ELSEVIER

International Journal of Pharmaceutics 154 (1997) 149–155

international journal of pharmaceutics

Bioactivation of morphine-3-propionate, a prodrug of morphine, in tissues from different species

Lotte Groth, Aksel Jørgensen, Bente Steffansen, Lona Louring Christrup *

Departments of Pharmaceutics, The Royal Danish School of Pharmacy, 2 Universitetsparken, DK-2100 Copenhagen, Denmark

Received 3 October 1996; received in revised form 18 April 1997; accepted 27 April 1997

Abstract

The bioactivation of the morphine prodrug, morphine-3-propionate, has been evaluated by determination of the first-order hydrolysis rate in different tissue homogenates and blood fractions from various mammal species, including man. The hydrolysis rates were determined in whole blood, serum and plasma from rat, rabbit and man, in serum from pig, in liver, kidney, brain, buccal mucosa, muscle and skin homogenate from rat, rabbit and pig and in skin homogenate from man. Within the same species there was no difference in the enzymatic activity in whole blood, serum and plasma. Comparing the enzymatic activity in blood fractions from the various species, the activity was higher in man followed by rabbit, rat and pig, respectively. The enzymatic activity in the tissue homogenates was general highest in liver followed by kidney, brain, buccal mucosa, muscle and skin. The tissue homogenate from rabbit had higher enzymatic activity than those from rat, which again showed higher activity than those from pig. Comparison of the Michaelis-Menten parameters, K_m and V_{max}, obtained using pig and rat serum respectively, suggested that morphine-3-propionate has a lower affinity for enzymes present in pig serum than in rat serum and that the enzymes found in pig serum has a lower hydrolytic capacity than those in rat serum. The results obtained in this study indicate that the three animal species investigated, can serve as a model for man in bioactivation studies on morphine-3-propionate and possibly for other short chain morphine-3-esters as well. © 1997 Elsevier Science B.V.

Keywords: Morphine prodrugs; Morphine-3-propionate; Rabbit; Pig; Rat; Human; Tissue homogenate; Blood fractions

1. Introduction

Morphine plays an essential role in the treatment of patients suffering from severe chronic pain. Unfortunately, due to the short elimination half-life and the extensive and variable first-pass

* Corresponding author. Tel.: +45 35 370850; fax: +45 35 2613.

0378-5173/97/$17.00 © 1997 Elsevier Science B.V. All rights reserved.

PII S0378-5173(97)00127-0

metabolism following peroral administration, a number of disadvantages, such as a high incidence of side effects due to high plasma peak levels, necessity of frequent dosing and unpredictable and incomplete bioavailability are associated with conventional morphine therapy.

To overcome these problems transdermal and buccal administration may be considered. Both routes offer a simple, painless method of morphine administration. A necessity for the development of a transdermal or buccal delivery system of morphine is, however, that the drug is capable of permeating the skin or buccal mucosa at a rate sufficiently high to obtain plasma levels within the therapeutic range.

Previous studies have, however, revealed that permeation of morphine through skin and buccal mucosa is very limited (Roy and Flynn, 1989; Weinberg et al., 1988; Al-Sayed-Omar et al., 1987), which may make the substance unsuited for buccal and transdermal delivery. The poor permeation properties of morphine are mainly ascribed to its low lipophilicity.

In order to improve the transdermal and buccal absorption of morphine, the prodrug approach has been examined. Using this approach the physico-chemical properties of morphine are changed in order to optimize the biphasic solubility and hence increase the diffusion rate through different biological barriers. In previous studies various aliphatic morphine esters have been prepared by esterification of the 3-phenolic or 6-alcoholic group or both and characterized with respect to solubility, lipophilicity, chemical stability, stability against enzymatic degradation and ability to permeate different biological barriers (Drustrup et al., 1991; Jørgensen et al., 1994)

In vitro diffusions experiments showed an improved permeation through skin and buccal mucosa (Drustrup et al., 1991; Christrup et al., 1997). Further it was found that esters formed at the 3-phenolic group were rapidly hydrolysed by enzymes in human serum both as 3-monoesters and as 3,6-diesters, whereas the esters formed at the 6-alcoholic group were quite resistant to attack by serum enzymes (Drustrup et al., 1991). It was concluded that monoesters formed at the 3-phenolic group were the most promising candidates for buccal and transdermal delivery since bioactivation of the morphine prodrugs should take place during or immediately after penetration by a quantitative cleavage to morphine by enzymes present in the skin, buccal mucosa or plasma.

The objective of the present study has been to examine the bioactivation of a morphine prodrug, morphine-3-propionate, in blood fractions and different tissue homogenates obtained from rat, rabbit, pig and man. The rates of hydrolysis were compared in order to point out a suitable model animal for in vivo studies of absorption and bioactivation of morphine prodrugs.

2. Materials and methods

2.1. Apparatus

High-performance liquid chromatography (HPLC) was performed with a system consisting of a Milton Roy constaMetric 3000 solvent delivery system, a Milton Roy spectroMonitor 3100 variable wavelength detector and a Hitachi autosampler Model 655A-40.The column was a RP-select B Lichrocart 75-4 column (69×4 mm, 5-μm particles) equipped with a synchropak RSC pre-column.

Homogenisation of the different tissue samples was performed using a B. Braun microdismembrator II.

2.2. Preparation of morphine-3-propionate

Morphine-3-propionate was prepared by reacting morphine hydrochloride with propionic anhydride in alkaline aqueous solution. The method is a modification of procedures previous described for the synthesis of morphine-3-acetate (Welsh, 1954) and morphine-3-propionate (Sy et al., 1986). In brief: morphine hydrochloride (3.75 g) was added to 200 ml of a 10% aqueous solution of sodium bicarbonate with a pH of 9.0. Propionic anhydride (13 ml) was added in one portion. The reaction mixture was stirred at room temperature for 2 h. The pH of the clear solution was adjusted to 8.0 with 2 N sodium hydroxide. The solution

was extracted with methylene chloride (2 × 150 ml). The combined extracts were dried over anhydrous sodium sulphate and evaporated in vacuo at 40–50°C to afford morphine-3-propionate as an oil in 95% yield. The compound crystallized from ether-petroleum ether at − 18°C.

The elemental analysis (C, H and N) was within ± 2% of the theoretical values. The melting point was 85–86°C.

2.3. *In vitro studies*

2.3.1. *Collection of samples*

Liver, kidney, brain, muscle of the femur, buccal mucosa and skin from the abdomen were isolated and plasma, serum, whole blood, erythrocytes and haemolysed erythocytes were obtained from Mol:Male Sprague–Dawley rats (200 g). Liver, kidney, brain, cerebellum, muscle of the femur, buccal mucosa, jejunum mucosa and skin from the abdomen, whole blood, plasma and serum were obtained from New Zealand white rabbits (2.5–3.0 kg). Liver, kidney, brain, muscle of the femur, buccal mucosa and skin from the abdomen and serum samples were obtained from domestic pigs (35 kg). Human serum was obtained from 13 healthy volunteers (age: 24–57 years). Human mammary skin was obtained from a single woman undergoing mastectomy.

2.3.2. *Handling of samples*

Before homogenisation in the Microdismembrator II, the tissues were cut into small pieces, then placed in the teflon chamber containing a metal ball, and lowered into liquid nitrogen. The frozen chamber was transferred to the microdismembrator II and vibrated, hereby powdering the tissue. The powdered tissue was added phosphate buffer (0.05 M; pH 7.4) and vibrated again. The final concentration of tissue in the homogenate varied from 0.5–10%. The tissue homogenate were kept on ice and used within 6 h of preparation. All blood fractions were used within six hours of collection and kept on ice until used. Samples of whole blood were collected in heparinised test tubes.

2.3.3. *Determination of K_m and V_{max}*

In order to determine an initial substrate concentration for the hydrolysis experiments, which ensures first-order kinetics, the Michaelis-Menten constant, K_m, and the maximal rate of hydrolysis, V_{max}, were determined using 10% rat serum, 10% porcine serum and 10% homogenate of porcine skin and porcine buccal mucosa. Tissue homogenate and serum were diluted with isotonic 0.05 M phosphate buffer pH 7.4. The initial substrate concentrations of morphine-3-propionate ranged from 31.25 μM to 2.0 mM.

The initial rates of hydrolysis, V_{init}, were calculated and a Michaelis-Menten plot was obtained by plotting V_{init} against the actual substrate concentration, S.

K_m and V_{max} were estimated from a Hanes plot, where S/V_{init} is plotted versus S; $1/V_{max}$ being the slope of the plot and K_m the intercept with the S axis.

2.3.4. *Hydrolysis of morphine-3-propionate*

In these experiments an initial concentration of 0.05 mM was used. Morphine-3-propionate was added to the different tissue homogenates and blood fractions. After mixing, the mixtures were incubated at 37°C. At appropriate intervals 500 μl aliquots of the medium were withdrawn and added to a mixture of 1.1 ml acetonitrile and DMSO, (10:1 v/v), in order to deproteinise the solution and release the morphine ester bound to the proteins (McDowall, 1989).

After mixing and centrifugation at 5000 rpm for 10 min, 20 μl of the clear supernatant were analyzed for content of morphine-3-propionate and morphine by HPLC.

The study was done in duplicate with each tissue homogenate and blood fraction. First-order rate constants for the hydrolysis of morphine-3-propionate were determined from the slopes of linear parts of the log-linear profile of the residual ester against time.

The different blood fractions and tissue homogenate were diluted with 0.05 M isotonic phosphate buffer pH 7.4. The degree of dilution was in each case adjusted in order to obtain a suitable rate of hydrolysis for the experiment.

In a preliminary study the individual variation in the hydrolysis rates were evaluated. Experiments were performed in liver, muscle, skin, buccal mucosa and serum from five rats and in serum from thirteen volunteers.

2.4. HPLC analysis of morphine-3-propionate

A reversed-phase HPLC procedure was used for the quantitative determination of morphine and morphine-3-propionate. The injection volume was 20 μl. The mobile phase was a mixture of acetonitrile, methanol and 10 mM phosphate buffer, pH 6.5 (30:10:60 v/v). The flow rate was 1.0 ml/min and the column effluent was monitored at 214 nm. Quantification of the compound was done by measurements of the peak heights in relation to those of standards chromatographed under the same conditions. The retention times of morphine and morphine-3-propionate were 1.3 and 5.1 min, respectively, and the detection limit for both compounds were 0.0001 mM.

3. Results and discussion

3.1. Individual variation in rate of hydrolysis in rats and human serum

The calculated first-order rate constants for the hydrolysis of morphine-3-propionate obtained in different tissue homogenates and blood fractions from five rats were subjected to a multifactor analysis of variance. No significant difference in hydrolysis rate was found between rats ($p < 0.05$). Thus, in the subsequent studies pooled samples of both tissues and blood fractions were used.

The mean first-order rate constant for the hydrolysis of morphine-3-propionate in human serum obtained from 13 individuals was found to be 0.24 ± 0.073 min^{-1} (mean ± S.D.), range 0.0612–0.336. The corresponding values obtained from rat serum ($n = 5$) were 0.23 ± 0.021 (mean ± S.D.), range 0.1995–0.2701. Thus, judged from the standard deviation and range the individual variation in humans is larger than the individual variation in rats. This might, however, be expected, since laboratory rats of the same strain constitute a much more homogeneous population than humans.

3.2. Determination of K_m and V_{max} in porcine tissues and in rat serum

Typical Michaelis-Menten curves were obtained when initial rates of hydrolysis of morphine-3-propionate in rat serum, homogenate of porcine skin, buccal mucosa and in porcine serum were plotted against substrate concentration. The Michaelis-Menten parameters K_m and V_{max} determined from Hanes plots, are shown in Table 1. The K_m value for the hydrolysis of morphine-3-propionate was higher and the V_{max} value lower in serum obtained from pig than in serum obtained from rat, indicating that morphine-3-propionate has a lower affinity for enzymes present in pig serum than in rat serum and that the enzymes found in pig serum has a lower hydrolytic capacity than those in rat serum. Consequently the enzyme or group of enzymes responsible for the hydrolysis of morphine-3-propionate may show a species difference.

Comparing the V_{max} and K_m values obtained for the hydrolysis in porcine serum and tissue, the hydrolytic capacity is within the same order of magnitude, whereas the affinity for the serum proteins seems lower than for proteins present in skin and buccal mucosa.

3.3. The initial substrate concentration

The morphine-3-propionate concentration, 0.05 mM, used for determination of hydrolysis rate in

Table 1
Michaelis-Menten parameters for the hydrolysis of morphine-3-propionate obtained in 10% serum and 10% tissue homogenates from rat and pig

	K_m (mM)	V_{max} (mM min^{-1})
Pig serum	0.67 ± 0.17	0.021 ± 0.0036
Pig buccal mucosa	0.35 ± 0.18	0.031 ± 0.0040
Pig skin	0.39 ± 0.12	0.020 ± 0.0037
Rat serum	0.40 ± 0.14	0.059 ± 0.0045

The parameters were estimated from Hanes plots.
Values are given as mean ± S.D.

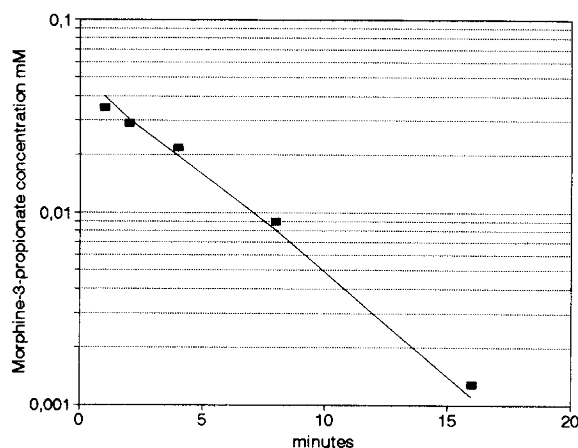

Fig. 1. Hydrolysis profile for morphine-3-propionate in 10% rat serum. The initial concentration of morphine-3-propionate was 0.05 mM.

the different tissues was chosen as a compromise between the sensitivity of the analytical method and the demand being below K_m to obtain first-order kinetics of the hydrolysis. First-order kinetics was obvious in all the experiments. A typical first-order hydrolysis curve for morphine-3-propionate in 10% rat serum is shown in Fig. 1.

In preliminary studies, where the remaining ester and the morphine formed during hydrolysis were both quantified, it was shown that the ester is quantitatively hydrolysed to morphine. Thus, for all following studies of the hydrolysis only the amount of remaining ester was measured.

3.4. Hydrolysis in different blood fractions

Hydrolysis studies with whole blood, serum and plasma from man and rabbit could not be performed in 10% dilutions, as the activities were too high to allow determination of the rate constants. More diluted fractions were therefore used. The rate constants obtained with whole blood, serum and plasma in each species are shown in Table 2 along with the actual dilutions used. The rate constants for whole blood, plasma, serum, erythrocytes and haemolysed erythrocytes obtained with rat serum were 0.23, 0.17, 0.22, 0.16 and 0.22 min^{-1} respectively, indicating that the enzymes involved in the hydrolysis of the morphine ester are equally distributed among all blood components.

From the data presented in Table 2 it can be seen that the enzymatic activity in the human blood fractions is highest; being approximately 2, 20 and 100 times higher than the activity in blood fractions from rabbit, rat and pig, respectively.

In accordance with our findings Owen and Nakatsu (1984) reported that the hydrolysis at the 3-position ester group of morphine diesters occurred faster in human than in rat blood. On the other hand, Oldendorf and Stoller (1989) showed that deacetylation of heroin at the 3-position in rat serum proceeded much faster than in human serum. Wright (1941) found that heroin is deacetylated at the 3-position by human serum at a much slower rate than in rabbit serum, but that acetylcholine is hydrolysed at a higher rate in human than in rabbit serum.

From the data in Table 2, it is also seen that within the same species no difference in the hydrolytic activity of whole blood, serum and plasma could be demonstrated.

This finding is contradictory to the results of Nakamura et al. (1975), who showed that in human whole blood deacetylation of morphine-3,6-diacetate (heroin) to morphine-6-acetate proceeds twice as rapidly as in 90% human serum and in rat whole blood deacetylation of heroin at the 3-position proceeds more rapidly than in rat serum, but comparable to the results of Oldendorf and Stoller (1989), who, contradictory to Nakamura et al. (1975), found a faster deacetylation of heroin in rat serum that in rat whole blood.

Table 2
First-order rate constants for the hydrolysis of morphine-3-propionate in different blood fractions from various species

K (min^{-1})	Whole blood	Plasma	Serum
Human (0.5%)	nd	0.23 ± 0.0082	0.24 ± 0.073
Rabbit (1%)	0.21 ± 0.0040	0.22 ± 0.0071	0.20 ± 0.012
Rat (10%)	0.23 ± 0.0036	0.17 ± 0.030	0.23 ± 0.021
Pig (10%)	nd	nd	0.043 ± 0.0043

Values are given as mean ± S.D.
nd, not determined.

Table 3
First-order rate constants for the hydrolysis of morphine-3-propionate in different tissue homogenates from various species

	Pig		Rat		Rabbit	
	Dilution (%)	K (min^{-1})	Dilution (%)	K (min^{-1})	Dilution (%)	K (min^{-1})
Liver	1	2.04 ± 0.03	1	1.35 ± 0.19	0.5	4.67 ± 0.79
Kidney	1	0.42 ± 0.11	1	0.25 ± 0.03	0.5	3.07 ± 0.06
Brain	5	0.078 ± 0.003	1	0.04 ± 0.02	1	0.11 ± 0.02
Buccal mucosa	5	0.07 ± 0.01	5	0.16 ± 0.08	0.5	0.35 ± 0.08
Muscle	10	0.033 ± 0.006	10	0.07 ± 0.02	10	0.11 ± 0.03
Skin	10	0.031 ± 0.005	10	0.08 ± 0.01	10	0.70 ± 0.06

Values are given as mean ± S.D.

This indicates that different esters (substrates) have different affinities for the respective blood enzymes responsible for their hydrolysis and/or that the different enzymes have different hydrolytic capacities.

3.5. *Hydrolysis in different tissue homogenate*

The hydrolysis studies with different tissue homogenates could not be performed with the same dilution factor, as the enzymatic activities were too different to allow determination of the rate constants. The rate constants obtained in the different tissue samples from different species are shown in Table 3, along with the actual dilutions used. In addition to the values listed in Table 3, rate constants were determined in human skin homogenate 10%, rabbit jejunum homogenate 0.5% and rabbit cerebellum homogenate 1%. The values (mean ± S.D.) being 0.46 ± 0.11, 0.35 ± 0.04 and 0.071 ± 0.006 min^{-1}, respectively.

In general, irrespective of species, liver, kidney and brain possess the highest enzymatic activity and muscle and skin the lowest enzymatic activity among the different tissues included in this study, indicating that the distribution pattern of hydrolytic enzymes for morphine-3-propionate in the organism are identical in different species. This finding is in accordance with results obtained by Wright (1942) who found that the hydrolysis of heroin proceeded most quickly in liver followed by kidney and brain, respectively, and most slowly in muscle.

A comparison of the activity in the different species shows that tissues from rabbit have the highest enzymatic activity followed by tissues from pig and rat.

In accordance with this Wright (1942) found that tissues from rabbit had higher enzymatic activity—judged by the rate of hydrolysis of heroin—than had the corresponding tissues from rat.

The study was not designed to allow identification of the esterases involved in the hydrolysis of morphine-3-propionate. However, if comparing the relative enzymatic activities found with the results obtained by Ecobichon (1972), who studied the relative hepatic and renal carboxylesterase activities in different mammalian species the same order of activity is seen, namely higher activity in liver than in kidney and highest activity in tissue from rabbit followed by human, pig and rat tissue, respectively. This may indicate that carboxylesterase plays a role in the bioactivation of morphine-3-propionate. Other esterases such as serum cholinesterase (Lockridge et al., 1980; Owen and Nakatsu, 1983) and arylesterase (Smith and Cole, 1976) have been found to be involved in the hydrolysis of morphine-3-esters. Thus, it seems likely that more than one enzyme is involved in the in vivo bioactivation of morphine-3-esters.

4. Conclusion

Judged on a comparison of the rates of hydrolysis obtained in blood fractions and skin it can be concluded that the esterase activity in human is higher or of the same magnitude as that of the corresponding tissue in the animals investigated. The hydrolytic activity of the organs in the three animal species investigated shows the same pattern, the enzymes of the liver have the highest activity followed by the kidney and brain. Since, there is no reason to expect man to differ in this respect it seems likely that morphine-3-propionate will be rapidly and efficiently bioactivated to morphine when given to man, irrespective of administration route. The relative high hydrolysis rate seen in skin and buccal mucosa indicates that bioactivation will be initiated already during the absorption process if morphine-3-propionate is given by the transdermal or the buccal route.

Among the investigated species the hydrolysis activity in rabbit tissue comes closest to that of man. However, in all cases the bioactivation takes place with a high rate. Thus, it seems reasonable to assume that any one of the three animal species can be used as an animal model for studies with morphine-3-propionate and other short chain morphine 3-esters.

Acknowledgements

This study was supported by the Danish Medical Research Council and by PharmaBiotec Research Center II.

References

Al-Sayed-Omar, O., Johnston, A., Tuner, P., 1987. Influence of buccal absorption of morphine sulphate and its major metabolite, morphine-3-glucuronide. J. Pharm. Pharmacol. 39, 934–935.

Christrup, L.L., Christensen, C.B., Friis, G.J. and Jørgensen, A., 1997. Improvement of buccal delivery of morphine using the prodrug approach. Int. J. Pharm. 154, 157–165.

Drustrup, J., Fullerton, A., Christrup, L.L., Bundgaard, H., 1991. Utilization of prodrugs to enhance the transdermal absorption of morphine. Int. J. Pharm. 71, 105–116.

Ecobichon, D.J., 1972. Relative amounts of hepatic and renal carboxylesterases in mammalian species. Res. Commun. Chem. Pathol. Pharmacol. 3 (3), 629–636.

Jørgensen, A., Christrup, L.L., Fullerton, A., Christensen, C.B., Bundgaard, H., 1994. Prolonged release from the injection site of morphine from morphine esters in an oil vehicle given by intramuscular injection to pigs. Pharmcol. Toxicol. 75, 319–320.

Lockridge, O., Mottershaw-Jackson, N., Eckerson, H.W., la Du, B.N., 1980. Hydrolysis of diacetylmorphine (heroin) by human serum cholinesterase. J. Pharmacol. Exp. Ther. 215, 1–8.

McDowall, R.D., 1989. Sample preparation for biomedical analysis. J. Chromatogr. 492, 3–58.

Nakamura, G.R., Thornton, J.I., Noguchi, T.T., 1975. Kinetics of heroin deacetylation in aqueous solution and in human serum and whole blood. J. Chromatogr. 110, 81–89.

Oldendorf, W.H., Stoller, B.E., 1989. Simple method for determination of heroin deacetylation in rat and human serum. Biomed. Med. Metab. Biol. 41, 246–255.

Owen, J.A., Nakatsu, K., 1983. Diacetylmorphine (heroin) hydrolases in human blood. Can. J. Physiol. Pharmcol. 61, 870–875.

Owen, J.A., Nakatsu, K., 1984. Morphine diesters II. Blood metabolism and analgesic activity in the rat in the rat. Can. J. Physiol. Pharmacol. 62, 452–456.

Roy, S.D., Flynn, G.L., 1989. Transdermal delivery of narcotic analgesics: Comparative permeabilities of narcotic analgesics through human cadaver skin. Pharm. Res. 6, 825–832.

Smith, D.A., Cole, W.J., 1976. Identification of an arylesterase as the enzyme hydrolysis diacetylmorphine (heroin) in human plasma. Biochem. Pharmacol. 25, 367–370.

Sy, W.W., By, A.W., Neville, G.A., Wilson, W.L., 1986. Syntheses of 3-*O*- and 6-*O*-propanoylmorphine: a reinvestigation and correction. J. Pharm. Sci. 75 (8), 787–789.

Welsh, L.H., 1954. O^3-Monoacetylmorphine. J. Org. Chem. 19, 1409–1415.

Weinberg, D.S., Inturrisi, C.E., Reidenberg, B., Moulin, D.E., Nip, T.J., Wallenstein, S., Houde, R.W., Foley, K.M., 1988. Sublingual absorption of selected opioid analgesics. Clin. Pharmacol. Ther. 44, 335–342.

Wright, C.I., 1941. The enzymatic deacetylation of heroin and related morphine derivatives by blood serum. J. Pharmacol. Exp. Ther. 71, 164–177.

Wright, C.I., 1942. The deacecylation of heroin and related compounds by mammalian tissues. J. Pharmacol. Exp. Ther. 75, 328–337.