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Morphine Metabolism in Human Skin Microsomes

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Key Words
Morphine · UGT2B7 · Morphine glucuronidation · Skin metabolism · Human skin microsomes

Abstract
For patients with severe skin wounds, topically applied morphine is an option to induce efficient analgesia due to the presence of opioid receptors in the skin. However, for topical administration it is important to know whether the substance is biotransformed in the skin as this can eventually reduce the concentration of the active agent considerably. We use skin microsomes to elucidate the impact of skin metabolism on the activity of topically applied morphine. We are able to demonstrate that morphine is only glucuronidated in traces, indicating that the biotransformation in the skin can be neglected when morphine is applied topically. Hence, there is no need to take biotransformation into account when setting up the treatment regimen.

Introduction

Severe skin wounds, as in the case of burn patients, are associated with strong pain and very often require treatment with systemically applied opioids. This treatment can cause typical opioid-related adverse effects such as constipation or sedation. The presence of opioid receptors in peripheral structures like sensory nerve endings, melanocytes, normal human keratinocytes and fibroblasts [1–4] in principle allows for a treatment with topically applied opioids in order to gain efficient analgesia and to avoid systemic side effects. Several clinical studies on topically applied opioids in severe skin wounds have shown that efficient analgesic effects can be obtained [for review, see 5, 6]. Nevertheless, the failing success in some studies raises the question of inadequate morphine delivery or local morphine biotransformation [for review, see 5]. Biotransformation of morphine in the skin has not yet been studied although skin metabolism has been shown to activate or inactivate xenobiotics [7–9], which is a relevant parameter for the efficiency and tolerability of a treatment regimen. In the liver, morphine is mainly metabolized to morphine-3- and morphine-6-glucuronide by UDP-glucuronosyltransferase (UGT)2B7 [10]. Expressed in traces in normal human dermal fibroblasts and normal human keratinocytes on the mRNA level [11, 12], UGT2B7 may also metabolize morphine when applied onto the skin. Moreover, the relevance of strongly expressed and active UGT1 [13] needs to be considered as a potential source of morphine glucuronidation.

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Materials and Methods

Materials/Equipment

Human skin microsomes were obtained from Biopredic International (Rennes, France); liver microsomes (BD UltraPool™ HLM 150) and UGT reaction mix solution were purchased from BD Biosciences (Woburn, Mass., USA); β-glucuronidase type HP-2 was obtained from Sigma-Aldrich (Schnelldorf, Germany), and morphine HCl and hydromorphone HCl were purchased from Fagron (Hamburg, Germany). Stock solutions for HPLC analysis and glucuronidation assay were prepared in a mixture of 0.05 M potassium hydrogen phosphate buffer (pH 4.9) and acetonitrile (90:10) and remained stable at –20°C for up to 3 months. The chemicals were of highest quality.

For morphine quantification HPLC instruments from Merck Hitachi (Tokyo, Japan) were used (autosampler L-7200, interface D-7000, pump L-7100, UV detector L-7400, fluorescence detector L-7480 and HSM software D-7000). Column (LiChhroCART® 250-4 RP-8.5 μm) and precolumn (LiChroCART 4-4) were purchased from Merck (Darmstadt, Germany).

Methods

Morphine Glucuronidation Assay

UGT reaction mix solution A (containing uridine 5′-diphospho-glucuronic acid, UDPGA, 2 mM), UGT reaction mix solution B (containing Tris HCl 50 mM, $MgCl_2$ 8 mM, alamethicin 25 μg/ml; final concentrations) and morphine stock solution (either 0.1 or 0.01 mM in incubation) were preincubated for 5 min at 37°C. Human skin microsomes (200 μg) were added for a final volume of 200 μl. After an incubation period of 2 h at 37°C the reaction was stopped by the addition of 100 μl ice-cold 18.5% HCl. After centrifugation at 10,000 *g* for 2 min, the supernatant was removed and neutralized using 1 N NaOH. Pooled liver microsomes were used as a positive control. Blank controls (no microsomes added) and negative controls (no morphine added) were also studied.

For the quantification of metabolites (morphine-3- and morphine-6-glucuronide) a β-glucuronidation assay was performed. The samples were divided into two aliquots. One aliquot was incubated for 72 h at 37°C with a double volume of 400 U/ml β-glucuronidase in McIlvaine's buffer (pH 5.0) and the other with McIlvaine's buffer only. Reaction was stopped by adding 1 N NaOH and the pH was adjusted to 9.5. The morphine content was determined after ethyl acetate extraction using HPLC UV/fluorescence measurement and hydromorphone as the internal standard. The liquid phase was potassium hydrogen phosphate buffer (pH 4.9; 92%) and acetonitrile (8%). Fluorescence intensity (extinction 235 nm, emission 345 nm) was measured for the quantification of morphine (retention time: 6.5 min) and UV detection (210 nm) was used for hydromorphone (retention time: 10.5 min). The range of linearity is 0.1–100 μM for morphine and 1–100 μM for hydromorphone ($R^2 \geq 0.99$), respectively. Interday and intraday variabilities are suitable ($SD_{rel} \leq 12\%$) [14]. For the quantification of the metabolites the following equation was used:

$$[ME] = ([Mor]_+ - [Mor]_-) \times 2$$

[ME] = amount of metabolites; $[Mor]_+/[Mor]_-$ = the amount of morphine with and without incubation with β-glucuronidase.

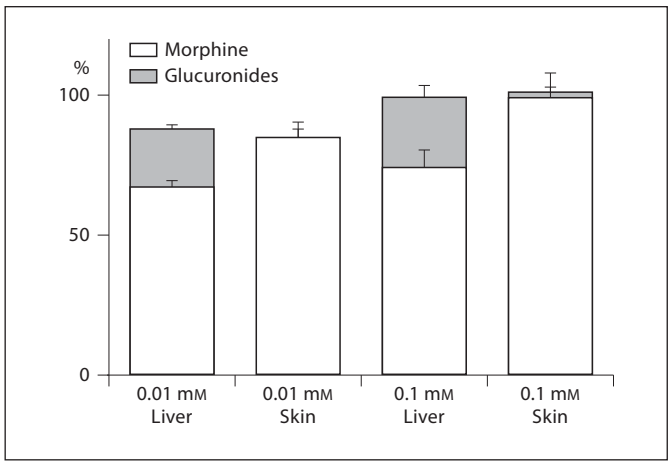

Fig. 1. Morphine glucuronidation after 2 h incubation with 200 μg of liver or skin microsomes. The liver microsomes metabolized morphine extensively, whereas the skin microsomes transformed morphine only to a minor extent.

Results and Discussion

Morphine Metabolism

For the development of a drug for topical use it is important to take the biotransformation in the skin into account. The biotransformation of morphine in the liver leads to the formation of the active metabolite morphine-6-glucuronide and the inactive morphine-3-glucuronide. UGT2B7 is the dominating enzyme responsible for the glucuronidation of morphine, but an involvement of UGT1A1, 1A3, 1A6, 1A8, 1A9 and 1A10 is also possible. Furthermore, the formation of normorphine by CYP3A4 and CYP2C8 has to be considered [10]. PCR studies revealed UGT2B7 expression in extra hepatic tissues like the kidneys, mammalian glands, lung and the small intestine [16]. To date almost nothing is known about the expression of UGT2B7 in the skin. The enzyme expression on mRNA level [11] in traces in normal human keratinocytes and normal human dermal fibroblasts, as well as in the human epidermis and in the Episkin™ skin model [16], indicates that morphine may be biotransformed in the skin – but most likely to a lower extent. Moreover, morphine is not biotransformed in human cadaver skin which was obtained 24–48 h postmortem [17]. Thus, the storage of the skin may reduce enzyme activity significantly.

In order to confirm the expected poor morphine metabolism in the skin we investigated morphine glucuronidation using human skin microsomes. One batch was

obtained from a female donor (66 a); the other batch represented a pool of two female donors (36 a and 56 a). In accordance with previous results [11], we investigated two morphine concentrations in the microsome assay. Microsomal glucuronidation assays are commonly used for biotransformation studies of chemicals and active pharmaceutical substances such as morphine, acetaminophen and testosterone [18, 19]. In order to ensure that both types of microsomes are active, testosterone was incubated to prove the CYP activity (data not shown). We performed a β-glucuronidase assay for the quantification of morphine-6- and morphine-3-glucuronide; the distinction between both metabolites is not possible with this method. Incubation (2 h) of microsomal protein 200 μg and 0.1 or 0.01 mM of morphine resulted in negligible metabolite formation (1.7 ± 4.5 and 0.2 ± 6.3%, respectively), whereas liver microsomes generated 24.6 ± 9.4 and 20.6 ± 2.8% metabolites (fig. 1). Thus, the results of the mRNA expression study were confirmed: morphine is only marginally biotransformed in human skin. However, the poor morphine glucuronidation in human skin clearly differs from the 4-methylumbelliferone metabolism [13], a preferential substrate of UGT1 which is well expressed in reconstructed human epidermis. Since skin microsomes are not only comprised of UGT2B7 but also of all other enzymes present in the skin, major morphine inactivation after topical application can be excluded. Yet hepatic biotransformation will metabolize percutaneously absorbed morphine. Although reconstructed human epidermis was proven to be predictive for human skin with respect to phase I [20] and phase II [13] biotransformation, morphine metabolism in the skin should be investigated further in in vivo studies to support our findings. This would involve using the microdialysis technique to measure the formation of morphine 3- and morphine-6-glucuronide after the topical application.

Conclusion

Topically applied morphine is an option to achieve efficient pain relief for patients with severe skin wounds associated with severe pain. Since morphine is only metabolized in traces in the skin, biotransformation can be neglected for the development of a topical morphine formulation.

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