to exhibit 65% and 21% of the analgesic activity of hydromorphone, respectively, as determined by mechanical clip test (Small et al., 1938). Here we would like to report the identification of a novel metabolite of hydromorphone, norhydromorphone, in a cancer patient's urine, and the antinociceptive activities of norhydromorphone and other structural analogs, namely hydromorphone, morphine, dihydromorphine and dihydroisomorphine determined in the rat formalin test. Initially developed by Dubuisson and Dennis (Dubuisson and Dennis, 1977), the formalin test has several important advantages over threshold thermal or mechanical nociceptive tests, including free movement of the animals during the test, a bi-phasic nociceptive response curve with the involvement of inflammation in the second phase, and nociceptive responses involving complex movement instead of simple reflex. The formalin test is believed to be a valid animal model for clinical pain (Dubuisson and Dennis, 1977; Tjølsen et al., 1992).



Hydromorphone



Norhydromorphone

Methods

Hydromorphone hydrochloride was a generous gift from Knoll Pharma Inc. (Mississauga, Ont., Canada). Morphine sulfate pentahydrate was obtained from BDH Inc. (Toronto, Ont.). Dihydromorphine hydrochloride and dihydroisomorphine hydrochloride were synthesized as previously described (Zheng et al., 2002a). Other reagents were of analytical grade and the solvents were of HPLC grade. Purified water was produced using a Milli-Q water purification system (Mississauga, Ont.).

Synthesis of norhydromorphone

The synthesis of norhydromorphone was accomplished by adaptation of the methods for syntheses of normorphine and nordihydrocodeine (Konishi et al., 1994; Montzka et al., 1974). To 1.1 g (3.8 mmol) of hydromorphone base was added 114 ml of chloroform, 6.8 g (68 mmol) of potassium bicarbonate and 4.8 ml (34 mmol) of 2,2,2-trichloroethyl chloroformate (Aldrich, Milwaukee, WI, USA), and was refluxed for 5 hr with magnetic stirring. The reaction mixture was allowed to cool, filtered, and washed successively with 50 ml of water and 50 ml of 0.1 N HCl. The chloroform phase was separated, dried over sodium sulfate anhydrous powder, evaporated using a rotary evaporator, and further condensed under vacuum overnight. The residue was a thick liquid. To this residue was added 50 ml of tetrahydrofuran, 4.3 g of zinc powder and 4.3 ml of 1 N NaH_2PO_4 solution, and the mixture was refluxed for 2.5 hr. The mixture was allowed to cool and filtered. The filtrate was evaporated using a rotary evaporator. The remaining liquid residue was diluted with 20 ml of water and the pH was adjusted to 2 with concentrated HCl. The solution was washed with 10 ml of ethyl ether. The acidic aqueous layer was separated and the pH was adjusted to pH 10 with ammonium hydroxide. Precipitation was completed at 4 °C overnight. The crystals were filtered, washed with methanol and dried under vacuum with a yield of 0.56 g (54%). The product did not melt up to 285 °C (Thomas Hoover capillary melting point apparatus, Philadelphia, PA, USA). The hydrochloride salt was obtained by suspending the base in methanol and treating with ethanolic HCl solution. The ethanolic HCl solution was prepared by bubbling HCl gas in ethanol. The ^{1}H NMR spectrum (Bruker WH-200 spectrometer) in d_2-H_2O exhibited signals at: δ 4.15 (m, 1H, 9-H); 5.09 (s, 1H, 5β-H); 6.80 (m, 2H, 1-H, 2-H). LC-MS: $[M+1]^+$ 272 (rel. 100%); IR (Bomem MB-100 FTIR spectrophotometer): 3187 cm^{-1} (bonded OH), 3123 cm^{-1} (secondary amine), 2726 cm^{-1} (secondary amine salt). UV λ_{max} (water): 280 nm; UV λ_{max} (0.1 N NaOH): 292 nm. *Anal*. Calcd. for $C_{16}H_{17}NO_3 \cdot HCl \cdot H_2O$: C, 58.99; H, 5.57; N, 4.30. Found: C, 58.66; H, 5.77; N, 4.04 (Microanalytical Service Laboratory, Department of Chemistry, University of British Columbia).

Isolation of norhydromorphone from patient urine

The same urine sample previously isolated (Zheng et al., 2002a) was used for the identification of norhydromorphone. Briefly, an aliquot of pooled urine sample from a cancer patient undergoing hydromorphone therapy (subcutaneous infusion, 4 mg every three hours) was passed through an Amberlite XAD-2 resin column (Supelco, Oakvill, Ont.). The column was eluted with methanol which was then concentrated under vacuum. The residue was reconstituted in water and the pH was adjusted to 9.5–10 with 5% ammonium hydroxide. The aqueous solution was extracted with ethyl acetate. The organic phase was separated and concentrated under vacuum. Prior to the LC-MS-MS analysis, the

residues from the organic phase were dissolved in methanol. An aliquot of 20 μl of the final solution was analyzed by LC-MS-MS.

LC-MS-MS system

The LC-MS-MS system consisted of a HP series II 1090 liquid chromatograph (Hewlett Packard, Avondale, PA, USA), a Zorbax SB-phenyl column (4.6 mm × 25 cm) (Rockland Technologies, Inc., Newport, DE, USA), and a VG Quattro triple quadrupole mass spectrometer equipped with an electrospray interface (Fisons, Altrincham, England).

A mobile phase consisting of 0.1% (v/v) formic acid in 18% of methanol (methanol-water, 18–82, v/v) was delivered isocratically at a flow rate of 0.7 ml/min. The LC effluent was split after the column and 10% of the effluent was introduced into the MS. The MS/MS analysis was performed in the positive ion mode using the Multiple Reaction Mode (MRM). The electrospray source was operated with a capillary voltage of 3.4 kV, a cone voltage of 40 V and a source temperature of 130 °C. The collision energy was −80 eV. Argon was used as a collision gas at a pressure of 3×10^{-4} mbar. The low mass and high mass resolutions for both MS1 and MS2 were set at 7.0. A dwell time of 0.3 sec was used for monitoring.

Determination of antinociceptive activities of morphine, hydromorphone, norhydromorphone, dihydromorphine, and dihydroisomorphine in male Sprague Dawley rats using the formalin test

The experimental protocol was approved by the Committee on Animal Care of the University of British Columbia (Vancouver, BC, Canada). Male Sprague Dawley rats were obtained from the Animal Care Center of UBC six days before the experiment. The rats were provided standard laboratory food (Purina Lab Chow, PMI Inc., St. Louis, MO, USA) and water ad libitum. The animal housing room was maintained at 24 °C, with a 12-hour light/dark cycle (lights on at 06:00 hr). All experiments were conducted between 11:00– 17:00 hr.

The test was conducted in a transparent polycarbonate cage (46 × 26 × 20 cm; Allentown Caging, Allentown, NJ, USA) with a mirror placed at a 45° angle underneath, allowing an unobstructed observation of the movement of the paws. The animal was habituated to the test environment for at least 15 min before the test. Each rat was tested once and was sacrificed by an overdose of sodium pentobarbital given i.p. after the completion of the test.

A 5% (v/v) formalin solution was freshly prepared by diluting 50 μl of 37% formaldehyde (Fisher Scientific, Nepean, Ont.) with 950 μl of 0.9% sodium chloride solution. Fifty μl of the 5% formalin solution were injected subcutaneously (s.c.) into the plantar surface of one of the hind paws using a half-ml LO-DOSE® insulin syringe (28G, Becton Dickinson and Co., Franklin Lakes, NJ). Following the injection, nociceptive responses were observed according to the four rating scales described by Dubuisson and Dennis (Dubuisson and Dennis, 1977) for 60 min:

0 = Both hind paws were placed on the floor and the body weight was evenly distributed between the two hind paws;
1 = The injected paw was placed lightly on the floor or on another part of the body with little or no weight bearing on it, and the animal was limping during locomotion;
2 = The injected paw was elevated and not in contact with any surface, whereas the uninjected paw was placed firmly on the floor. During the later stage of the test (the last 10 − 15 min), the animals attempted

to sleep by curling up the injected paw off the floor or tucking the injected paw under the body, but not in contact with any surface. This behavior was also given the rating of 2;
3 = The injected paw was licked, bitten, or shaken.

The time spent in each of the four categories was recorded at 3-min intervals using a computer program (Burton, 1996). The computer program assigned each of the four arrow keys on the computer keyboard to one of the four behavioral categories, and the time spent in a particular behavioral category was recorded by pressing the specifically assigned key. The nociceptive rating was calculated as follows (Dubuisson and Dennis, 1977):

$$\text{Nociceptive rating} = \frac{T_1 + 2T_2 + 3T_3}{180}, \quad (1)$$

where T_1, T_2 and T_3 are the time spent in categories 1, 2 and 3, respectively, at each of the 3-min (180-sec) intervals.

Seven groups of rats were tested: (1) a formalin control group, in which the animals received an injection of 5% formalin solution only; (2) a formalin plus saline control group, in which the animals received an i.p. administration of 0.3 ml of 0.9% sodium chloride solution immediately before the injection of 5% formalin solution; in groups (3) to (7), the animals received i.p. administration of morphine sulfate, hydromorphone hydrochloride, norhydromorphone hydrochloride, dihydromorphine hydrochloride, and dihydroisomorphine hydrochloride, respectively, at various doses immediately before the injection of 5% formalin solution. Drug substances were dissolved in 0.9% sodium chloride solution and administered at 1 ml/kg. Due to inadequate solubility of norhydromorphone hydrochloride in 0.9% sodium chloride solution, the same solution (20 mg/ml) was used in the injection of the two highest doses (20 and 40 mg/kg).

Preliminary tests were also conducted for hydromorphone-3-glucuronide, dihydromorphine-3-glucuronide and dihydroisomorphine-3-glucuronide at the doses of 17 mg/kg, 18 mg/kg and 16 mg/kg, respectively. Hydromorphone-3-glucuronide, dihydromorphine-3-glucuronide and dihydroisomorphine-3-glucuronide were synthesized as previously described (Dubuisson and Dennis, 1977). The glucuronides were dissolved in 0.9% sodium chloride solution and administered (i.p.) immediately before the injection of 5% formalin solution.

The above study design is summarized in Table 1.

Calculation of antinociceptive activities of drug substances in the formalin test

Calculated nociceptive ratings at 3-min intervals were plotted against time and the areas under the nociceptive rating versus time curves (AUC_N) were calculated for the first and second phases, respectively, using the trapezoidal rule. The first and second phases were defined according to the results obtained from the formalin only group. The percentage of maximum possible effect (MPE%) at a given dose was thus calculated as follows:

$$\text{MPE\%} = \left(1 - \frac{\text{mean } AUC_N \text{ of the testing group}}{\text{mean } AUC_N \text{ of the formalin plus saline group}}\right) \times 100\%, \quad (2)$$

The doses at 50% of MPE (ED_{50}), 95% confidence limits of ED_{50}, and potency ratios were calculated from plots of MPE% against log doses according to the method by Litchfield and Wilcoxon (Litchfield and Wilcoxon, 1949).

Table 1
Summary of the doses and concentrations of the drug substances used in the formalin test

Group numbers/drugs	Doses		Concentrations	
	μmol/kg[a]	mg/kg[b]	μmol/ml[c]	mg/ml[d]
(1)/Formalin only	–	–	–	–
(2)/Formalin plus saline	–	–	–	–
(3)/Morphine sulfate	10	4.0	10	4.0
	16	6.0	16	6.0
	21	8.0	21	8.0
	32	12	32	12
(4)/Hydromorphone hydrochloride	2.5	0.80	2.5	0.80
	3.1	1.0	3.1	1.0
	3.7	1.2	3.7	1.2
(5)/Norhydromorphone hydrochloride	28	9.0	28	9.0
	61	20	61	20
	123	40	61	20
(6)/Dihydromorphine hydrochloride	10	3.3	10	3.3
	18	6.0	18	6.0
	28	9.0	28	9.0
	37	12	37	12
(7)/Dihydroisomorphine hydrochloride	25	8.0	25	8.0
	50	16	50	16
	99	32	99	32
Hydromorphone-3-glucuronide	37[e]	17[e]	18[f]	8.5[f]
Dihydromorphine-3-glucuronide	39[e]	18[e]	20[f]	9.0[f]
Dihydroisomorphine-3-glucuronide	33[e]	16[e]	33[f]	16[f]

[a] The doses are expressed as base equivalent.
[b] The doses are expressed as salt equivalent.
[c] The concentrations are expressed as base equivalent in 0.9% sodium chloride.
[d] The concentrations are expressed as salt equivalent in 0.9% sodium chloride.
[e] The doses are expressed as anhydrous equivalent.
[f] The concentrations are expressed as anhydrous equivalent.

Statistical analysis

Statistical analysis was performed using a statistical software package programmed by the Departments of Botany and Zoology, Faculty of Science, the University of British Columbia. In cases where two sample means were compared, two-tailed t-tests were performed, while in cases where more than two samples means were compared, one-way ANOVA was performed. The Tukey test was conducted if a significant difference was detected. Differences were considered statistically significant at the level of $P \leq 0.05$.

Results

Synthesis and identification of norhydromorphone

N-Demethylation of hydromorphone was accomplished by using 2,2,2-trichloroethyl chloroformate as a dealkylation reagent which formed the intermediate N-carbtrichloroethoxynorhydromorphone

(Montzka et al., 1974; Abdel-Monem and Portoghese, 1972) followed by hydrolysis to give rise to norhydromorphone. The success of the N-demethylation was confirmed by the result from the ^{1}H NMR experiment which did not show a singlet due to the N-methyl group in hydromorphone.

Norhydromorphone was previously tentatively identified as a metabolite of hydromorphone in the same cancer patient's urine (Dubuisson and Dennis, 1977). It is confirmed in this study by comparing the characteristics of chromatography and ion fragmentation pattern with the synthetic standard. As shown in Figs. 1 and 2, the HPLC retention times and ion transition of norhydromorphone in the urine sample and synthetic standard are in good agreement. The same LC-MS-MS systems and conditions developed in this study have also been utilized for the analysis of hydromorphone and its metabolites in plasma samples (Zheng et al., 2002b).

As shown in Fig. 1, the MS-MS responses of hydromorphone and norhydromorphone standards were similar at 5 μg/ml, with 8.49×10^5 and 6.60×10^5, respectively. Therefore, norhydromorphone detected in this patient's urine (4.58×10^4) was a minor metabolite, only representing less than 2% of hydromorphone (4.19×10^6) (Fig. 2).

Antinociceptive activities of morphine, hydromorphone, norhydromorphone, dihydromorphine, and dihydroisomorphine in the rat formalin test

In the group of rats which only received an injection of 50 μl of 5% formalin solution in the hind paw, a typical bi-phasic response curve, in which the nociceptive ratings were plotted against time, was

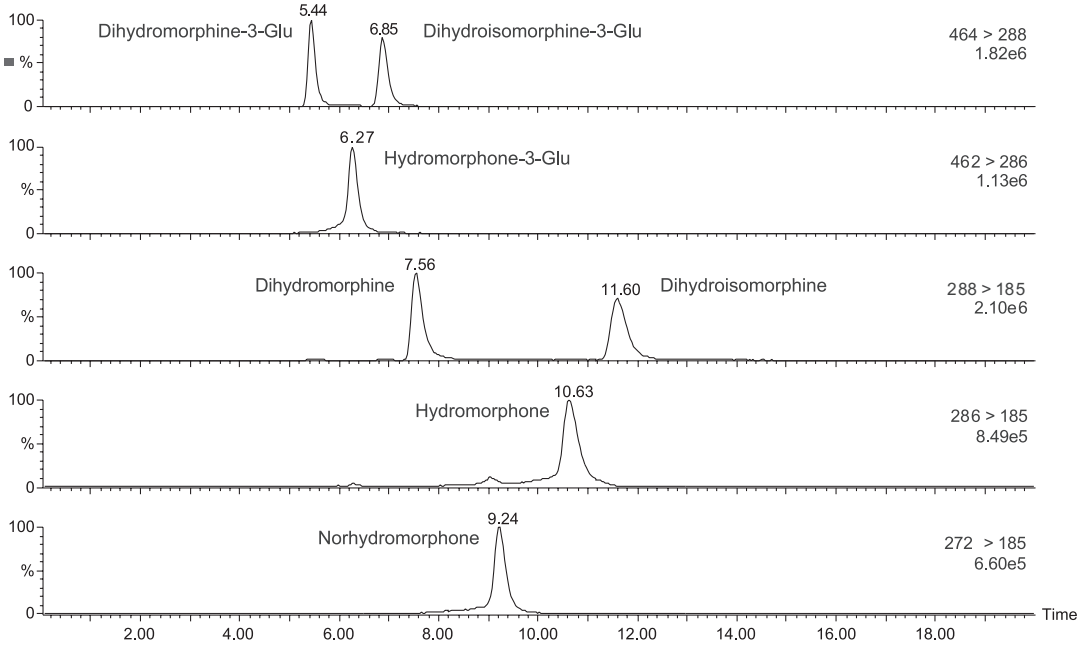


Fig. 1. LC-MS-MS chromatograms of a standard solution containing the standards of hydromorphone and its metabolites. The standard solution was prepared at 5.0 μg/ml for all standards. On the right corner of each chromatogram, e.g., 464 > 288, lists the parent to daughter ion transition used for MS-MS analysis. The values below the ion transition are absolute intensity of the most intense peak in that particular ion transition channel. The Y-axis indicates relative intensities. The number on the top of each peak is the retention time for that compound. Glu is an abbreviation for glucuronide.

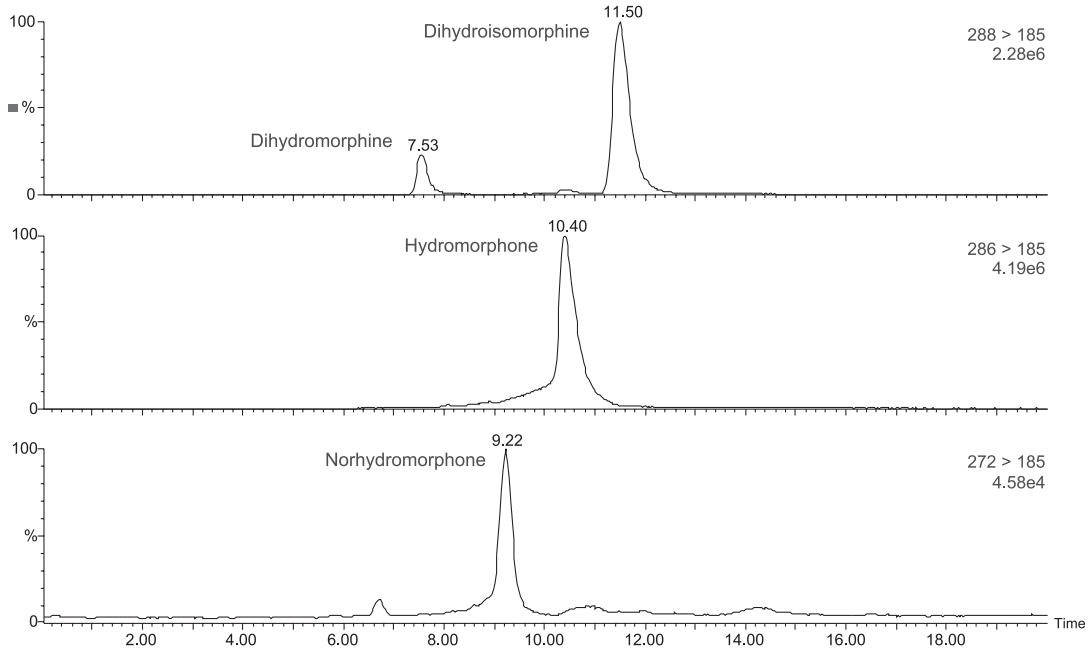


Fig. 2. LC-MS-MS chromatograms of unconjugated metabolites of hydromorphone in urinary extract dissolved in methanol. Please refer to Fig. 1 for details of legends.

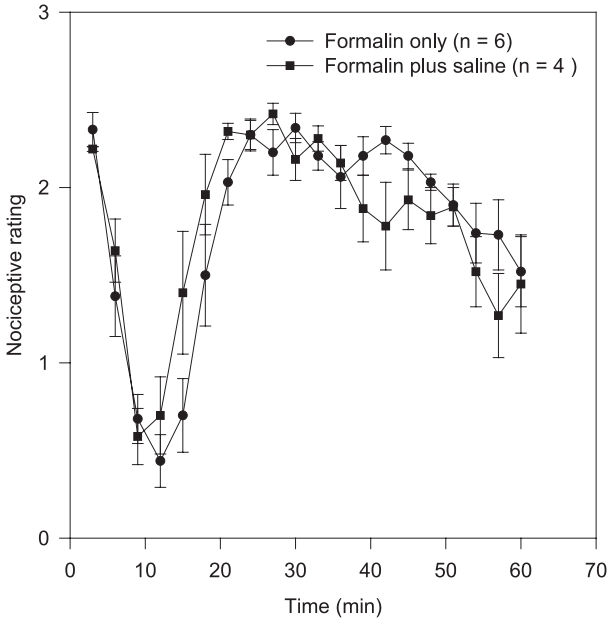


Fig. 3. The response curves of the formalin only group and the formalin plus saline group in the formalin test. Standard error bars are shown at each data point.

Table 2
The values of AUC_N[a] and the percentages of maximum possible effect (MPE%) determined in the formalin test

Groups/drugs	$AUC_{N\ (0–9\ min)}$[b]	$AUC_{N\ (15–60\ min)}$[b]	MPE%[b,c]
Formalin only (n = 6)	8.6 ± 0.74	89 ± 2.0	NA[d]
Formalin plus saline (n = 4)	9.0 ± 0.70	87 ± 3.9	NA
Morphine sulfate[e]			
10 μmol/kg (n = 5)	7.3 ± 0.75	69 ± 7.4[f,*]	21 ± 8.5
16 μmol/kg (n = 4)	8.9 ± 1.1	46 ± 3.4**	47 ± 3.9
21 μmol/kg (n = 5)	9.6 ± 1.1	21 ± 9.0 **	76 ± 10
32 μmol/kg (n = 3)	6.0 ± 1.2	0.087 ± 0.087**	100 ± 0.10
Hydromorphone hydrochloride[e]			
2.5 μmol/kg (n = 4)	6.9 ± 1.0	65 ± 5.2*	25 ± 5.8
3.1 μmol/kg (n = 3)	7.4 ± 0.84	58 ± 5.0**	33 ± 5.7
3.7 μmol/kg (n = 3)	7.6 ± 3.2	0.26 ± 0.26**	100 ± 0.33
Norhydromorphone hydrochloride[e]			
28 μmol/kg (n = 4)	7.4 ± 1.5	72 ± 4.5 *	17 ± 5.0
61 μmol/kg (n = 4)	7.6 ± 0.86	74 ± 2.9 *	15 ± 3.3
123 μmol/kg (n = 5)	8.1 ± 1.2	66 ± 6.3 *	25 ± 7.3
Dihydromorphine hydrochloride[e]			
10 μmol/kg (n = 4)	7.4 ± 0.32	61 ± 7.0*	30 ± 7.8
18 μmol/kg (n = 4)	9.4 ± 0.20	45 ± 8.6**	49 ± 9.9
28 μmol/kg (n = 4)	6.4 ± 0.67	25 ± 2.4**	71 ± 2.6
37 μmol/kg (n = 3)	7.4 ± 1.7	6.1 ± 3.5**	93 ± 4.0
Dihydroisomorphine hydrochloride[e]			
25 μmol/kg (n = 4)	7.8 ± 0.92	63 ± 4.8*	27 ± 5.5
50 μmol/kg (n = 4)	9.0 ± 1.8	38 ± 3.7**	56 ± 4.3
99 μmol/kg (n = 4)	8.4 ± 0.60	12 ± 5.0**	86 ± 5.6
Hydromorphone-3-glucuronide			
37 μmol/kg (n = 1)	10	94	NA
Dihydromorphine-3-glucuronide			
39 μmol/kg (n = 1)	9.6	94	NA
Dihydroisomorphine-3-glucuronide			
33 μmol/kg (n = 1)	10	83	NA

[a] Areas under the nociceptive rating versus time curves in the formalin test.

[b] The results are presented as mean ± standard error of the mean.

[c] The percentage of maximum possible effect calculated according to the AUC_N values in the second phase.

[d] Not applicable.

[e] Doses of the drug substance tested in the formalin test. The doses are base equivalent.

[f] The results are significantly different from the control groups ($P < 0.05$); the results are significantly different from those obtained at the previous lower doses within the same treatment group ($P < 0.05$).

* The results are significantly different from the control groups ($P < 0.05$).

** The results are significantly different from those obtained at the previous lower doses within the same treatment group ($P < 0.05$).

observed (Fig. 3). The nociceptive ratings at 9, 12 and 15 min were not significantly different ($P > 0.05$). Thus the first phase was defined from the beginning of the test to the end of the 8th min, and the second phase from the 15th min to the end of the test. The period between the first and second phases was defined as the interphase. The responses of the formalin group were indistinguishable from those of the formalin plus saline group as measured by AUC_N ($P > 0.05$; Table 2).

Morphine is the gold standard against which the analgesic or antinociceptive activities of other compounds are compared. In the present study, morphine also served as an indicator of the feasibility of the formalin test. Fig. 4 shows the response curves in the formalin test following i.p. administration of morphine at the four different doses. The initial dose of 10 μmol/kg (4 mg/kg) for morphine was chosen based on the work by Dubuisson and Dennis (Dubuisson and Dennis, 1977) showing that morphine administered at 6 mg/kg was able to effectively control the formalin-induced nociception. The values of AUC_N for the first phase at the four doses tested were not significantly different from the values of AUC_N for the first phase obtained in the control groups ($P > 0.05$; Table 2), thus the results obtained from the first phase could not be used for the evaluation of antinociceptive activities. The values of AUC_N for the second phase, however, steadily decreased ($P < 0.05$) as the doses of morphine were increased (Table 2). During the second phase, the frequencies of licking and elevating the injected paw were significantly decreased in the morphine treated group as compared to the control groups. At the highest dose tested, almost 100% antinociception was achieved in the second phase. The results indicated that the AUC_N of the second phase was a valid measure for antinociceptive activities of the test compounds. The percentages of MPE% were thus calculated by equation (2) using the values of AUC_N for the second phase in the treatment and the formalin plus saline groups.

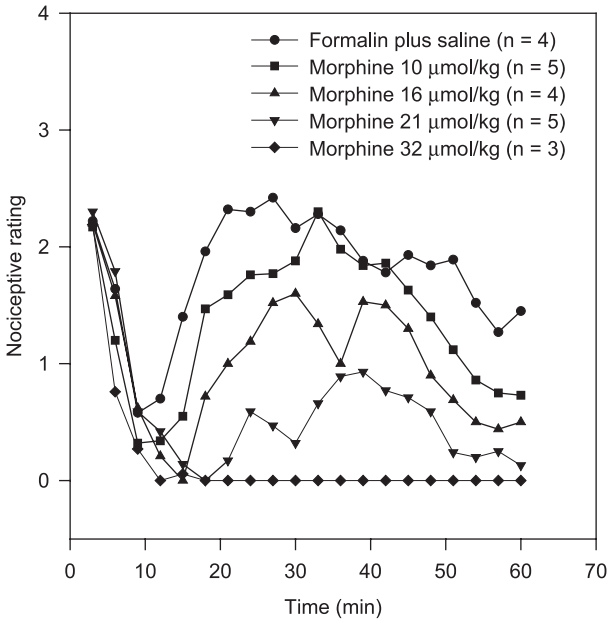


Fig. 4. The response curves in the formalin test following i.p. administration of morphine sulfate at four different doses. The doses listed are base equivalent. The response curve of the formalin plus saline group is also shown for comparison. For clarity of the presentation, the error bars are not shown.

Fig. 5(a) shows the response curves in the formalin test following administration of hydromorphone hydrochloride. As in the case of morphine, the values of AUC_N for the first phase at the three doses tested were not significantly different from the values of AUC_N for the first phase of the control groups ($P > 0.05$; Table 2). In the second phase, hydromorphone produced 100% antinociception at less than one-eighth of the dose for morphine.

Fig. 5(b) shows the response curves in the formalin test following administration of norhydromorphone hydrochloride. The values of AUC_N for the first phase at the three doses tested were not

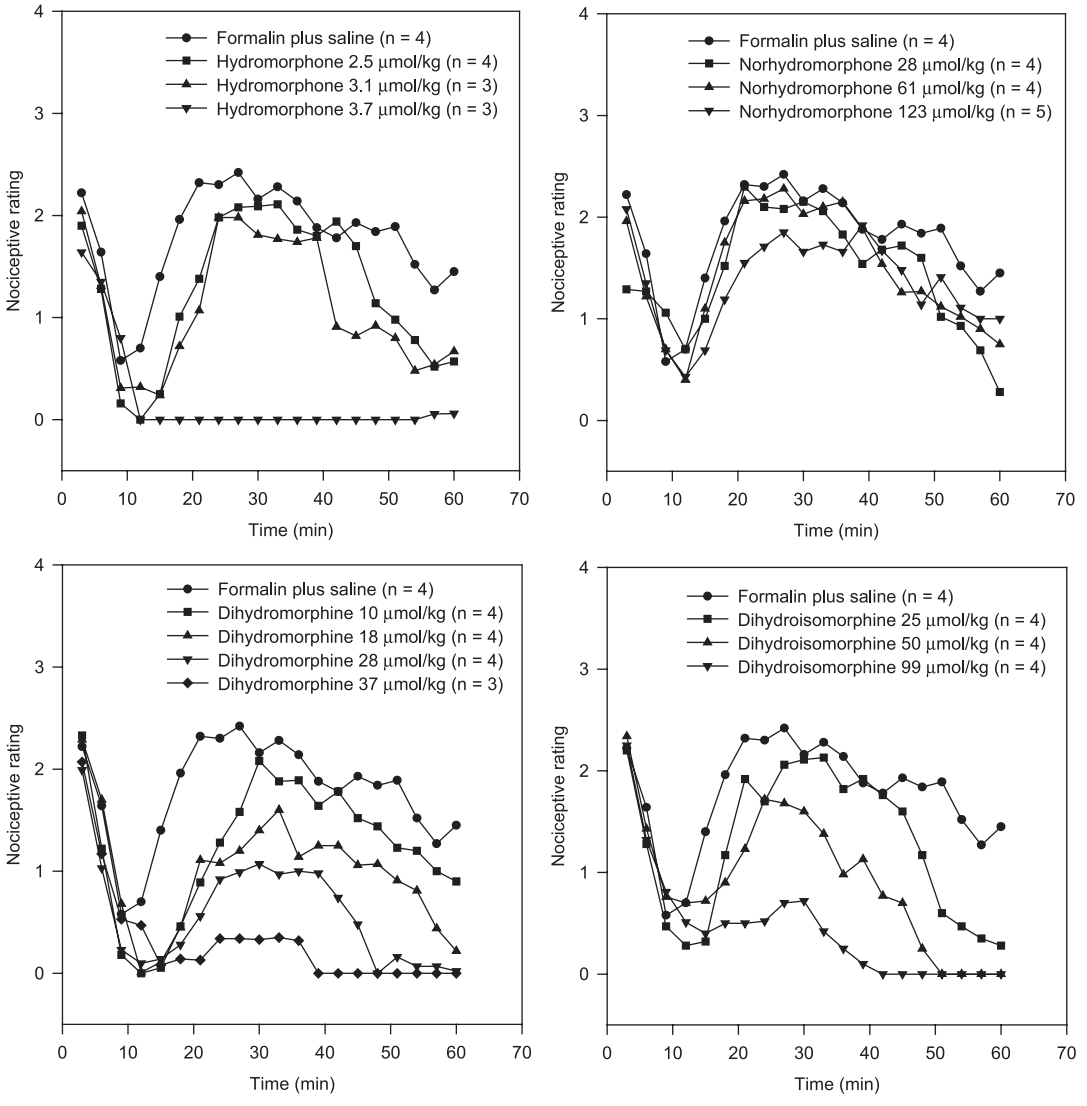


Fig. 5. The response curves in the formalin test following i.p. administration of (clockwise from top-left): (a) hydromorphone hydrochloride, (b) norhydromorphone hydrochloride, (c) dihydromorphine hydrochloride, and (d) dihydroisomorphine hydrochloride at different doses. The doses listed are base equivalent. The response curve of the formalin plus saline group is also shown for comparison. For clarity of the presentation, the error bars are not shown.

significantly different from the values of AUC_N for the first phase of the control groups ($P > 0.05$; Table 2). Although the values of AUC_N for the second phase of the norhydromorphone treated group were significantly different from the values of AUC_N for the second phase of the control groups ($P < 0.05$), the results of the norhydromorphone treated groups were not significantly different from each other ($P > 0.05$), which indicated that norhydromorphone produced the same degree of antinociception at the three doses tested.

Fig. 5(c) and (d) show the response curves in the formalin test following i.p. administration of dihydromorphine hydrochloride and dihydroisomorphine hydrochloride, respectively. Similarly, the values of AUC_N for the first phase of both compounds were not significantly different from the values of AUC_N for the first phase of the control groups ($P > 0.05$; Table 2). It was also evident that at similar doses dihydromorphine produced similar responses as morphine, whereas dihydroisomorphine required much higher doses to achieve the similar responses produced by morphine (Table 2).

A preliminary study was also conducted for hydromorphone-3-glucuronide, dihydromorphine-3-glucuronide, and dihydroisomorphine-3-glucuronide at the doses of 37 μmol/kg (17 mg/kg; n = 1), 39 μmol/kg (18 mg/kg; n = 1), and 33 μmol/kg (16 mg/kg; n = 1), respectively. The results did not indicate that the glucuronides induced any antinociception at the doses tested (Fig. 6 and Table 2).

The overall dose-response curves for the drug substances tested in the formalin test, excluding the glucuronides, are presented in Fig. 7. The values of ED_{50} for the drug substances (Table 3) were calculated according to the method by Litchfield and Wilcoxon (Litchfield and Wilcoxon, 1949), in which the results of MPE% were plotted against the common log of the corresponding doses followed by linear regression analysis. Since the results of MPE% for the second phase of the norhydromorphone

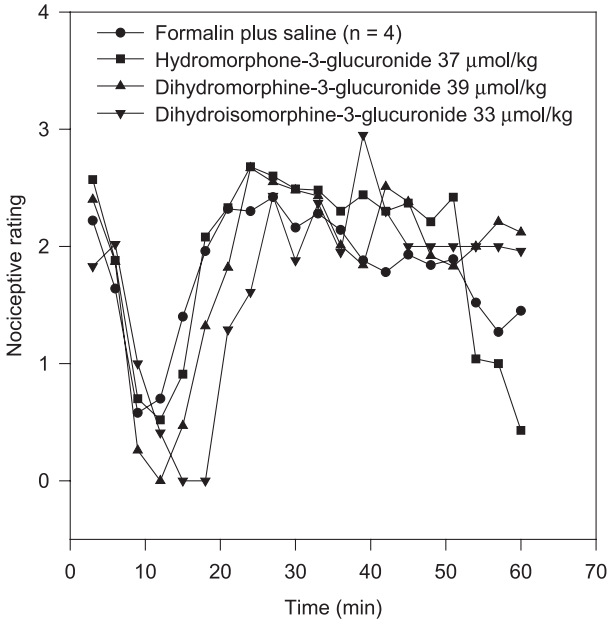


Fig. 6. The response curves in the formalin test following i.p. administration of hydromorphone-3-glucuronide (n = 1), dihydromorphine-3-glucuronide (n = 1), and dihydroisomorphine-3-glucuronide (n = 1). The response curve of the formalin plus saline group is also shown for comparison. For clarity of the presentation, the error bars are not shown for the formalin plus saline group.

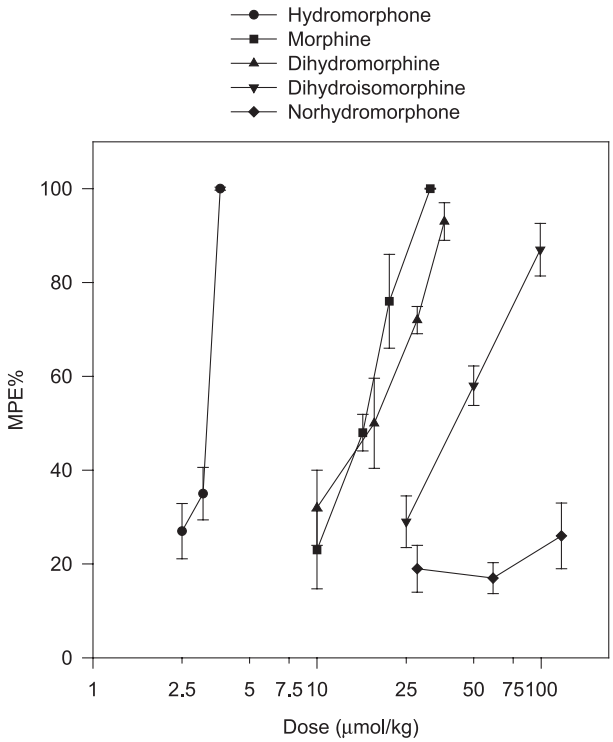


Fig. 7. The dose-response curves of hydromorphone, morphine, dihydromorphine, dihydroisomorphine and norhydromorphone in the formalin test. Standard error bars are shown at each data point.

treated group were not significantly different at the three doses tested, ED_{50} could not be calculated for norhydromorphone.

The dose-response curves for morphine, hydromorphone, dihydromorphine and dihydroisomorphine are parallel (P = 0.05) (Litchfield and Wilcoxon, 1949). Thus the potency ratios of hydromorphone, dihydromorphine and dihydroisomorphine to morphine were calculated and are presented in Table 3. The results indicate that hydromorphone is five times as potent as morphine, whereas morphine is approximately three times as potent as dihydroisomorphine. Morphine and dihydromorphine are equipotent in the present study (P = 0.05) (Litchfield and Wilcoxon, 1949).

Table 3
ED_{50}, 95% confidence limits of ED_{50} and potency ratios of hydromorphone, morphine, dihydromorphine and dihydroisomorphine determined in the formalin test

Drugs	ED_{50} (μmol/kg)[a]	95% Confidence limits of ED_{50} (μmol/kg)[a]	Potency ratio[b]
Hydromorphone	3.0	2.5–3.6	5.0
Morphine	15	10–22	1.0
Dihydromorphine	16	8.8–29	0.94
Dihydroisomorphine	42	19–92	0.36

[a] The results were calculated according to the method by Litchfield and Wilcoxon (Litchfield and Wilcoxon, 1949), and are base equivalent.

[b] The potency ratios were calculated relative to the potency of morphine.

Discussion

Initially developed by Dubuisson and Dennis (Dubuisson and Dennis, 1977), the formalin test has several important advantages over threshold thermal or mechanical nociceptive tests. First, unlike the tail-flick and tail-clip tests, the formalin test allows free movement of the animal during the experiment. It is known that opioid-induced antinociception is potentiated by restraint stress, possibly through the release of endogenous opioids (Woolfolk and Holtzman, 1993). Second, the noxious stimulus in the formalin test is prolonged, continuous and moderate generated by tissue injury (Tjølsen et al., 1992). In control rats, bi-phasic nociceptive response curves were observed (Fig. 3), in which the second phase involves processes of inflammation and central sensitization that are more clinically relevant than brief noxious stimuli (Vaccarino and Chorney, 1994; Abram et al., 1996). Third, the responses in the formalin test involve more complex movements of different parts of the body rather than simple spinally mediated reflexes, and thus additional brain regions are involved as compared to the tail-flick and tail-clip tests (Manning and Mayer, 1995; Abbott et al., 1996). Because of these advantages, the formalin test is believed to be a valid animal model for clinical pain.

In the original formalin test in rats by Dubuisson and Dennis (Dubuisson and Dennis, 1977), 50 μl of 5% formalin solution was injected into the dorsal surface of one of the forepaws and the nociceptive behaviors thus produced were observed for 60 min. Since then, several modifications have been made in the experimental procedures of the test. Grooming is a natural behavior frequently observed in rats, particularly with the forepaws (Tjølsen et al., 1992), which can be mistaken for nociceptive behaviors. To decrease the interference introduced by the natural grooming behavior, hindpaws were used for the injection of the formalin solution by other investigators (Vaccarino and Chorney, 1994; Manning and Mayer, 1995; Abram et al., 1996), as well as in the present study. During the habituation period before the test, grooming with the hindpaws was not observed in the present study.

The antinociception produced by morphine in the formalin test was used as a reference for comparison of antinociception produced by the other four drug substances. One hundred percent inhibition of the second phase was achieved at the dose of 32 μmol/kg (12 mg/kg; as sulfate salt) (Table 2). In comparison, morphine sulfate at a dose of 6 mg/kg was reported to provide close to 100% inhibition of the second phase by Dubuisson and Dennis (Dubuisson and Dennis, 1977). Older rats weighing 300 − 500 g were used in this reported work, whereas younger rats weighing 284 g were used in the present study. It has been reported that aging is a factor in the manifestation of nociceptive responses in rats (Bhargava and Villar, 1991; Chung et al., 1995). Younger rats (40–50 days; 141 g) were found to react much more vigorously than older rats (100–120 days; 390 g) in a peripheral neuropathic pain model (Chung et al., 1995). Greater antinociception following morphine administration was also observed in 24-week-old rats as compared to 8-week-old rats in the tail-flick test (Bhargava and Villar, 1991). In postoperative patients over the age of 20 years, it was found that the requirements for morphine decreased with the increase in patient age (Macintyre and Jarvis, 1995). Therefore, it is not unreasonable to suspect that aging might also be a significant factor on the final outcomes of the formalin test.

The dose-response curves of hydromorphone, morphine, dihydromorphine and dihydroisomorphine are parallel (Fig. 7). The relative potency ratio of hydromorphone to morphine was determined as 5 : 1 in the present study (Table 3). This potency ratio is in general agreement with the results obtained in patients for the management of cancer and postoperative pain (Mahler and Forrest, 1975; Houde, 1986; Collins et al., 1996; Dunbar et al., 1996). However, the binding affinity of hydromorphone to the μ-receptors is only twice that of morphine (Chen et al., 1991). A higher intrinsic activity of

hydromorphone than morphine may account for the observed differences between the receptor binding affinities and the analgesic potencies of the two opioids. The higher analgesic potency of hydromorphone as compared to morphine is clinically important. As hydromorphone hydrochloride is also four times more water soluble than morphine sulfate (Budavari et al., 1996), concentrated parenteral preparations of hydromorphone are available for the relief of severe pain in opioid-tolerant patients (Gillis, 1996), or for the application of subcutaneous infusion which requires small volume delivery (Bruera et al., 1993).

The relative potency ratios of dihydromorphine (equipotent) and dihydroisomorphine (0.36 : 1) to morphine were lower than the results reported in cats using the tail-clip test (Small et al., 1938), which were 2.9 : 1 and 0.94 : 1, respectively. The differences in mechanisms involved in the two tests may be responsible for the discrepancies between the two studies. As discussed earlier, the tail-clip test measures transient and threshold responses of the animal, whereas the formalin test assesses responses of the animal to continuous and moderate stimulation, and involves processes of inflammation and central sensitization in the second phase. In addition, it has been reported that the response profiles of cats and rats in the formalin test are different. When cats were tested in the formalin test, the characteristic bi-phasic response curves seen in rats were not observed (Dubuisson and Dennis, 1977). Furthermore, even two different species of rats may respond to the formalin injection differently. For example, Wistar rats obtained from the University of New South Wales (Australia) responded more vigorously to the formalin injection than Long-Evans rats obtained from Charles River (Quebec, Qué) (Abbott et al., 1995). Formalin solution at 1.5% produced the same intensity of nociception in Wistar rats as that produced by 2.5% formalin solution in Long-Evans rats. Due to these reasons, direct comparison of the results between the two studies is difficult and inappropriate.

The antinociceptive activities of norhydromorphone were not significantly different between the three doses tested (28, 61 and 123 μmol/kg). Based on the available results, it is not possible to determine whether they represent the lower portion of the dose-response curve, i.e., below 20% of MPE%, or they represent a ceiling effect of norhydromorphone.

It is known that the replacement of the N-methyl group in morphine alkaloids has profound effects on the agonistic and antagonistic properties of the compounds. Substitution on morphine-like molecules was proposed and shown to have long-range effects (Kolb, 1979; Darling et al., 1982). For example, substitution of the piperidine nitrogen can distort the bond angles and bond distances of the entire molecule via long-range inductive effects. These changes can alter the electron distribution throughout the molecule, the pK_a and lipophilicity of the compound, the binding affinity to the receptor, and, ultimately, the pharmacological properties of the compound. Extension of the N-methyl group in morphine to a N-ethyl group reduces the antinociceptive potency of morphine and reveals antagonistic properties (Lenz et al., 1986). Further extension to a N-propyl group produces antagonism only, while a N-phenylethyl substituent increases the antinociceptive potency of morphine by 6-fold. Other well-known examples include the mixed agonist-antagonist nalorphine which is a N-propylene substituted derivative of morphine, and the pure antagonists naloxone and naltrexone which are N-propylene and N-cyclopropyl substituted derivatives of oxymorphone, respectively.

The low antinociceptive activity of norhydromorphone observed in the present study may also in part be explained by the relatively high polarity due to the secondary amine group and hence decreased lipophilicity of the molecule, which makes distribution across the BBB difficult. In addition, receptor

binding affinity and intrinsic activity, which remain to be determined, may also contribute to the low antinociceptive activity of norhydromorphone.

The absence of antinociception following administration of hydromorphone-3-glucuronide, dihydromorphine-3-glucuronide and dihydroisomorphine-3-glucuronide is not surprising. Morphine-3-glucuronide was reported to be devoid of antinociceptive activity (Gong et al., 1992). A similar result was also obtained with oxymorphone-3-glucuronide (Cramond et al., 1996). The lack of binding affinity of the 3-*O*-glucuronides for opioid receptors, which was observed for morphine-3-glucuronide (Bartlett and Smith, 1995; Löser et al., 1996), is the most likely explanation. The results emphasize that the presence of a free C_3-OH is essential for the analgesic effect of morphine alkaloids. In fact, central excitatory activities were observed in rats following intracerebraoventricular administration of hydromorphone-3-glucuronide (Wright et al., 2001), morphine-3-glucuronide (Hashiguchi et al., 1995), and oxymorphone-3-glucuronide (Cramond et al., 1996). Central excitation caused by opioid metabolites may be one of the explanations for myoclonus associated with opioid therapy (Holdsworth et al., 1995).

Conclusions

Norhydromorphone was identified as a novel but minor metabolite of hydromorphone in a cancer patient's urine. Its antinociceptive activities were determined in the rat formalin test following i.p. administration. Only limited antinociception was observed and no significant increase in antinociception was detected at the three doses tested. The presence of the secondary amine group in norhydromorphone may increase the polarity of the molecule and thus makes it less favorable than its parent tertiary amine drug, hydromorphone, to cross the BBB, which may in part be responsible for the low antinociception seen following i.p. administration. In addition, decreased intrinsic antinociceptive activities might also be responsible and remains to be determined.

For comparison, the antinociceptive activities of morphine, hydromorphone, dihydromorphine and dihydroisomorphine were also determined using the formalin test. Our results indicate that morphine, hydromorphone, dihydromorphine and dihydroisomorphine were equally effective in the formalin test. However, hydromorphone was five times as potent as morphine in the formalin test, while dihydromorphine and dihydroisomorphine were equipotent to and 36% as potent as morphine, respectively. The antinociceptive potencies of dihydromorphine and dihydroisomorphine from this study were found to be lower than those previously reported for cats using the tail-clip test. The different underlying mechanisms of nociception between the two tests and the different animal species used in the two studies may be responsible for the different results. Hydromorphone-3-glucuronide, dihydromorphine-3-glucuronide and dihydroisomorphine-3-glucuronide did not exhibit any antinociceptive effect at the doses tested. The results further underscore the importance of a free C_3-OH to the analgesic effect of morphine alkaloids.

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