Biochemical Pharmacology, Vol. 42, Suppl., pp. S55–S60, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

INFLUENCE OF MORPHINE CONCENTRATION ON DETERGENT ACTIVATION OF RAT LIVER MORPHINE-UDP-GLUCURONOSYLTRANSFERASE

L. LEAR, R.L. NATION and I. STUPANS*

School of Pharmacy, University of South Australia, North Terrace, Adelaide, South Australia, Australia 5000

(*Received* 29 *July* 1991; *accepted* 9 *September* 1991)

Abstract—The effect of two detergents, Triton X-100 and Brij 58, on the production rate of morphine-3-glucuronide by rat hepatic microsomes has been investigated over a range of detergent and substrate concentrations, using a specific HPLC assay. Activation of morphine-UDP-glucuronosyltransferase (morphine-UDPGT) by Triton X-100 was more complex than that shown by Brij 58. At the optimal concentration of Triton X-100 (0.1–0.125 mg Triton X-100/mg microsomal protein), relative metabolic activity (activity of morphine-UDPGT in the activated state/activity of morphine-UDPGT in the native state; RMA) was 0.9, 1.3 and 2.5 at morphine concentrations of 0.05, 0.5 and 2.5 mM, respectively. Analysis of results from six individual rats in the native and maximally activated state (0.125 mg Triton X-100/mg microsomal protein) showed that RMA was highly dependent upon substrate concentration ($P < 0.0001$). Activation produced by the optimal concentration of Brij 58 (0.15 mg Brij 58/mg microsomal protein) was also dependent upon substrate concentration with values for RMA of 3.3, 6.4 and 9.3 at morphine concentrations of 0.05, 0.5 and 2.5 mM, respectively. Analysis of kinetic data is complicated by substrate concentration-dependent detergent activation. It is proposed that factors contributing to substrate concentration-dependent variable activation may include micellar solubilization of substrate by detergent and/or the presence of at least two enzyme forms capable of glucuronidating morphine with differential effects of detergents on these forms.

The activity of UDP-glucuronosyltransferase (UDPGT†) enzymes is dependent upon the integrity of the phospholipid membrane in which they are bound [1–5]. The rate of glucuronidation in native microsome preparations is relatively low so various methods which perturb the lipid phase of the biomembrane have been employed in an effort to decrease latency. These methods include sonication [4, 6], alterations in pH [6], treatment with phospholipases [6], proteases [4, 7], bilirubin [8] and UDP-*N*-acetylglucosamine [4, 5, 9], or the use of organic solvents [7]. However, it appears that the most commonly used means of altering microsomal UDPGT activity is by the use of detergents, such as Triton X-100 or Brij 58 [10].

To determine the optimal concentration of detergent required to activate a microsomal system, enzyme activity is measured in the presence of varying detergent concentrations, usually at a single substrate concentration. There is little agreement in the literature concerning the magnitude of activation produced by detergents and whether activation or deactivation of the enzyme, in fact, occurs. Some workers have observed an increase in glucuronidation rate with the use of Triton X-100 [9, 11–13] or Brij 58 [14, 15] while others have reported either no appreciable effect [9, 16], or a deactivation [17, 18]. It has been suggested that factors contributing to these apparent discrepancies include a species difference in the levels of UDPGT activity, variations in the method of microsome preparation and a dependence upon UDPGA concentration in the assay [19]. Some investigators have commented that optimal detergent concentration is dependent upon substrate [20].

Morphine is a compound which may undergo glucuronidation at either the 3 or 6 position. In some species, such as the rat [21, 22], only M3G is formed. Using the rat as an animal model, the production rate of M3G by hepatic microsomes was measured under conditions of varying substrate and detergent concentration. In this communication, we present evidence that substrate concentration is an important factor in determining the degree of microsomal UDPGT activation in the presence of detergent.

MATERIALS AND METHODS

Chemicals and reagents. Morphine-3-β-glucuronide, polyoxyethylene 20-cetyl ether (Brij 58) and UDPGA (sodium salt) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Morphine HCl was obtained from Macfarlan Smith Ltd (Edinburgh, U.K.) and Triton X-100 from BDH Chemicals (Poole, U.K.). HPLC solvents were from Waters Associates (Lane Cove, Australia). All other reagents and solvents were of analytical reagent grade.

Preparation of microsomes. Adult male Hooded Wistar rats (180–200 g) were killed by cervical dislocation and exsanguination. All further steps were carried out at 4°. Livers were removed, finely

* Corresponding author. FAX (61) 8-223-6511.

† Abbreviations: UDPGT, UDP-glucuronosyltransferase; M3G, morphine-3-glucuronide; UDPGA, UDP-glucuronic acid; RMA, relative metabolic activity; CMC, critical micelle concentration.

minced and homogenized in phosphate buffer (0.1 M, pH 7.4) with KCl (1.15%) using a motor driven Potter–Elvehjem homogenizer. The homogenate was centrifuged at 10,000 *g* for 20 min. The supernatant was centrifuged at 105,000 *g* for 50 min. The microsomal pellet was resuspended in the same buffer and centrifuged at 105,000 *g* for 50 min. The pellet was resuspended in 20% glycerol in phosphate buffer (0.1 M, pH 7.4) and stored at −70°. Microsomes were thawed only once prior to use. Microsomal protein was prepared as either pooled (N = 6) or individual rat liver samples.

Measurement of morphine glucuronidation by rat liver microsomes. Microsomal incubations contained UDPGA (3 mM), $MgCl_2$ (5 mM), Tris–HCl (0.1 M, pH 7.4), microsomal protein (1 mg/mL), Triton X-100 (0–0.05% w/v; equivalent to 0–0.50 mg Triton X-100/mg microsomal protein) or Brij 58 (0–0.4% w/v; equivalent to 0–4.0 mg Brij 58/mg microsomal protein) and morphine (0.05–5 mM) in a total volume of 0.4 mL. Reactions were started by the addition of UDPGA and incubations were performed at 37° for 30 min. Reactions were shown to be linear for up to an incubation time of 1 hr and for microsomal protein content up to 2 mg/mL. The reaction was stopped by the addition of 1 mL acetonitrile containing phenytoin (42 μg/mL) as the internal standard and cooling on ice. Each incubation was mixed briefly and centrifuged at 2000 *g* for 20 min. The supernatant was diluted 1 in 3 with phosphate buffer (10 mM, pH 2.1) and 200 μL injected onto the HPLC column.

HPLC conditions for determination of M3G. HPLC conditions were based upon a modification by Milne *et al.* [23] of a method originally described by Svensson *et al.* [24]. The HPLC pump (Model M45), automatic injector (WISP, Model 712) and UV detector (Model 481), set at 210 nm, were from Waters Associates. The output from the detector was connected to a Shimadzu (Kyoto, Japan) C-R3A Chromatopac integrator. A guard column (23.2 × 3.6 mm i.d.; Waters Associates) packed with C_{18}/Corasil (37–50 μm; Waters Associates) was connected in line prior to the HPLC column. The reverse phase radially compressed 4 μm Nova-Pak C_{18} cartridge (100 × 5 mm i.d.; Waters Associates) was eluted with the mobile phase, 25% acetonitrile in phosphate buffer (10 mM, pH 2.1) containing sodium dodecyl sulphate (0.8 mM), at a flow rate of 0.8 mL/min. Under these conditions, the retention times of M3G, phenytoin and morphine were 6.9, 9.7 and 16.9 min, respectively. M3G was quantified by constructing standard curves based on peak height ratio of authentic standards in the range of 0.4 (minimum quantifiable concentration) to 150 μM.

Other assays. Protein determination was by the method of Lowry *et al.* [25] with bovine serum albumin as standard.

Analysis of results. Incubations were carried out in two replicates and the mean used to calculate results. The rate of M3G production was expressed in terms of RMA (activity of morphine-UDPGT in the activated state/activity of morphine-UDPGT in the native state). Group data are presented as means ± SD. ANOVA was used to assess whether statistical differences exist between groups.

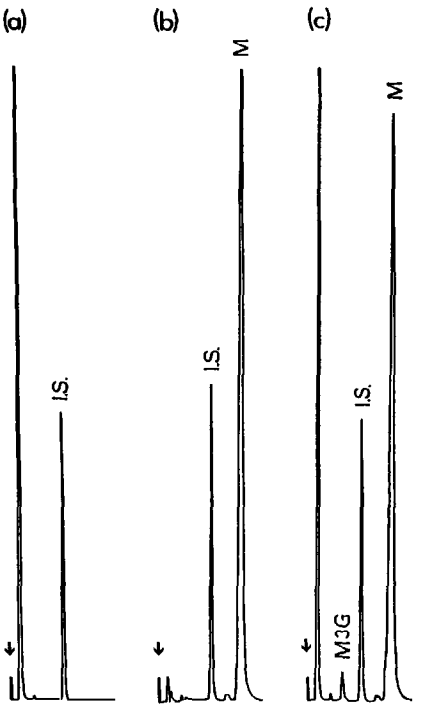

Fig. 1. Representative HPLC chromatograms of rat hepatic microsomal incubations for measuring production of M3G: (a) incubation in the absence of morphine; (b) incubation in the absence of UDPGA (morphine 2.5 mM); and (c) incubation in the presence of morphine (2.5 mM) and UDPGA. Retention times of M3G, phenytoin (I.S.) and morphine (M) are 6.9, 9.7 and 16.9 min, respectively. The arrow marks the time of injection.

RESULTS

Analysis of M3G

Representative chromatograms for microsomal incubations are shown in Fig. 1. Figure 1a and b represent incubations in the absence of morphine or UDPGA, respectively, which produced no peak eluting at the retention time of M3G. Figure 1c illustrates a representative chromatogram for measuring M3G production following microsomal incubation in the presence of both morphine (2.5 mM) and UDPGA. The within day reproducibility of the assay was determined by measuring M3G production at a substrate concentration of 0.05 mM in eight separate incubations and was found to be 3.3% (coefficient of variation).

Effect of Triton X-100 on M3G production in rat liver microsomes

The relationship between RMA and Triton X-100 concentration in microsomal mixtures was determined using pooled microsomes at morphine concentrations of 0.05, 0.5 and 2.5 mM (Fig. 2a). At all substrate concentrations, maximal activation occurred at a Triton X-100 concentration of 0.01–0.0125% (w/v). The degree of activation at the optimal detergent concentration varied markedly with substrate concentration with values for RMA

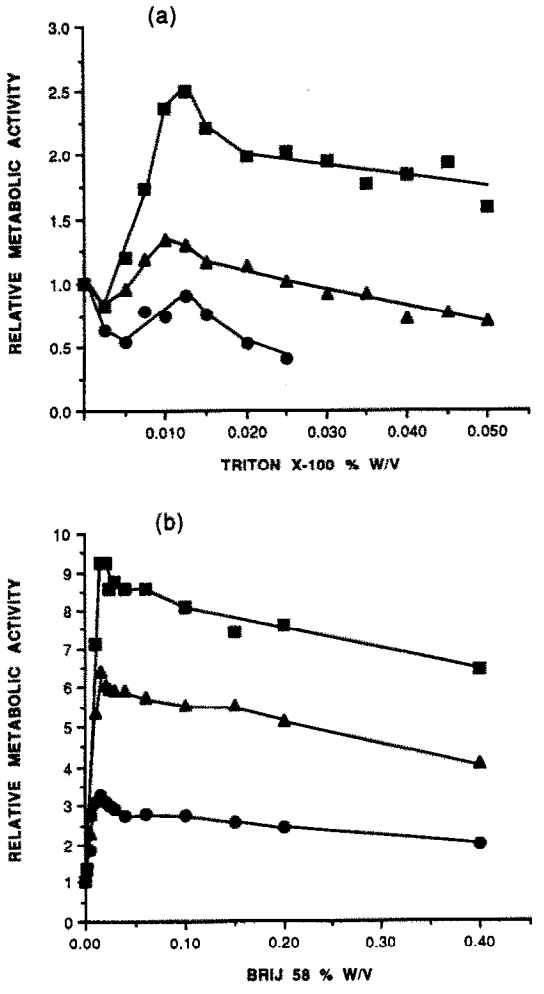

Fig. 2. RMA of pooled rat hepatic microsomes as a function of (a) Triton X-100 or (b) Brij 58 concentration at morphine concentrations of 0.05 mM (●), 0.5 mM (▲) and 2.5 mM (■).

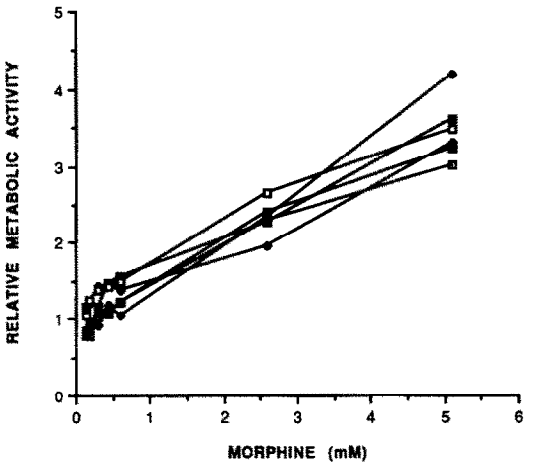

Fig. 3. RMA of hepatic microsomes from six individual rats as a function of morphine concentration at a fixed concentration (0.0125% w/v) of Triton X-100.

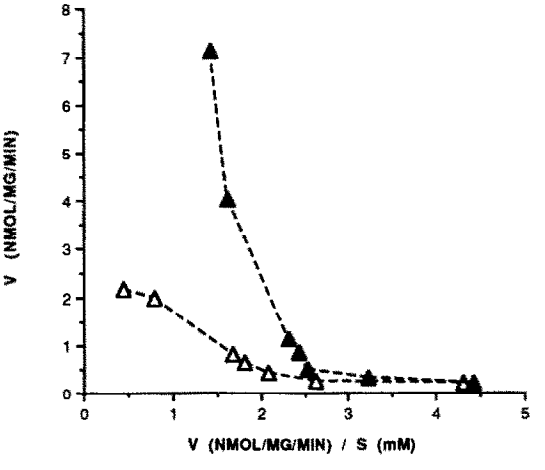

Fig. 4. Eadie–Hofstee plot of morphine glucuronidation for an individual rat. Microsomes were in the native (△) or maximally activated (Triton X-100, 0.0125% w/v; ▲) state.

of 0.9, 1.3 and 2.5 at morphine concentrations of 0.05, 0.5, and 2.5 mM, respectively. At all substrate concentrations, as Triton X-100 concentration increased, a decrease in activity preceded the increase to maximal activation. At the lowest substrate concentration, the RMA was less than unity at all detergent concentrations and M3G production was not quantifiable at Triton X-100 concentrations greater than 0.025% (w/v).

Microsomes from six individual rats were incubated in the native and maximally activated states (0.0125% w/v Triton X-100; equivalent to 0.125 mg Triton X-100/mg microsomal protein) over a range of morphine concentrations (Fig. 3). Variable activation occurred with RMA increasing ($P < 0.0001$, ANOVA) from 0.862 ± 0.169 at 0.05 mM morphine to 3.383 ± 0.410 at 5 mM morphine. Data obtained from one individual rat in the native and maximally activated states, plotted in the form of an Eadie–Hofstee plot, are shown in Fig. 4.

Effect of Brij 58 on M3G production in rat liver microsomes

Using the pooled microsomes employed in the Triton X-100 experiments described above, the relationship between RMA and Brij 58 concentrations in microsomal mixtures was determined at morphine concentrations of 0.05, 0.5 and 2.5 mM (Fig. 2b). At all substrate concentrations, maximal activation occurred at a Brij 58 concentration of 0.015% (w/v) (equivalent to 0.15 mg Brij 58/mg microsomal protein). The RMA in the presence of optimal detergent concentration was found to be dependent upon substrate concentration with values of 3.3, 6.4 and 9.3 obtained at morphine concentrations of 0.05, 0.5, and 2.5 mM, respectively. The RMA in the presence of Brij 58 was greater than unity at all substrate and Brij 58 concentrations.

DISCUSSION

Previous investigators of the effects of optimal Triton X-100 concentration on the activity of microsomal UDPGT have reported a range of activation values. For example, using the substrates estradiol [13], estrone [13], bilirubin [9], phenolphthalein [9], tetrahydrocortisone [9] and 4-methylumbelliferone [9], activation in the presence of optimal detergent concentration was 1.2-, 3.5-, 4.5-, 5-, 5- and 12-fold, respectively. Different laboratories using the same substrate have also reported markedly different degrees of enzyme activation. Using *p*-nitrophenol at 0.17 mM [11], 0.5 mM [9] and 0.4 mM [12] a 3-, 4-, and 20-fold activation, respectively, was observed. A 2-fold increase of UDPGT activity has been observed using 0.2 mM *o*-aminophenol [12], contrasting with the results of Wisnes [9] who observed no appreciable activation at 0.5 mM of the same substrate. Using rat intestinal microsomes, del Villar *et al.* [16] reported also that Triton X-100 produced no appreciable activation of the glucuronidation of 1.5 mM morphine or 4.0 mM *p*-nitrophenol. Inhibition of glucuronidation in the presence of Triton X-100 has been observed in both rat [17] and dog [18] hepatic microsomes using 4.0 and 1.5 mM propranolol, respectively, as substrate. In the present study, at a given morphine concentration, the degree of UDPGT activation varied substantially with Triton X-100 concentration (Fig. 2a) as has been reported previously with a number of other substrates [9, 11–18]. Moreover, and importantly, at the optimal concentration of Triton X-100 the magnitude of RMA was highly dependent upon morphine concentration (Figs 2a and 3). At the lowest morphine concentration (0.05 mM), RMA was less than unity indicating that Triton X-100 caused a relative deactivation of morphine-UDPGT. As morphine concentration increased so did RMA which reached a value of approximately 3.4 at a morphine concentration of 5.0 mM, indicating relative activation of the enzyme.

As with Triton X-100, a range of activation values has been reported with Brij 58. Nemoto *et al.* [14] observed a 5.6-fold increase in rat hepatic microsomal glucuronidation of 3-hydroxybenzo[*a*]pyrene in the presence of optimal Brij 58. Miners *et al.* [15] found that human hepatic microsomal glucuronidation of paracetamol was increased by about 50% by the optimal concentration of Brij 58 while glucuronidation of 4-methylumbelliferone, morphine and *p*-nitrophenol was increased by about 3-fold. We observed that Brij 58 led to an increase in rat hepatic morphine glucuronidation and, as with Triton X-100, the magnitude of the activation was dependent upon morphine concentration. At the optimal Brij 58 concentration, RMA increased from 3.3 to 9.3 over the morphine concentration range of 0.05 to 2.5 mM (Fig. 2b).

Triton X-100 has been considered the preferred detergent for UDPGT assays due to general availability [26]. In the present studies, the effects of Triton X-100 and Brij 58 differed in a number of respects. As detergent concentration increased, Triton X-100 produced an initial decrease in RMA prior to an increase whereas Brij 58 caused an increase in morphine-UDPGT activity over all detergent concentrations. At the respective optimal detergent concentrations, Triton X-100 produced a RMA of less than unity, at a morphine concentration of 0.05 mM, whereas Brij 58 produced a RMA greater than unity. For all morphine concentrations, the degree of maximal activation in the presence of Triton X-100 was less than that observed with Brij 58. Following maximal activation, Triton X-100 produced a more rapid decline in RMA at all substrate concentrations when compared to Brij 58. At a morphine concentration of 0.05 mM, M3G production was not quantifiable at Triton X-100 concentrations greater than 0.025% (w/v) whereas Brij 58 produced activation of morphine-UDPGT at all substrate and detergent concentrations. Thus, Brij 58 led to greater activation at its optimal concentration and less inhibition at higher concentrations, consistent with earlier observations [27]. It is apparent that Triton X-100 acts on microsomal UDPGT by more complex mechanisms than Brij 58. It has been suggested that, despite the extensive use of Triton X-100 as an activator of UDPGT, it may not be the ideal detergent to use because in solubilizing the enzyme the detergent may induce damage to the active site [7, 10].

One of the key observations in the present study was the substrate concentration dependence of the degree of detergent activation of microsomal UDPGT. At morphine concentrations of 0.05, 0.5 and 2.5 mM, values for RMA in the presence of optimal Triton X-100 concentration were 0.9, 1.3 and 2.5, respectively. These compare to values of 3.3, 6.4 and 9.3 at the optimal Brij 58 concentration. The variation in the value of RMA at the respective optimal detergent concentrations may be explained in part by detergent mediated micellar solubilization of the substrate. The CMC of Triton X-100 and Brij 58 is 0.019% (w/v) [28] and 0.0085% (w/v) [29], respectively, although the presence of any type of electrolyte will alter the CMC [30]. It is of interest that these CMC values are in the vicinity of the concentration values of Triton X-100 (0.0125% w/v) and Brij 58 (0.015% w/v) that we observed as causing maximal activation of morphine-UDPGT. Since the velocity of glucuronidation would be expected to be dependent upon non-micellar substrate concentration, a decrease in free substrate concentration would lead to an apparent decrease in enzyme activity. Thus, at low morphine concentrations a greater percentage of substrate may be solubilized in the presence of detergent in comparison to high substrate concentrations, leading to lower values for RMA.

The existence of more than one UDPGT enzyme capable of glucuronidating morphine at the 3 position may have contributed to the variation in RMA observed with morphine concentration in the presence of optimal concentrations of Triton X-100 and Brij 58. As illustrated by the non-linearity of Eadie–Hofstee plots (Fig. 4) for both the native and maximally activated microsomes in the presence of Triton X-100, rat liver morphine glucuronidation appears to involve at least two enzymes. Evidence

for the existence of more than one morphine-UDPGT enzyme has been demonstrated in both rats [21, 22] and humans [22, 31]. The detergents may have a differential effect on the morphine-UDPGT enzymes. For example, in the presence of Triton X-100 the high affinity, low capacity enzyme may be inhibited whereas the low affinity, high capacity form may be activated. Whatever the cause of the substrate concentration-dependent detergent activation, the Eadie–Hofstee plots for the detergent-treated and native microsomes differ markedly, illustrating that this phenomenon may complicate interpretation of kinetic data.

In conclusion, we have observed that activation of morphine-UDPGT by the detergents Triton X-100 and Brij 58 is highly dependent upon substrate concentration. This phenomenon may explain in part the variability that exists in the literature as to the magnitude of UDPGT activation produced by detergents.

REFERENCES

1. Dutton GJ, Factors affecting UDPGlucuronyltransferase activity *in vitro*. In: *Glucuronidation of Drugs and Other Compounds*, pp. 29–52. CRC Press, Florida, 1980.
2. Burchell B and Coughtrie MWH, UDP-glucuronosyltransferases. *Pharmac Ther* **43**: 261–289, 1989.
3. Singh OMP, Graham AB and Wood GC, The phospholipid dependence of UDP-glucuronyltransferase: conformation/reactivity studies with purified enzyme. *Biochem Biophys Res Commun* **107**: 345–349, 1982.
4. Vanstapel F and Blanckaert N, Topology and regulation of bilirubin UDP-glucuronyltransferase in sealed native microsomes from rat liver. *Arch Biochem Biophys* **263**: 216–225, 1988.
5. Vanstapel F, Hammaker L, Pua K and Blanckaert N, Properties of membrane-bound bilirubin UDP-glucuronyltransferase in rough and smooth endoplasmic reticulum and in the nuclear envelope from rat liver. *Biochem J* **259**: 659–663, 1989.
6. Vessey DA and Zakim D, Regulation of microsomal enzyme by phospholipids. II. Activation of hepatic uridine diphosphate-glucuronyltransferase. *J Biol Chem* **246**: 4649–4656, 1971.
7. Illing HPA and House ESA, Effects of detergents and organic solvents on rat liver microsomal UDP-glucuronosyltransferase activity towards phenolic substrates. *Xenobiotica* **11**: 709–718, 1981.
8. Sanchez E and Tephly TR, Activation of hepatic microsomal glucuronyl transferase by bilirubin. *Life Sci* **13**: 1483–1490, 1973.
9. Wisnes A, Studies on the activation *in vitro* of glucuronyltransferase. *Biochim Biophys Acta* **191**: 279–291, 1969.
10. Illing HPA, Choice of detergent for activating UDP-glucuronosyltransferase. *Biochem Soc Trans* **8**: 116–117, 1980.
11. Graham AB and Wood GC, Studies of the activation of UDP-glucuronyltransferase. *Biochim Biophys Acta* **276**: 392–398, 1972.
12. Zakim D, Goldenberg J and Vessey DA, Regulation of microsomal enzymes by phospholipids. VI. Abnormal enzyme–lipid interactions in liver microsomes from Gunn rats. *Biochim Biophys Acta* **297**: 497–502, 1973.
13. Miller KW, Heath EC, Easton KH and Dingell JV, Effect of Triton X-100 on the conjugation of tetrahydrocortisone, *in vitro*. *Biochem Pharmacol* **22**: 2319–2327, 1973.
14. Nemoto N, Kawana M and Takayama S, Effects of activation of UDP-glucuronyl transferase on metabolism of benzo[*a*]pyrene with rat liver microsomes. *J Pharm Dyn* **6**: 105–113, 1983.
15. Miners JO, Lillywhite KJ, Yoovathaworn K, Pongmarutai M and Birkett DJ, Characterization of paracetamol UDP-glucuronosyltransferase activity in human liver microsomes. *Biochem Pharmacol* **40**: 595–600, 1990.
16. Del Villar E, Sanchez E and Tephly TR, Morphine metabolism. II. Studies on morphine glucuronyltransferase activity in intestinal microsomes of rats. *Drug Metab Dispos* **2**: 370–374, 1974.
17. Thompson JA, Hull JE and Norris KJ, Glucuronidation of propranolol and 4′-hydroxypropranolol. Substrate specificity and stereoselectivity of rat liver microsomal glucuronyltransferases. *Drug Metab Dispos* **9**: 466–471, 1981.
18. Wilson BK and Thompson JA, Glucuronidation of propranolol by dog liver microsomes. Effects of enantiomeric inhibition and detergent treatment on stereoselectivity. *Drug Metab Dispos* **12**: 161–164, 1983.
19. Graham AB and Wood GC, Factors affecting the response of microsomal UDP-glucuronyltransferase to membrane perturbants. *Biochim Biophys Acta* **311**: 45–50, 1973.
20. Jacobson MM, Levin W and Conney AH, Studies on bilirubin and steroid glucuronidation by rat liver microsomes. *Biochem Pharmacol* **24**: 655–662, 1975.
21. Rane A, Gawronska-Szklarz B and Svensson J-O, Natural (−)- and unnatural (+)-enantiomers of morphine: comparative metabolism and effect of morphine and phenobarbital treatment. *J Pharmacol Exp Ther* **234**: 761–765, 1985.
22. Coughtrie MWH, Ask B, Rane A, Burchell B and Hume R, The enantioselective glucuronidation of morphine in rats and humans. Evidence for the involvement of more than one UDP glucuronosyltransferase isoenzyme. *Biochem Pharmacol* **38**: 3273–3280, 1989.
23. Milne RW, Nation RL, Reynolds GD, Somogyi AA and Van Crugten JT, High-performance liquid chromatographic determination of morphine and its 3- and 6-glucuronide metabolites: improvements to the method and application to stability studies. *J Chromatogr* **565**: 457–464, 1991.
24. Svensson J-O, Rane A, Säwe J and Sjöqvist F, Determination of morphine, morphine-3-glucuronide and (tentatively) morphine-6-glucuronide in plasma and urine using ion-pair high-performance liquid chromatography. *J Chromatogr* **230**: 427–432, 1982.
25. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
26. Bock KW, Burchell B, Dutton GJ, Hänninen O, Mulder GJ, Owens IS, Siest G and Tephly TR, UDP-glucuronosyltransferase activities. Guidelines for consistent interim terminology and assay conditions. *Biochem Pharmacol* **32**: 953–955, 1983.
27. Bock KW, Huber E and Schlote W, UDP-glucuronyltransferase in perfused rat liver and in microsomes. Effects of CCl_4 injury. *Naunyn Schmiedebergs Arch Pharmacol* **296**: 199–203, 1977.
28. Helenius A, McCaslin DR, Fries E and Tanford C, Properties of detergents. *Methods Enzymol* **56**: 734–749, 1979.
29. Schick MJ, *Nonionic Surfactants (Surfactant Science Series)*, Vol. 1. Dekker, New York, 1966.
30. McDonald C and Richardson C, The effect of added

salts on solubilization by a non-ionic surfactant. *J Pharm Pharmacol* **33**: 38–39, 1981.
31. Miners JO, Lillywhite KJ and Birkett DJ, *In vitro* evidence for the involvement of at least two forms of human liver UDP-glucuronosyltransferase in morphine-3-glucuronidation. *Biochem Pharmacol* **37**: 2839–2845, 1988.