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Research Paper

The pharmacokinetics of morphine and its glucuronide conjugate in a rat model of streptozotocin-induced diabetes and the expression of MRP2, MRP3 and UGT2B1 in the liver

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Abstract

Objectives The aim was to investigate the pharmacokinetics of morphine and its metabolite, morphine-3-glucuronide (M3G), in a rat model of streptozotocin (STZ)-induced diabetes.
Methods Morphine (15 mg/kg) was administered intravenously, and the concentrations of morphine and M3G in the plasma, urine and bile were measured by HPLC. Changes in the expression of multidrug resistance-associated proteins (MRP2 and MRP3) and UDP-glucuronosyltransferase 2B1 (UGT2B1) mRNA in the liver were also estimated by reverse-transcriptase PCR.
Key findings Plasma morphine concentrations were lower in the STZ-diabetic rats than controls although the elimination half-life of morphine was similar in the two groups (47.9 ± 10.7 min and 47.2 ± 8.6 min, respectively). The concentration of M3G in plasma was higher in STZ-diabetic than control rats, and the biliary excretion of M3G was lower in the STZ-diabetic rats (7.4 ± 2.3% vs 13.3 ± 2.0%). The urinary excretion of M3G was similar in the two groups (10.1 ± 6.8% vs 10.9 ± 4.9%). The expression of MRP3 and UGT2B1 mRNA was increased in STZ-diabetic rats, whereas expression of MRP2 mRNA was decreased.
Conclusions In STZ-diabetic rats, the distribution volume of morphine increased, the glucuronidation rate and M3G transportation into the blood were enhanced, and the excretion of M3G was decreased, leading to an increase in the plasma M3G concentration.
Keywords diabetes; morphine; morphine-3-glucuronide; pharmacokinetics; transporter

Introduction

Morphine is a potent analgesic that acts by binding to μ-opioid receptors.[1] It is primarily eliminated in the bile after glucuronidation in the liver. In humans, morphine is mainly metabolised to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) by UDP-glucuronosyltransferase (UGT) 2B7, whereas in rats it is metabolised to M3G by UGT2B1.[2] The pharmacological effects of M3G and M6G are profoundly different. For example, M6G has an analgesic effect whereas M3G does not.[3] In addition, M6G is excreted in urine and bile, whereas M3G is almost entirely excreted in urine. M3G is transported across the sinusoidal and canalicular membranes of hepatocytes by ATP-binding cassette transporters belonging to the multidrug resistance-associated protein (MRP) family. MRP2 transports morphine conjugates across the canalicular membrane into the bile; MRP3 transports them across the sinusoidal membrane into the blood.[1,4] If the expression of these hepatic enzymes and transporters changes in a diseased state, the pharmacokinetics of morphine and its glucuronide conjugate will also change.

Diabetes is a metabolic disorder caused by the absence of insulin and/or by the response of organs to this lack of insulin. In 1999, the Japan Diabetes Society classified diabetes into type 1, type 2, gestational diabetes and other groups.[5] In diabetes, the expression of metabolic enzymes and transporters in the liver is altered. Shimojo demonstrated that the expression of cytochrome P450 1A2, 2B1 and 4A were altered in rat models of type 1 diabetes induced by streptozotocin (STZ) treatment.[6] Furthermore, van Waarde *et al*. reported that the

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expression of MRP2 was decreased and the expression of multidrug resistance 2 (MDR2) was increased, while that of the bile salt export pump remained unchanged.[7]

In recent years, both diabetes- and cancer-related morbidities have increased.[8] This trend may lead to a greater use of morphine in diabetic patients. There have been clinical reports that the analgesic effect of morphine is decreased in diabetic patients, and this has also been demonstrated in rat and mouse models.[9,10] Results from these studies suggest that differences in the μ-opioid receptor between diabetic and non-diabetic mice are primarily responsible for the weak effect of morphine in diabetic mice. In addition, changes in morphine pharmacokinetics in the diabetic state might be an important determinant of the analgesic effect of morphine. However, few studies have examined the pharmacokinetics of morphine in the diabetic state.

The primary objective of the present study was to examine the pharmacokinetics of morphine and M3G (plasma concentration, urinary excretion and biliary excretion) in a rat model of STZ-induced diabetes. We also evaluated changes in the expression of UGT2B1, MRP2 and MRP3 mRNA in the liver and the relationships of these changes to alterations in the pharmacokinetics of morphine and M3G.

Materials and Methods

Materials

Morphine was from Takeda Chemical Inc. Ltd (Osaka, Japan). M3G was kindly provided by Shionogi Co. Ltd (Osaka, Japan). Naloxone was from Daiichi Sankyo Co. Ltd (Tokyo, Japan). Sepasol RNA I Super was from Nacalai Tesque Inc. (Kyoto, Japan). Glycogen solution and the SuperScript III First-Strand Synthesis System for reverse-transcriptase PCR (RT-PCR) were from Invitrogen Life Technologies Inc. (Tokyo, Japan), and the KOD Dash kit was from Toyobo Inc. (Osaka, Japan). Sense and antisense primers for UGT2B1 were purchased from Sigma Inc. (Kanagawa, Japan), and those for MRP2 and MRP3 were purchased from Bex Inc. (Tokyo, Japan). Sense and antisense primers for β-actin were obtained from Life Technologies Inc. (Carlsbad, CA, USA). All other reagents were of molecular biology grade and of the highest quality available.

Animals

Twenty 6-week-old male Donryu rats were purchased from Japan SLC Inc. (Shizuoka, Japan). The rats were housed for 1 week to exclude any abnormal animals. Animals were maintained one per cage in a 12 h light–dark cycle (lights on 8:00 am to 8:00 pm) under controlled temperature (22 ±2°C) and humidity (50 ± 5%). Food and water were available ad libitum.

All rats were handled in accordance with the Guidelines for the Care of Laboratory Animals established by Kobe Gakuin University. The protocol for this animal study was approved by the Animal Experimentation Ethics Committee of Kobe Gakuin University.

Experimental protocol

After an overnight fast, the STZ-diabetic group (n = 10) were given an intraperitoneal injection of STZ, 50 mg/kg. The control group (n = 10) were given an i.p. injection of saline. Animals were then housed under normal conditions for 4 weeks.

Blood samples from three rats in each group were collected under pentobarbital anaesthesia. The rats were then killed and the liver harvested. Blood and liver samples were stored at −80°C until analysis. The liver tissue was stored in an RNA stabilisation solution to prevent the decomposition of total RNA by RNase. Blood samples were centrifuged at 12 000 rpm for 10 min to obtain plasma samples, which were dispatched to Falco Biosystems Ltd (Kyoto, Japan) for measurement of the concentrations of total bile acid, free fatty acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and albumin. The blood glucose level was measured using Precision QID (Abbott Japan Co. Ltd, Tokyo, Japan). The remaining seven rats in each group were used to investigate the pharmacokinetics of morphine and M3G.

Pharmacokinetics of morphine and M3G

Four rats from each group were given an intravenous infusion of morphine 15 mg/kg over 5 min via the leg vein. Blood samples (0.5 ml) were taken from the arteries of the contralateral limb under isoflurane anaesthesia. Samples were obtained at 0, 5, 10, 15, 25, 45, 65, 95, 125, 185, 245 and 365 min from the start of morphine administration and were centrifuged at 12 000 rpm for 10 min to obtain the plasma.

Urine excreted during the experiments was collected, and bladder urine was collected after the experiments. Plasma and urine were stored at −80°C until analysis.

In the other three rats from each group, the abdomen was opened under isoflurane anaesthesia, and the bile duct was cannulated using a 1 mm diameter polyethylene tube. These rats were given an intravenous injection of morphine, 15 mg/kg over 5 min via the leg vein. Bile excreted over the 365 min from the start of morphine administration was collected via the bile duct. Bile samples were stored at −80°C until analysis.

Measurement of morphine and M3G

Morphine and M3G concentrations were extracted by solid-phase extraction and measured by HPLC, as described by Tan *et al.*[11] Solid-phase extraction was performed using Inertsep C18-C FF cartridges (GL Science Inc., Tokyo, Japan.). The cartridges were conditioned with 3 ml methanol, 3 ml water, 2 ml acetonitrile/10 mmol/l phosphate buffer (10 : 90 v/v; pH 2.1), and 3 ml 0.5 mol/l ammonium sulfate buffer (pH 9.3). After this conditioning, 200 μl of the samples were added to 20 μl naloxone (internal standard) and loaded onto the cartridges. The plasma samples were loaded undiluted; urine samples were loaded after dilution in saline. The cartridges were then washed twice with 2 ml 5 mmol/l ammonium sulfate buffer (pH 9.3). Samples were eluted with 2 ml acetonitrile/10 mmol/l phosphate buffer (10 : 90 v/v; pH 2.1), and the eluent was evaporated at 50°C under reduced pressure. The residue was dissolved in 300 μl mobile phase, and an aliquot injected onto the HPLC system.

The reverse-phase column (Inertsil ODS-3; GL Sciences Inc., Tokyo, Japan) was maintained at 50°C. The mobile phase was 0.1 mol/l phosphate buffer (pH 2.1), acetonitrile and methanol (72 : 24 : 2 v/v), delivered at a constant flow run of 1 ml/min for a total run time of 70 min. Morphine was detected using an electrochemical detector (Coulochem

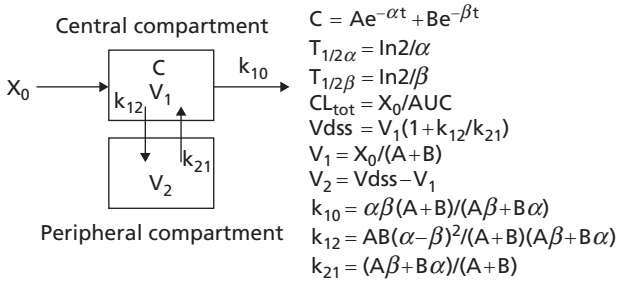

Figure 1 The two-compartment model. C, plasma concentration; $T_{1/2\alpha}$, distribution half-life; $T_{1/2\beta}$, elimination half-life; CL_{tot}, total body clearance; AUC, area under the blood concentration–time curve; Vdss, steady-state volume of distribution; V_1, distribution volume in central compartment; V_2, distribution volume in peripheral compartment; k_{10}, k_{12} and k_{21}, kinetic constants

II; Esa Inc., Chelmsford, MA, USA). M3G was detected at 214 nm using an ultraviolet–visible spectrophotometer (SPD-10A; Shimadzu Corp., Kyoto, Japan).

The pharmacokinetic parameters of morphine in plasma were calculated using a two-compartment model fitted using Multi software[12] (Figure 1).

Analysis of mRNA expression by RT-PCR

Total RNA was extracted from liver tissue (100 mg) using Sepasol RNA I Super, chloroform and isopropanol. Synthesis of cDNA was performed by RT-PCR with the SuperScript III First-strand synthesis system using KOD Dash DNA polymerase. Sense and antisense primers, together with 0.5 μl synthetic cDNA, were added to 24.5 μl reaction mixture containing KOD Dash DNA polymerase. Denaturation, annealing and extension were performed for 23–35 cycles using the iCycler system (DM9110; Bio-Rad Laboratories, Hercules, CA, USA).

Degenerate PCR primers were designed and synthesised chemically on the basis of the amino acid sequences of rat UGT2B1 (255 bp; sense primer, 5′-TGT TGG TAT TCC CTT GTT TGC-3′; antisense primer, 5′-GTG CTT GGC TCC TTT GTG ACG-3′); MRP2 (421 bp; sense primer, 5′-ATC CTC AGC TGC TGA AGT TG-3′; antisense primer, 5′-CTG ATC TTG GAT GCC AGA AC-3′); MRP3 (423 bp; sense primer, 5′-TCA AAG AGG AGA TCG CAG AG-3′; antisense primer, 5′-AGC ATG AGG ATG GGG GCC AG-3′) and β-actin (646 bp; sense primer, 5′-ATG TAC GTA GCC ATC CAG GC-3′; antisense primer, 5′-TCC ACA CAG AGT ACT TGC GC-3′).

The PCR products were resolved by electrophoresis on 2% agarose gels for 50 min. The gels were stained with ethidium bromide and the bands visualised using a UV transilluminator (Atto Corp., Tokyo, Japan). Specific bands were quantified by densitometric analysis.

Statistical analysis

Data are presented as means ± SD. Significant differences were evaluated using the Mann–Whitney U test; $P < 0.05$ was considered significant.

Results

Streptozotocin-induced diabetes

Blood chemistry values are shown in Table 1. Blood glucose levels were significantly higher in the STZ-diabetic rats than the controls. The plasma concentrations of total bile acids and free fatty acids were significantly higher in the STZ-diabetic group. In addition, the volume of drinking water consumed was higher in the STZ-diabetic rats than the control group (693.6 ± 163.7 vs 102.4 ± 11.7 ml/kg per day). These results indicate that the rats given STZ could be used as a model of type 1 diabetes.

The concentrations of serum albumin, AST and ALT were similar in the STZ-diabetic group and control group, indicating that STZ does not induce liver damage.

Pharmacokinetics of morphine and its glucuronide conjugates

The plasma concentration–time profiles of morphine are shown in Figure 2a. The plasma concentration of morphine at the end of morphine administration (in the first blood sample taken) was lower in the STZ-diabetic group than the control group (14.4 ± 10.8 vs 37.7 ± 33.6 μg/ml), and the concentration remained lower in the STZ-diabetic group than the control group over the course of the experiment. The pharmacokinetic parameters calculated using the two-compartment model are shown in Table 2. Distribution half-life and elimination half-life were similar in the two groups. Steady-state volume of distribution, central compartment volume and peripheral compartment volume were higher in the STZ-diabetic group, and the area under the blood concentration–time curve was lower.

The plasma concentration–time profiles of M3G are shown in Figure 2b. In the control group, the M3G concentration increased until 65 min after the end of morphine administration and decreased thereafter. Concentrations in the STZ-diabetic group also increased until 65 min after the end of morphine administration and were higher than in the control group for the remainder of the experiment.

The urinary and biliary excretion ratios of morphine and M3G are shown in Table 3. The urinary excretion ratios of morphine and M3G were similar in the two groups of rats. However, the biliary excretion ratio of both morphine and M3G was significantly lower in the STZ-diabetic group.

mRNA expression

We also investigated the mRNA expression of UGT2B1, which metabolises morphine into M3G, MRP2, which

Table 1 Blood chemistry values

	Control	STZ-diabetic rats
Glucose (mmol/l)	7.7 ± 0.58	23.4 ± 2.3*
Serum albumin (g/dl)	2.3 ± 0.1	2.2 ± 0.1
Aspartate aminotransferase (IU/l)	210.7 ± 17.0	162.3 ± 37.4*
Alanine aminotransferase (IU/l)	61.3 ± 7.6	77.0 ± 16.1
Total bile acids (mg/dl)	15.1 ± 6.4	30.6 ± 15.6
Free fatty acids (mEq/l)	0.6 ± 0.1	2.0 ± 0.1*

Values are means ± SD ($n = 3$). STZ, streptozotocin. *$P < 0.05$ vs control rats.

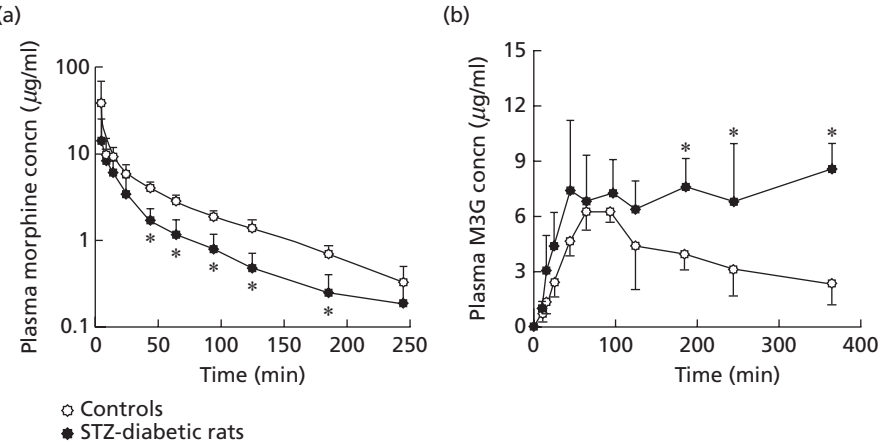

Figure 2 Pharmacokinetics of (a) morphine and (b) morphine-3-glucuronide (M3G) in control and diabetic rats given an intravenous injection of morphine, 15 mg/kg over 5 min. Values are means ± SD ($n = 4$). $^{*}P < 0.05$ vs control groups

Table 2 Pharmacokinetic parameters determined using a two-compartment model, calculated from the plasma concentration–time profiles of morphine

	Controls ($n = 4$)	STZ-diabetic rats ($n = 3$)
$T_{1/2\alpha}$ (min)	2.5 ± 1.4	4.5 ± 1.8
$T_{1/2\beta}$ (min)	47.2 ± 8.6	47.9 ± 10.7
$AUC_{0\rightarrow\infty}$(mg·min/l)	732.1 ± 19.1	421.4 ± 138.5
CL_{tot} (ml/min)	9.5 ± 3.0	13.5 ± 3.8
Vdss (l/kg)	1.4 ± 0.3	1.8 ± 0.2
V_1 (l/kg)	0.51 ± 0.044	0.60 ± 0.028*
V_2 (l/kg)	0.93 ± 0.31	1.2 ± 0.17
k_{10} (min^{-1})	0.047 ± 0.0052	0.063 ± 0.014
k_{12} (min^{-1})	0.22 ± 0.17	0.080 ± 0.033
k_{21} (min^{-1})	0.13 ± 0.11	0.040 ± 0.015

$T_{1/2\alpha}$, distribution half-life; $T_{1/2\beta}$, elimination half-life; AUC, area under the blood concentration–time curve; CL_{tot}, total body clearance; Vdss, steady-state volume of distribution; V_1, distribution volume in central compartment; V_2, distribution volume in peripheral compartment; k_{10}, k_{12} and k_{21}, kinetic constants. Values are means ± SD. $^{*}P < 0.05$ vs control group.

Table 3 Urinary and biliary excretion ratio in control and diabetic rats over 360 min, expressed as a percentage of the dose administered, following an intravenous injection of morphine, 15 mg/kg over 5 min

		Control	Diabetic rats
Urinary excretion (%) ($n = 4$)	Morphine	9.8 ± 6.4	9.8 ± 5.5
	M3G	10.9 ± 4.9	10.1 ± 6.8
Biliary excretion (%) ($n = 3$)	Morphine	1.3 ± 0.94	0.60 ± 0.28
	M3G	13.3 ± 2.0	7.4 ± 2.3*

Values are means ± SD ($n = 3$). M3G, morphine-3-glucuronide. $^{*}P < 0.05$ vs control group.

transports M3G from hepatocytes into bile, and MRP3, which transports M3G from hepatocytes into blood. The corresponding electrophoretic profiles and band strengths are shown in Figure 3. The expression of UGT2B1 mRNA was approximately 1.6-fold higher in the STZ-diabetic group than the control group (Figure 3a). The expression of MRP2 mRNA was approximately 2-fold lower and the expression of MRP3 mRNA approximately 2.4-fold higher in the STZ-diabetic group (Figures 3b and 3c).

Discussion

It is well known that morphine is generally ineffective for nerve pain. Thus, it is possible that morphine is also ineffective for a diabetic patient who is experiencing nerve pain. Furthermore, it has been reported that morphine is often ineffective in diabetic animals, which is related to alteration in the μ-opioid receptor function.[9,10] Husamettin *et al.* have suggested that the decreased analgesic effect of morphine in diabetes may be related to interleukin-1β.[13] Because it is likely that the weak analgesic effect of morphine in diabetes is controlled by a complex mechanism, it is necessary to investigate this phenomenon from the perspective of a variety of disciplines such as pharmacodynamics and pharmacokinetics. In the present study, we investigated the alteration in morphine pharmacokinetics in the STZ-induced diabetic rat model.

The plasma morphine concentration was lower in the STZ-diabetic group than the controls. As the albumin levels were comparable in the two groups, the lower plasma concentration of morphine in the STZ-diabetic group was due to an increased volume of distribution. The elimination processes were similar in the two groups, as was the elimination half-life. These results suggest that morphine concentration is likely to be lower in diabetic patients than in non-diabetic patients, which may partially explain why morphine exhibits a weaker analgesic effect in diabetic patients.

The pharmacokinetics of M3G was markedly changed in the STZ-diabetic rats: the M3G concentration was higher than in the controls, and the biliary excretion ratio of M3G was significantly lower (Figure 2b and Table 3). To explain these changes in M3G pharmacokinetics, we investigated the alterations in UGT2B1, MRP2 and MRP3 mRNA expression in the liver. Morphine is mainly metabolised to M3G by UGT2B1 in the rat liver. M3G is transported into the bile by MRP2 and into the blood by MRP3. Although the expression of

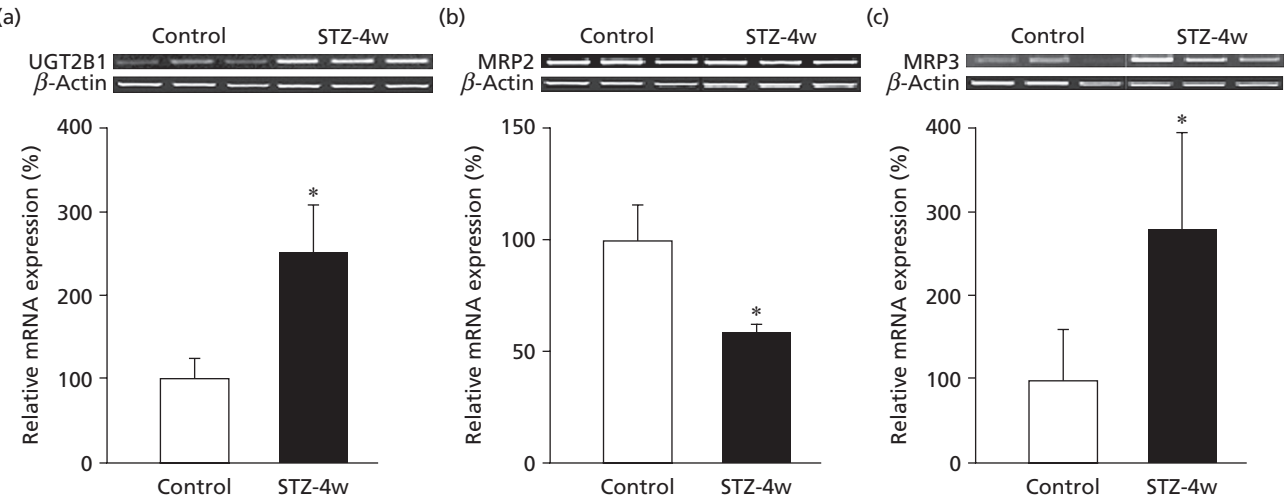

Figure 3 Electrophoretic profiles of (a) UGT2B1, (b) MRP2 and (c) MRP3 mRNA in the livers after streptozotocin (STZ) administration (expressed as a percentage of the control value). Values are expressed as means ± SD ($n = 3$). $^{*}P < 0.05$ vs control rats

UGT2B1 and MRP3 mRNA was increased in the STZ-diabetic group, expression of MRP2 mRNA was lower (Figure 3). These results suggest that plasma concentrations of M3G were increased in STZ-diabetic rats by three mechanisms: increased glucuronidation of morphine due to increased expression of UGT2B1, increased transport of M3G into the blood due to increased expression of MRP3, and decreased biliary excretion ratio of M3G because of decreased expression of MRP2.

In the present study, we examined mRNA expression by RT-PCR and found indications that the altered expressions of UGT2B1, MRP2 and MRP3 explain the altered M3G pharmacokinetics in diabetes. To clarify these phenomena, a future study will analyse mRNA expression by real-time PCR and measure the amount and activity of proteins.

Morphine is metabolised to the inactive metabolite M3G by UGT2B1 in rats, whereas morphine is metabolised to M3G and to the active metabolite M6G by UGT2B7 in humans. MRP3 also transports M6G across the sinusoidal membrane into the blood.[1] Thus, diabetic patients may show alterations in M6G pharmacokinetics in addition to the changes in M3G pharmacokinetics observed in STZ-diabetic rats. Further studies on the plasma concentration of M6G in diabetic patients are necessary to confirm this hypothesis.

Conclusions

We have investigated the pharmacokinetics of morphine and its metabolites in a rat model of STZ-induced diabetes and examined changes in the expression of UGT2B1, MRP2 and MRP3, which are largely concerned with the hepatic metabolism of morphine. The morphine plasma concentration was lower in the STZ-diabetic rats due to an increase in the distribution volume, which may contribute to the decreased analgesic effect of morphine seen in diabetes. The expression of UGT2B1 in the liver was enhanced, leading to enhanced glucuronidation. The expression of MRP3 was increased and the expression of MRP2 decreased in diabetic rats, and the biliary excretion ratio of M3G was reduced, all of which contributed to a sustained higher concentration of M3G in the diabetic rats than the control rats.

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