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The synthesis of 17α-ethynylestradiol glucuronides by mammalian microsomes immobilised in hollow-fibres

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Abstract

Uridine 5′-diphosphate glucuronosyl transferases (UGTs, E.C.2.4.1.17) catalyse the glucuronidation of xenobiotics. The oral contraceptive 17α-ethynylestradiol is partly cleared by glucuronidation on the 3 and 17 positions. These glucuronides are not commercially available and were synthesised as standards for characterisation of the metabolic profile of the drug. (1) Rat and dog microsomal suspensions were investigated, and the rates of glucuronide formation were found to be < 0.09 and 0.49 nmol min^{-1} mg^{-1} protein, respectively. Therefore, dog microsomes were selected and immobilised in hollow-fibre membrane bioreactors. (2) Two bioreactors (15-ml capacity) were run at 20°C and fed with a saturated solution (3.3 pmol/mg protein) of the sparingly soluble drug and co-factor UDPGA (0.15 mmol/mg protein) in recycle and single pass modes, at a flow rate of 20 ml/h. The recycle mode produced more glucuronide than the single pass up to 46 h (substrate to product peak area ratios of 1.6 and 5.9, respectively). The recycle mode was adopted and the bioreactors run continuously for 88 h. (3) A second batch using the same bioreactor was run for 120 h. The estimated conversion reduced from 50% on the first run to 25% on the second run. (4) A primary clean up was effected by adsorption on octadecyl silyl resin. Separation of the glucuronides from 17α-ethynylestradiol was on a normal phase high pressure liquid chromatography (HPLC) system. After collection of the glucuronide fraction, the solvents were evaporated off providing 1 mg of the two glucuronides (recovery efficiency > 90%).

Keywords: 17α-Ethynylestradiol glucuronides; Immobilisation; Hollow-fibres; Uridine 5′-diphosphate glucuronosyl transferases (UGTs); Glucuronidation

1. Introduction

Clinical trials of potential drug–drug interactions are an inherent part of drug development programmes. Specifically interactions with 17α-ethynylestradiol, a major component of contraceptive medications. This interaction can be monitored by the measurement of 17α-ethynylestradiol metabolism. Therefore, standards of 17α-ethynylestradiol and all its metabolites were required for this investigation. 17α-ethynylestradiol is partly cleared by glucuronidation in the 3 and 17 positions [1]. These glucuronides however, are not commercially available, so the aim of this project was to synthesise 17α-ethynylestradiol glucuronides.

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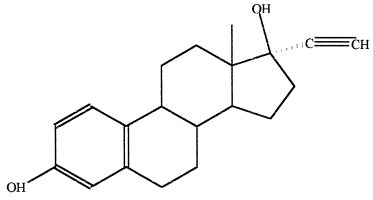

Uridine 5′-diphosphate glucuronosyl transferases (UGTs, E.C.2.4.1.17) catalyse glucuronidation, one of the major reactions involved in the phase II metabolism of xenobiotics, including 17α-ethynylestradiol. Rat and dog hepatic microsomal UGTs were investigated for glucuronidation activity towards 17α-ethynylestradiol. Dog microsomes were immobilised in hollow-fibre membrane bioreactors, the advantages of this immobilisation technique have previously been demonstrated for other drug-metabolising enzymes, in this case cytochrome P-450s [2]. Typically, the immobilisation is relatively easy to carry out, the stability of the enzyme system is improved, and the product yield is large and extraction facilitated [3].

Two bioreactors were used and their operational modes evaluated with respect to glucuronide productivity. The 17α-ethynylestradiol glucuronides were extracted from the bioreactor effluents by adsorption on octadecyl silyl resin, followed by fraction collecting from a normal phase high pressure liquid chromatography (HPLC) system.

2. Materials and methods

Rat and dog hepatic microsomal suspensions were prepared [4], and protein determined [5]. The suspensions were used to study in vitro glucuronidation activity towards 17α-ethynylestradiol. In vitro glucuronidation assays were performed as follows: (1) 1 mg of microsomal protein was incubated with the detergent digitonin (0.75 μmol/mg protein) so as to disrupt the microsomal membranes and expose the UGTs. (2) Preincubation of the microsomes with the co-substrate, UDP-glucuronic acid (1.5 μmol/mg protein), and the reaction buffer (7.5-mM $MgCl_2$, 75-mM Tris–HCl, pH 7.1) solution. (3) Addition of 17α-ethynylestradiol (0.2 μmol/mg protein), and the reaction monitored at 37°C for the allocated time. The final samples were analysed on a reversed-phase HPLC system (Table 1).

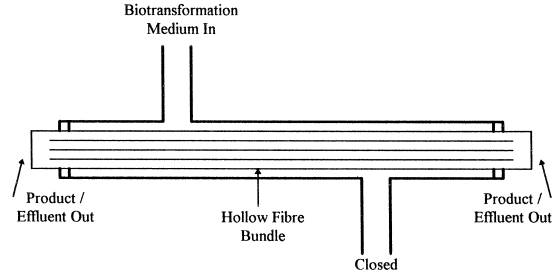

Fig. 1. Hollow-fibre membrane bioreactor.

The hollow-fibre membrane bioreactor (Fig. 1) incorporates seven individual hollow-fibres, providing an active surface area of 65.94 cm^2. Microsomal protein loading achieved was 10 ± 1 mg when 55 mg were introduced into the bioreactor ($n = 2$). An operation protocol has been developed involving four steps.

1. Initial fibre preparation
2. Immobilisation procedure
3. Biotransformation
4. Bioreactor recycling

Two bioreactors (15-ml capacity) were run at 20°C and fed with a saturated solution (3.3

Table 1
HPLC conditions

Reaction monitoring reversed phase HPLC	HICHROM INERTSIL S5ODS2 0.8 ml/min	0.5% ammonium phosphate pH 3 40%:60% methanol	$\lambda = 285$ nm
Glucuronide separation normal phase HPLC	HICHROM Spherisorb S5CN 1 ml/min	Gradient system 100% ethyl acetate to 40%:60% methanol over 30 min	$\lambda = 285$ nm

Table 2
The ratios from the recycle and single pass bioreactors

Time (h)	Recycle bioreactor S:P peak area ratio	Single pass bioreactor S:P peak area ratio
2	7.16	9.08
24	2.02	5.58
46	1.61	5.85

pmol/mg protein) of the sparingly soluble drug and co-factor UDPGA (0.15 mmol/mg protein) at a flow rate of 20 ml/h. One bioreactor was run in the recycle mode, and the other bioreactor in the single pass mode. It should be noted that a higher 17α-ethynylestradiol concentration than 3.3 pmol/mg protein, was originally administered to the bioreactor. Due to the instability of the UDPGA the bioreactor reservoirs were placed on ice, this however reduced the solubility of the substrate. During the whole biotransformation, the reservoir was maintained at ambient room temperature (19–21°C) and a saturated substrate solution administered.

The bioreactor effluent was initially cleaned up by adsorption on octadecyl silyl resin. The 17α-ethynylestradiol and glucuronides were extracted with a methanol wash, leaving reaction buffer and leaked protein on the resin. The final methanol sample was concentrated 10-fold by evaporation.

A TLC assay sensitive enough to extract the glucuronides could not be developed, therefore, the 17α-ethynylestradiol glucuronides were separated from the parent by a normal phase HPLC system (Table 1). 17α-ethynylestradiol had a retention time of 3 min and the glucuronides gave a single peak at 18 min. The glucuronides were collected from the HPLC and samples blown down under nitrogen to dryness.

The glucuronides were characterised by HPLC.

3. Results

Using the reversed-phase HPLC method, the synthesis of 17α-ethynylestradiol glucuronide over 1 h was followed. Dog hepatic microsomal UGTs had a glucuronidation rate of 0.49 nmol min^{-1} mg^{-1} protein, a greater activity than rat UGTs, which had a glucuronidation rate of $<$ 0.09 nmol min^{-1} mg^{-1} protein. Therefore, dog microsomes were used for the immobilised biotransformations.

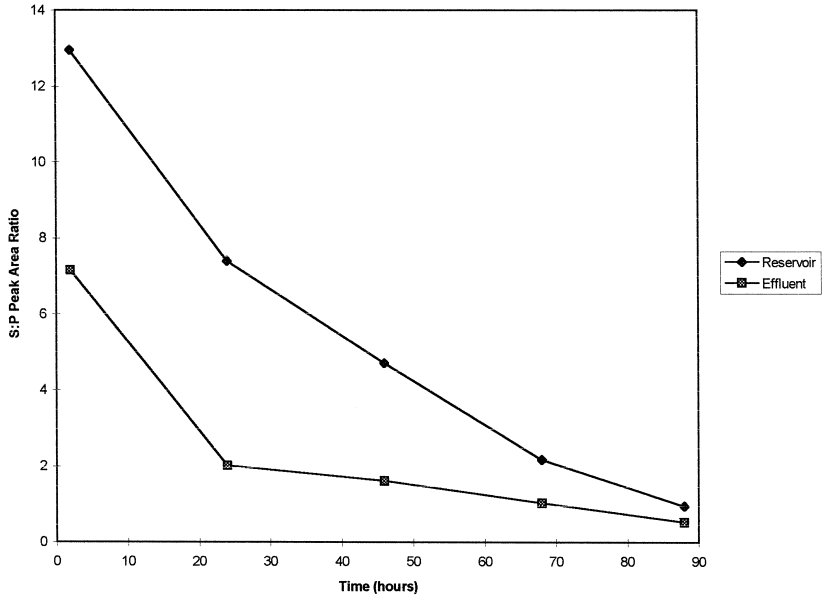

Fig. 2. Time course of reservoir and effluent of the recycle bioreactor.

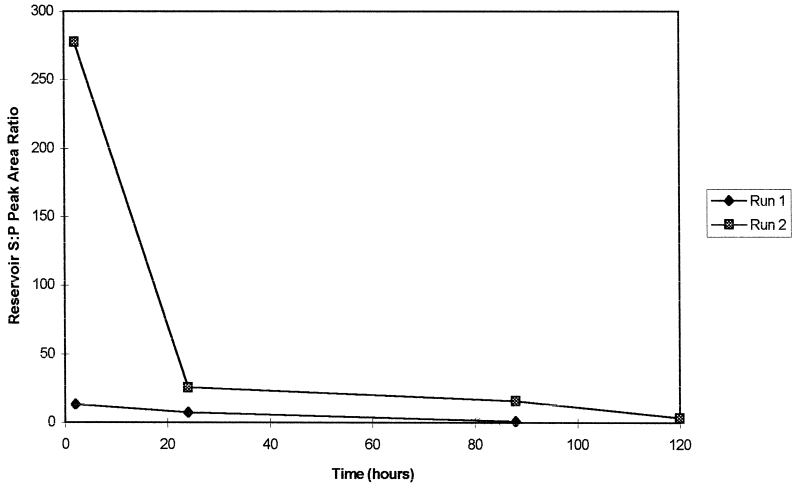

Fig. 3. The re-use of the recycle bioreactor.

Two bioreactors were run, one in recycle, the other in single pass mode for up to 46 h. The efficiency of the bioreactors was monitored by the substrate to product peak area ratio. From Table 2, it can be seen that the bioreactor ran in the recycle mode is more efficient than the single pass mode, as the ratio is much lower.

Both bioreactors were therefore run in the recycle mode for 88 h. Fig. 2 shows the time course of reservoir and effluent S:P Peak Area Ratios of the recycle mode bioreactor. The recycle bioreactor was rerun a second time for 120 h. Fig. 3 shows the comparison between run 1 and 2.

At the end of run 1, the S:P peak area ratio was 1, and for run 2, it was 3. From this, an estimated percentage conversion can be calculated using the following equation.

$$\text{Estimated\%Conversion} = \frac{[P]}{[P] + [S]}$$

Run 1—Peak area ratio of 1,10-μM Substrate, therefore, 10-μM Product.

$$\frac{10}{20} = 50\%\,\text{conversion}.$$

Run 2—Peak area ratio of 3,10-μM Substrate, therefore, 3.33-μM Product

$$\frac{3.33}{13.33} = 25\%\,\text{conversion}.$$

Fig. 4 shows the reversed-phase HPLC system run of the bioreactor effluent. The effluent

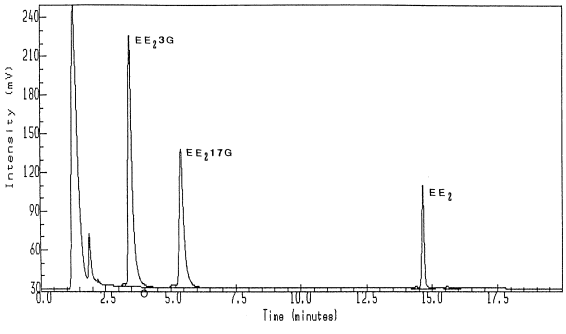

Fig. 4. Bioreactor effluent sample.

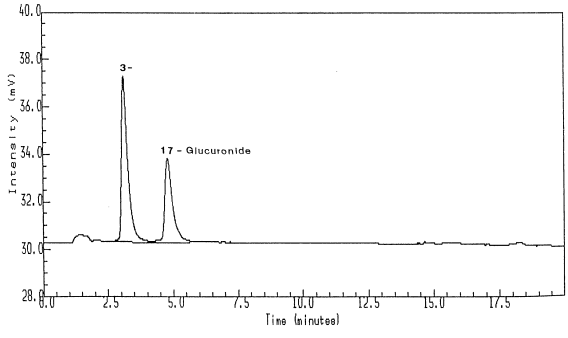

Fig. 5. Isolated glucuronides sample.

was then treated as described in Section 2. Fig. 5 shows the solution after isolation of products run on the same system. The final sample was very clean and clearly shows the presence of the 3- and 17-glucuronides only.

4. Discussion

Rat and dog hepatic microsomal UGTs both glucuronidate 17α-ethynylestradiol in the 3 and 17 positions; however, dog UGTs exhibited a 5-fold greater glucuronidation rate than rat. Dog UGTs, unlike rat UGTs, have not been extensively investigated to date, but there could be several reasons that may explain the difference in glucuronidation rates. The dog may express more UGT activities, due to being larger in size than the rat, also, the dog may have more UGT isoforms that are active towards 17α-ethynylestradiol than the rat.

The mode in which the bioreactors operated appeared to affect the rate of glucuronidation of 17α-ethynylestradiol. Under single pass conditions, where the biotransformation medium passes through the bioreactor once, the substrate to product peak area ratio was 5.85 after 46 h. However, when run in recycle, where the bioreactor effluent feeds back into the reservoir forming a closed loop system, the substrate to product peak area ratio was 1.61. Therefore, the recycle mode proved to be more efficient than the single pass mode for 17α-ethynylestradiol glucuronide synthesis.

The recycle bioreactor was run initially for 88 h, and then rerun a second time for 120 h, also in recycle. Run 1 and run 2 produced different estimated percentage conversions, 50% and 25%, respectively. Therefore, upon re-use of a hollow-fibre membrane bioreactor the estimated percentage conversion was halved with respect to 17α-ethynylestradiol glucuronidation. This result could possibly be due to the age of the microsomal UGTs immobilised. Previous research has shown that hollow-fibre membrane immobilised UGTs lose glucuronidating activity with time [3].

17α-Ethynylestradiol glucuronides were successfully synthesised and isolated using this technology. Previously, up to 15 mg of two drug glucuronides have been synthesised in a non-optimised bioreactor [6]. The bioreactors adopted for this research were partially optimised, with respect to operational mode, and biotransformation medium. Other factors to investigate for a fully optimised bioreactor are temperature and flow rate. However, with reference to 17α-ethynylestradiol, and other substrates like this, temperature could significantly affect their availability to the immobilised UGTs.

The use of hollow-fibre membrane immobilised UGTs to synthesise drug glucuronides has been successful here and previously [3,6]. Drug glucuronides are of paramount importance if they are pharmacologically or toxicologically active, e.g., morphine glucuronide(s) possess analgesic properties [7]. 17α-Ethynylestradiol glucuronides have been shown to possess cholestatic activity [8], and are therefore, important with respect to 17α-ethynylestradiol metabolism. The synthesis of these important glucuronides in a quick and effective manner, as shown here, is a great advantage to drug metabolism studies, especially in this particular case where the glucuronides of interest were commercially unavailable.

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References

[1] F.P. Guengerich, Life Sci. 47 (1990) 1981.
[2] P. Fernandez-Salguero, C. Gutierrez-Merino, A.W. Bunch, Enzyme Microb. Technol. 15 (1993) 100.
[3] V. Gilder, A.W. Bunch, S.G. Jezequel, Poster Presentation at Stowe Symposium for Drug Metabolism, Stoke Rochford Hall, August, 1996.
[4] H. Remmer, J.B. Schenkman, R.W. Estabrook, H.A. Sasame, J.R. Gillette, S. Narasimhulu, D.Y. Cooper, O. Rosenhal, Mol. Pharmacol. 2 (1966) 187.
[5] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[6] R. Webster, A.W. Bunch, S.G. Jezequel, Poster Presentation at the Biotransformation Meeting, Institute of Biotechnology, University of Cambridge, July, 1994.
[7] H.K. Kroemer, U. Klotz, Clin. Pharmacokin. 23 (4) (1992) 292.
[8] M. Vore, W. Slikker, TIPS, June (1985) 256.