Chimia 53 (1999) 590–593

ISSN 0009–4293

Enzymatic Synthesis of β-D-Glucuronides in an Enzyme Membrane Reactor

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Abstract. The production of two *O*-glucuronides in an enzyme membrane reactor on a 100 to 200 mg scale was examined. The aglycons were conjugated with the co-substrate β-D-uridine diphosphoglucuronic acid (UDPGA) in the presence of a guinea-pig liver preparation. The continuous synthesis, which was run in an enzyme membrane reactor, was followed depending on the substrate up to 118 hours or 24 hours, respectively. The reaction was monitored by TLC or HPLC. The purification of the two glucuronides was carried out by ion-exchange chromatography and by reversed-phase HPLC.

Introduction

The conjugation with β-D-glucuronic acid is a major pathway involved in phase-II metabolism, thus contributing to detoxification and elimination of xeno- and endobiotics in animals and man [1]. Consequently, the glucuronidation may have an important influence on the disposition, metabolism, and excretion of drugs, since this biotransformation increases the polarity and the molecular weight of the compound administered. Therefore, larger amounts of glucuronides, radioactively labelled and unlabelled, respectively, might be required for toxicological, pharmacological, and analytical studies. Recently, new prodrug strategies for cancer chemotherapy were published [2]. This interesting new approach focuses on the reduced toxicity of the glucuronide derivative of the drug as compared to its aglycon which is the pharmacologically effective agent. Toxicity is reduced since the aglycon is selectively liberated in the target tissue *i.e.*, the tumor. Therefore, overall systemic exposition is lowered without reducing the pharmacological effect. Preparation of such prodrugs for the use in antibody-directed enzyme prodrug therapy, activated by human β-glucuronidase, for instance, requires an efficient synthetic access to glucuronides.

The enzymatic glucuronidation of substrates, such as phenols, alcohols [3], carboxylic acids [4], thiols [5], and amines [6] or carbamates [7], is catalyzed by the uridine 5'-diphosphoglucuronyl transferases, a multigenic family of membrane-bound enzymes (UGTs). Research on UGTs increased drastically within the last years due to the availability of numerous UGTs by recombinant expression and the increasing knowledge on substrate specificity of the isoenzymes [8] as well as the knowledge on genetic variants among the different species. For example, polymorphism was found for the human UGT-1 gene [9]. Since recent reports from several authors have documented covalent binding of carboxylic acids to proteins mediated *via* their corresponding acyl glucuronide metabolites [10], interest in the formation, stability, and reactivity of acyl glucuronides [11] has increased. The formation of acyl glucuronides is a major metabolic pathway of nonsteroidal antiinflammatory drugs [12], mediated in humans mostly *via* UGT2B7 [13]. These acyl glucuronides can undergo rearrangement reactions to positional isomers which are resistant to β-glucuronidase degradation of the glucuronide to the parent compound [14].

The members of the UGT family are located in the liver, the hepatic endoplasmatic reticulum [15], and various extrahepatic tissues [16], such as kidney [17], intestine [18], spleen [19], lung [20], skin [21], prostate [21] [22], and placenta [23]. Several isoenzymes of UGT have been separated from rat liver [24]. In contrast to the biosynthesis of glucuronides, the chemical synthesis requires well-planned strategies and tactics of functional-group protection for both, the glucuronic acid and the aglycon. However, the most important step on a synthetic route to glucuronides is the development of suitable combinations of protecting groups which can be cleaved under mild non-degradative conditions. The synthesis of several types of *N*-, *O*- and *C*-β-D-glucuronides using different β-coupling reactions with protected glucuronides has been reviewed by *Kaspersen* and *Boeckel* [25]. The most common chemical synthesis is based on the *Mitsunobu* reaction [26].

The synthesis of glucuronides was also performed with glucuronyl transferases either in batch or in a hollow fibre, or with immobilized glucuronyl transferases [27], whereby the glucuronidation of phenol [28], naphthol [29], phenolphthalein [30], and bilirubin [31] was investigated profoundly. These aglycons were used to optimize and elaborate glucuronidation reactions and serve as reference glucuronides, *e.g.*, for enzyme-activity determinations.

Enzyme membrane reactors have been used for the continuous stereoselective preparation of amino acids [32], alcohols [33], and *N*-acetyl-neuraminic acid [34].

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The present study describes the continuous preparation of β-D-glucuronides using an enzyme membrane reactor.

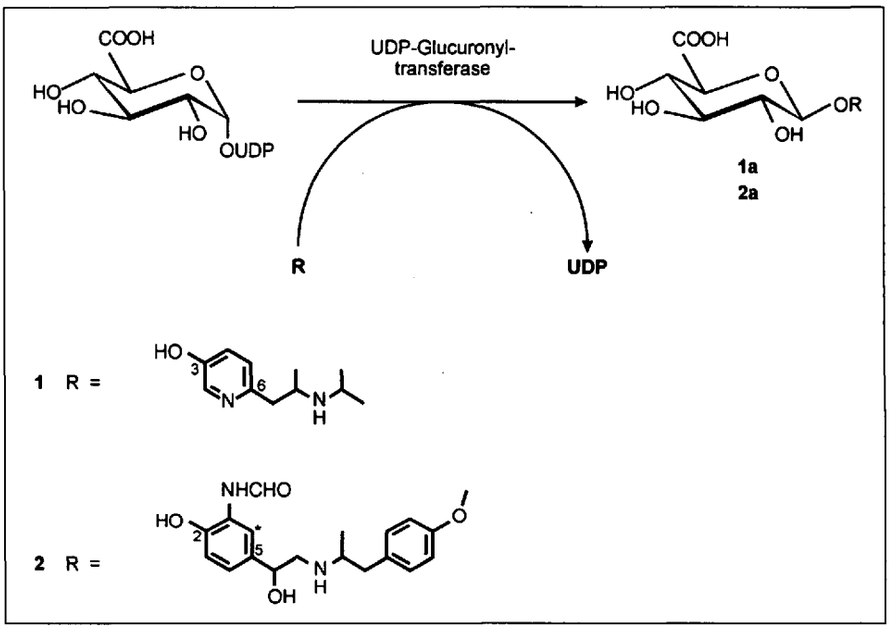
Results and Discussion

The enzyme-catalyzed transfer of glucuronic acid requires the presence of a suitable aglycon and the co-substrate β-D-UDP-glucuronic acid. The glucuronidation of a substrate is stereoselective with inversion of the anomeric center. The reaction of the two educts **1** and **2** (formoterol) to their corresponding *O*-glucuronides is shown in the *Scheme*. The degree of conversion was influenced by the residence time of the reactants, the amount of glucuronyl transferase, as well as by a β-D-glucuronidase activity which was also present in the liver-homogenate preparation. The formation of the glucuronide **1a** reached a steady state after about 8 h which was constant at least up to 24 hours (*Fig. 1*). Due to the efficient conversion of **1** to its glucuronide **1a**, the reaction could be run for up to 118 hours (Not shown in the graph). For the formanilide derivative **2**, however, maximum conversion to its glucuronide was observed after 3–7 h, thereafter, the conversion decreased slightly due to the back-reaction catalyzed by the β-D-glucuronidase.

The continuous enzymatically catalyzed glucuronidation of **1** and **2** was carried out in an enzyme membrane reactor with a volume of 10 ml. The reactor (setup), as shown in *Fig. 2*, was sterilized with 0.1% peracetic acid before use. The substrate solution was pumped into the reactor through a sterile filter at a constant rate of 6 ml/h, which corresponds to a residence time of 1.67 hours. The glucuronyl transferase, which was obtained from guinea-pig liver homogenate supernatant *S4.5*, was directly injected into the reactor. The homogenous enzyme preparation was retained in the reactor by an ultrafiltration membrane (cut-off: 10000 Da) that is permeable only to the substrates and products (*Fig. 2*).

The solution which passed the membrane was collected in fractions, the glucuronide formation was monitored in these fractions qualitatively and quantitatively by TLC (detection with thymol/sulfuric acid for **1a**) or with radioactivity detection (radioscanning for **2a**). After about 7 h, the optimal reaction conditions in the bioreactor were reached. The educt **1** was converted constantly to about 95% to its corresponding glucuronide **1a**. Compound **2**, however, was found to be a less suitable substrate for the guinea-pig glucuronyl transferase since no stable steady-state could be reached. The formation of the glucuronide **2a** decreased almost linearly in the time period of 4 to 21 h. Nevertheless, the conversion of **2** was about 50%. The glucuronide-containing fractions were lyophilized rapidly to avoid degradation of **1a** and **2a**. The products were isolated by HPLC in consecutive runs yielding mg amounts of the glucuronides (**1a**: 184.9 mg, equal to 64% overall yield; **2a**: 96 mg, equal to 37% overall yield).

Scheme. *UDP-Glucuronyl Transferase-Catalyzed Synthesis of Aryl Glucuronides with Guinea-Pig Liver Homogenate Supernatant* S4.5. The asterisk marks the position of the [^{3}H]-label for **2**. UDP: Uridine-5'-diphosphate. Site of glucuronidation of **1** at position 3 and of **2** at position 2.



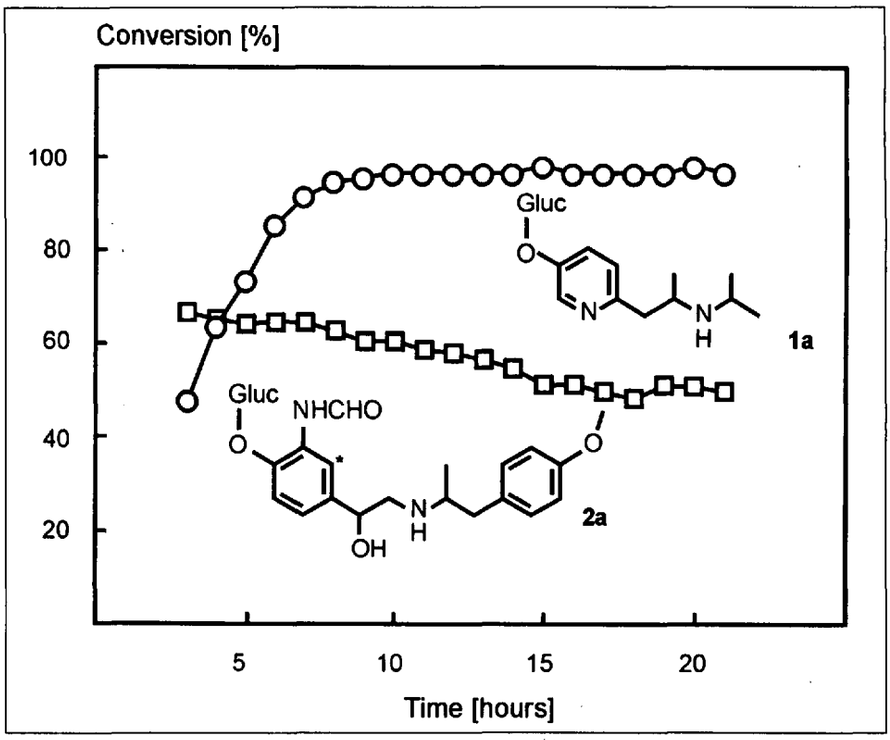


Fig. 1. *Conversion of 6-{2-[(1-methylethyl)amino]propyl}pyridin-3-ol* **1** *and [^{3}H]-labelled N-[2-hydroxy-5-(1-hydroxy-2-{[2-(4-methoxyphenyl)-1-methylethyl]amino}ethyl)phenyl]formamide* **2** *to their O-glucuronides* **1a** (–○–○–) *and* **2a**. (–□–□–). The asterisk marks the position of the [^{3}H]-label.

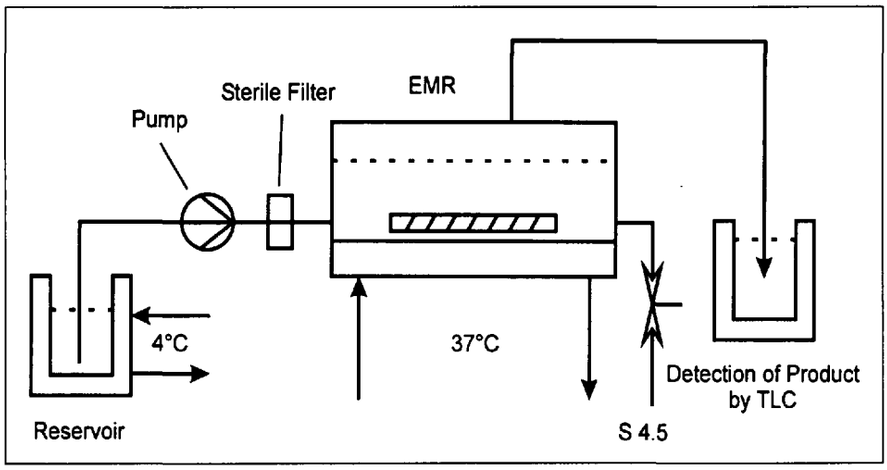


Fig. 2. *Apparatus for the continuous glucuronidation of different substrates.* The substrate and uridine 5'-diphosphoglucuronic acid (UDPGA) are pumped continuously from the reservoir *via* a sterile filter through the enzyme membrane reactor containing guinea-pig liver homogenate supernatant *S4.5*. The enzyme preparation was retained in the reactor by an ultrafiltration membrane (cut-off: 10000 Da). Formation of glucuronide was monitored by radio-TLC.

The structures of the β-D-glucuronides **1a** and **2a** were elucidated by means of FAB-MS and ^{1}H-NMR.

To assess the optimal conditions for the *in vitro* glucuronidation with the enzyme membrane reactor, a numerous factors have to be considered. These include the concentrations of UDPGA and the aglycon, the amount of protein, the residence time in the reactor, the pH, and the temperature. The liver homogenate used for glucuronidation contains both the UDP-glucuronyl transferase and the β-glucuronidase, which catalyzes the hydrolysis of glucuronides. Therefore, the equilibrium between glucuronide synthesis and cleavage should be carefully examined and investigated in a batch synthesis before performing the preparative synthesis in the enzyme membrane reactor. In conclusion, this method for the synthesis of glucuronides is a helpful tool for the preparation of 100 to 200 mg amounts of glucuronides in a reasonable period of time.

Experimental

Chemicals. **1** and **2** were obtained from *Novartis Pharmaceuticals Research Laboratories* (formerly *Ciba-Geigy*). HEPES Buffer ([4-(2-hydroxyethyl)piperazine-1-yl]sulfonic acid) was purchased from *Fluka*, UDPGA (uridine-5'-diphosphoglucuronic acid) and albumin from *Boehringer-Mannheim*. All solvents were of reagent grade.

HPLC Apparatus. A *Spectraphysics 8100* pump was used. The eluted compounds were detected by a *Spectraphysics* UV detector operating at 275 nm (**1a**) and 260 nm (**2a**).

Monitoring of the Glucuronide Formation by HPLC. The reaction was monitored by HPLC with the same column which was used for preparative isolation (10 μm, 10 × 230 mm; semi-preparative column filled in-house with the *Lichrosorb RP18* adsorption material from *Merck*) and eluting with 20 mM NH_4Ac in H_2O (acidified to pH 5.0 with AcOH) and MeCN (8:2, *v/v*). The flow rate was 2 ml/min, UV detection at 275 nm for **1a** and 260 nm for **2a**. In addition, **2a** was chromatographed using a different eluent (see synthesis and isolation of **2a**). For monitoring, the same method as for **1a** was used.

Monitoring of the Glucuronide Formation by TLC. Formation of glucuronide was followed by TLC (silica gel *60*, F_{254} 0.25 mm, *Merck*) with BuOH/acetone/AcOH/NH_3 (25%)/H_2O 70:50:18:1.5:60. The glucuronides **1a** and **2a** were detected using thymol/H_2SO_4 as a spray reagent, R_f(**1a**) = 0.36, R_f(**2a**) = 0.69.

Preparation of Liver Homogenate (S4.5). Fresh liver from guinea-pig (6 g) was homogenized in a *Potter-Elvehjelm* homogenizer (16'000 rpm, 0°, 1 min) in 19 ml ice-cold sucrose solution (0.25M) and then centrifuged (4'500 g, 5 min). The supernatant, which contains UDP-glucuronyl transferase activity, was stored at –20°.

Preparation of the Enzyme Membrane Reactor. For continuous production of the glucuronide, a 10-ml enzyme membrane reactor was used, equipped with an ultrafiltration membrane *YM10* (62 mm) from *Amicon* (Witten, Germany). The sterilization of the whole system was carried out by continuously flushing (or rinsing) with 0.1% peracetic acid for 12 h, followed by sterile water for removal of peroxides. Thereafter, 200 ml sterile HEPES buffer (50 mM, pH 7.4) were pumped through the membrane reactor. Then, a sterile solution of 50 mg albumin in 1 ml HEPES buffer was injected directly into the reactor to cover the membrane with a protein layer. After that, the continuous flow of 1 ml/min of HEPES buffer through the reactor was resumed. 4.5 ml of the liver fraction *S4.5* of guinea-pig liver homogenate in 0.25 M glucose solution were injected *via* the septum into the bioreactor. The flow rate of the peristaltic pump was reduced to 0.2 ml/min. Before the glucuronidation reaction was started, HEPES buffer was pumped through the reactor for about 30 min.

Synthesis of 2-{2-[(1-Methylethyl)amino]propyl}pyridin-5-yl β-D-Glucopyranosiduronic Acid (**1a**). UDPGA (20 mM) and **1** (5 mM) were dissolved in 50 mM HEPES buffer (pH 7.4) containing 10 mM $MgCl_2$. The mixture was kept at 0–5°. The total amounts of substrates used for the synthesis were 1963 mg UDPGA, 256.5 mg of **1**, and 675 ml HEPES buffer. This mixture was prepared freshly in six portions to avoid degradation. The reaction took place in the enzyme membrane reactor at 37° with a flow rate of 0.2 ml/min. The solution leaving the reactor was collected in 1 h-fractions. The total reaction time was 118 h. The reaction was monitored by TLC and HPLC. The glucuronide conversion varied between 76 and 90%, as indicated by concomitant HPLC analysis of the collected fractions.

Isolation of **1a**. All collected fractions (0–118 h) were combined, and the volume was reduced to 125 ml by lyophilization. The reaction mixture was first purified by ion-exchange chromatography (*DOWEX* 1 × 4, Cl^- form; 3 × 50 cm) using H_2O as an eluent. Ten fractions of 120 ml each were collected. The glucuronide-containing fractions were further purified on *AVICEL* (350 ml; 3 × 50 cm) with MeCN/H_2O (80:20, *v/v*; 7 fractions of 100 ml each; thereafter, with 60:40, *v/v*, 8 fractions). A further purification on *AVICEL* with 1,4-dioxane, MeOH, H_2O (70:10:20; *v/v*) was performed before the final purification step on HPLC (*RP-18*) was carried out using NH_4Ac (20 mM, pH 4.4) and acetonitrile (97:3; *v/v*). The glucuronide was recrystallized from MeOH to yield of 184.9 mg (63.9%, theoretical yield: 289.3 mg).

Synthesis and Isolation of 2-(Formylamino)-4-(1-hydroxy-2-{[2-(4-methoxyphenyl)-1-methylethyl]amino}ethyl)phenyl β-D-Glucopyranosiduronic Acid (**2a**; = *Formoterol Glucuronide*). Conditions for the synthesis were analogous as for **1a**. The [^{3}H]-labelled fumarate salt of **2** (211.4 mg with a specific radioactivity of 29 kBq/mg; radiolabelled **2** was synthesized in the Isoptope Laboratories of *Ciba-Geigy AG*, legal successor *is Novartis Pharma AG*) was dissolved in HEPES buffer, and the glucuronidation reaction was carried out as described for the synthesis of **1a**. For purification of **2a**, the crude reaction mixture was lyophilized, purified by ion-exchange chromatography on *DOWEX* (as described for purification of **1a**) and finally on HPLC (*RP-18*; 10 × 230 mm) in isocratic runs with a 4:1 (*v/v*) mixture of phosphate buffer pH 7.0 (41 mM Na_2HPO_4 + 28 mM KH_2PO_4 diluted with H_2O (1:6, v/v)) and MeOH at a flow rate of 3 ml/min. The overall yield of **2a** was 96 mg (37% theoretical yield).

Structure Elucidation. Data of **1a**. FAB-MS (positive-ion mode): *m/z* 371 $(M+H)^+$; ^{1}H-NMR (D_2O): 8.33 (H–C(6), pyridine); 7.59 (H–C(4), pyridine); 7.37 (H–C(3), pyridine); 5.15 (H–C(1), anomeric proton glucuronic acid). The position of glucuronidation was determined by NOE.: Saturation of the glucuronyl H–C(1) resulted in a strong NOE for pyridine H-C(4) and H–C(6).

Data for **2a**. FAB-MS (positive-ion mode): *m/z* 521 $(M+H)^+$; ^{1}H-NMR (CD_3OD, assignment and downfield shifts upon glucuronidation, as compared with the parent molecule: 8.23 (H–C(2), 0.14); 7.30 (H–C(5), 0.45); 7.08 (H–C(6), 0.04); 4.82 (H–C(1), anomeric proton glucuronic acid).

In the year 1987, Dr. *Hiltrud Stierlin* (*Ciba-Geigy AG*, legal successor is *Novartis Pharma*

AG) initiated the preparative glucuronide batch synthesis of radiolabelled and non-labelled drug candidates using liver homogenates from a large number of different animal species. She also introduced the enzyme membrane reactor as an efficient tool for the preparation of glucuronides.

Dr. *Gerhard Gross* and Dr. *Johannes Paul Pachlatko* are acknowledged for the critical reading of the manuscript and for helpful discussions. We are grateful to Mr. *Matthias Kuhn* for his assistance in preparing this paper.

Received: October 5, 1999

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