Development of an Automated Synthesis System for Preparation of Glucuronides Using a Solid-Phase Extraction Column Loaded with Microsomes

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An automated synthesis system using a solid-phase extraction (SPE) system and column packed with octadecylsilica (ODS), which was coated with phospholipid and loaded with dog liver microsomes, was developed for synthesis of glucuronides. Preparation of the microsome-immobilized SPE column, glucuronidation of drugs to synthesize the glucuronides and elution of the products were performed by an automated synthesis system. The phospholipid-coated SPE column and then the microsome-immobilized SPE column were readily prepared by allowing a solution containing L-α-dipalmitoylphosphatidylcholine to flow through the SPE column, and then by recycling a buffer solution containing dog liver microsomes through the resulting phospholipid-coated SPE column. The microsome-immobilized SPE column exhibiting the uridine diphosphate (UDP)-glucuronosyltransferase activity catalyzed the glucuronidation of mefenamic acid and estradiol to the corresponding glucuronides in the presence of UDP-glucuronic acid, and three glucuronides of mefenamic acid and estradiol were synthesized using the automated synthesis system, by simply recycling a buffer solution containing UDP-glucuronic acid through the microsome-immobilized SPE column loaded with the substrate. We used β-cyclodextrin as a solubilizing agent for the synthesis of the glucuronides of estradiol that is practically insoluble in aqueous solutions. The productivity of these glucuronides using the microsome-immobilized SPE column was higher than that using the free microsomes (batch method). Furthermore, we developed a fully automated synthesis-isolation system by coupling the automated synthesis system to an automated preparative HPLC system. The automated synthesis system as well as the fully automated synthesis-isolation system should be very useful for synthesizing glucuronides for drug development.

Key words automated synthesis; glucuronide; microsome: microsome-immobilized solid-phase extraction column

Since glucuronidation, which is catalyzed by uridine diphosphate glucuronosyltransferase (UDPGT)[1] that is located mainly in the liver and other tissues, is one of the main metabolic pathways for drugs *in vivo*, authentic samples of glucuronides of drugs are often needed for pharmacokinetics, pharmacological or toxicological study of drugs during preclinical or clinical drug development stages.

Glucuronides have been obtained by tedious isolation and purification of metabolites from biological samples,[2,3] by limited chemical synthesis,[4] by complicated chemo-enzymatic synthesis,[5] and by inconvenient biosynthesis using free microsomes[6,7] or immobilized microsomes.[1,8—11]

Recently, an efficient and useful synthesis of acylglucuronides of drugs using immobilized dog liver microsome octadecylsilica (ODS) particles coated with phospholipid (microsome-immobilized particles) has been reported from our laboratories.[12] However, this synthesis is still inconvenient, because preparation of the phospholipids-coated ODS particles, immobilization of microsomes on the particles, and glucuronidation of drugs must be conducted by rather tedious, batch-wise procedures including filtration steps. Moreover, preparation of glucuronides of water-insoluble drugs is not practicable by this synthesis.

To overcome these disadvantages, we developed a new automated process for the synthesis of authentic samples of glucuronides of drugs, using a solid-phase extraction (SPE) column loaded with the immobilized dog liver microsomes ODS coated with phospholipid (microsome-immobilized SPE column). This automated process was applied to synthesis of glucuronides of mefenamic acid and estradiol. For a water-insoluble substrate such as estradiol, we used β-cyclodextrin (β-CD) as a solubilizing agent. The microsome-immobilized SPE column and free microsomes were compared with respect to their productivity of glucuronides. Furthermore, we developed a fully automated synthesis-isolation system by coupling the automated synthesis system to an automated preparative HPLC system, isolating the pure product from the synthesis mixture, and applied to preparation of flufenamic acid 1-*O*-acylglucuronide.

Experimental

Materials SPE column (Bond Elut-C18, 500 mg, 3 ml) was obtained from Varian (Harbor City, CA, U.S.A.). β-CD and L-α-dipalmitoylphosphatidylcholine (DPPC) were purchased from Wako (Osaka, Japan). Uridine 5′-diphosphate-glucuronic acid (UDPGA) was obtained from Nacalai Tesque (Kyoto, Japan). Mefenamic acid, estradiol and flufenamic acid were purchased from Sigma (St. Louis, MO, U.S.A.). Acetonitrile, methanol, water and formic acid were of HPLC grade, and all other chemicals were of reagent grade.

HPLC and LC/MS Analyses The HPLC system consisted of a Shimadzu LC-20AD (Kyoto, Japan) as a pump, a Shimadzu SIL-HTc automatic injector as an injector, a Shimadzu SPD-20AV spectrophotometer as a UV detector and a Shimadzu CTO-20AC as a column oven. The chromatographic data were analyzed using a Waters Empower (Milford, MA). The HPLC column was a Cadenza 5CD-C18 (75×10 mm i.d., 5 μm, Imtakt, Kyoto, Japan). The mobile phase was 0.1% formic acid/acetonitrile solution (70 : 30) or (40 : 60) at a flow rate of 2 ml/min, and the detector was set at a wavelength of 230 nm or 320 nm. LC/MS experiments were conducted using a Waters Acquity Ultra Performance LC system coupled to a Waters LCT-Premier (Milford, MA, U.S.A.).

Preparation of Dog Liver Microsomes Dog liver samples were obtained from female beagle dogs without drug treatment in our laboratories. The microsomes were prepared in a conventional manner as described previously.[13] The microsomal pellets were stored in a freezer at −80 °C until use.

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The microsomal protein concentration was determined by using the Micro Bicinchoninic Acid Protein Assay Reagent Kit (Pierce, Rockford, IL, U.S.A.) with bovine serum albumin as a standard.[14] The microsomal protein concentration in our study was approximately 25 mg/ml.

Automated Synthesis System Preparation of the microsome-immobilized SPE column, glucuronidation of drugs to synthesize drug glucuronides and the elution of products were performed using the automated synthesis system depicted in Fig. 1a. The automated synthesis system consisted of an ASPEC XL SPE system (Gilson, Villiers le Bell, France) equipped with a peristaltic pump, two 10-port switching valves (Gilson), two 3-port switching valves (Takasago Electric, Nagoya, Japan), a thermostatic jacket (M&S Instruments, Osaka, Japan) and a bath circulator (Thermo Fisher Scientific, MA, U.S.A.). All steps were programmed with appropriate software. Only the SPE column must be renewed in every synthesis.

Automated Preparation of Microsome-Immobilized SPE Column and Automated Synthesis of Glucuronides The procedure was conducted at the ambient temperature of 25±1 °C. To prepare SPE column coated with phospholipid (phospholipid-coated SPE column), a solution of 65 mg of DPPC dissolved in 100 ml of 80% methanol was pumped through an SPE column with 500 mg of ODS at a flow rate of 6 ml/min for 12.5 min. After pumping, 30 ml of water was sent through the phospholipid-coated SPE column at a flow rate of 6 ml/min for 5 min to wash the column. The microsome-immobilized SPE column was prepared by recycling a solution of approximately 12.3 mg of microsomes in 30 ml of a 50 mM Tris–hydrochloric acid buffer (pH 7.4) through the phospholipid-coated SPE column at a flow rate of 3 ml/min for 60 min. To load the substrate, 50 ml of 50 mM Tris–hydrochloric acid buffer (pH 7.4) containing 50 μmol of the substrate dissolved in 1.5 ml of *N*,*N*-dimethylformamide or methanol was pumped through the microsome-immobilized SPE column at a flow rate of 3 ml/min for 35 min in a continuous circulation. To synthesize the glucuronides, 5 ml of 50 mM Tris–hydrochloric acid buffer (pH 7.4) containing 50 mM UDPGA and 10 mM magnesium chloride was pumped through the microsome-immobilized SPE column loaded with the substrate at a flow rate of 0.01 ml/min in a continuous circulation. The synthesis was achieved at 37 °C. After the reaction, the desired compound was eluted from the microsome-immobilized SPE column with 10 ml of 70% methanol.

Evaluation of Prepared Microsome-Immobilized SPE Column The amount of DPPC coated on the SPE column was calculated from the difference between DPPC concentrations of the solution before and after the preparation, where the DPPC concentrations were determined by HPLC as described previously.[15]

The amount of immobilized microsomes, estimated as the protein content, on the phospholipid-coated SPE column was calculated from the difference in the concentrations of the solution containing microsomes before and after the preparation, where the concentrations of the solution were determined, as described above, using the Micro Bicinchoninic Acid Protein Assay Reagent Kit.

Evaluation of Glucuronide Production The productivity of glucuronides using the automated synthesis system was calculated from the peak area of the products compared with that of the standard solution of the substrate injected onto the HPLC system under the same analysis conditions. HPLC with photodiode array detection confirmed no significant UV spectral shift between each glucuronide and its substrate. The productivity of glucuronides by using free microsomes was estimated similarly.

Coupling of Automated Preparative HPLC System to Automated Synthesis System to Constitute Fully Automated Synthesis-Isolation System (Fig. 1b) The automated synthesis system was directly connected to an automated preparative HPLC system. The Gilson preparative HPLC system used for the coupling consisted of a model 322-H2 pump and controller, a model 155 UV/Vis detector and a model GX-271 ASPEC autosampler and fraction collector. Chromatographic data were collected using Trilution LH software. The preparative column was a Cadenza 5CD-C18 (100×20 mm i.d., 5 μm). The mobile phases used were 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at a flow rate of 20 ml/min, and the detector was set at a wavelength of 320 nm. The proportion of solvent B was programmed at 10% for 0—3 min, and at 55% for 3—23 min. The composition of solvent B was then programmed to increase to 100% in 1 min and this composition was maintained for an additional 3 min.

NMR Analysis NMR spectra were recorded on a Varian INOVA-500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C) in CD_3OD. ^{1}H and ^{13}C signals were assigned on the basis of the ^{1}H–^{1}H correlation spectroscopy (COSY) and heteronuclear single-quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) spectra, respectively.

Mefenamic Acid 1-*O*-Acylglucuronide (MFAG) ^{1}H-NMR (500 MHz, CD_3OD) δ: 2.14 (3H, s), 2.32 (3H, s), 3.55 (1H, br t, *J*=*ca.* 9 Hz), 3.59 (1H, br t, *J*=*ca.* 8 Hz), 3.60 (1H, br t, *J*=*ca.* 9 Hz), 3.95 (1H, d, *J*=9.2 Hz), 5.78 (1H, d, *J*=7.9 Hz), 6.63 (1H, dd, *J*=8.6, 1.0 Hz), 6.67 (1H, ddd, *J*=8.0, 7.1, 1.0 Hz), 7.05 (1H, m), 7.09 (1H, m), 7.10 (1H, m), 7.27 (1H, ddd, *J*=8.6, 7.1, 1.6 Hz), 8.07 (1H, dd, *J*=8.0, 1.6 Hz). ^{13}C-NMR (125 MHz, CD_3OD) δ: 14.13, 20.65, 73.22, 73.79, 77.79, 77.80, 95.62, 110.89, 114.51, 117.20, 124.76, 127.23, 128.35, 133.02, 133.87, 136.01, 139.46, 139.65, 151.52, 168.45, 173.44. The ^{13}C-NMR data of MFAG prepared in our study were not fully consistent with those reported by Baba and Yoshioka.[5] We believe that the MFAG synthesized by Baba and Yoshioka was the carboxylic acid sodium salt in the glucuronic acid moiety.

Estradiol 3-*O*-Glucuronide ^{1}H-NMR (500 MHz, CD_3OD) δ: 0.77 (3H, s), 1.20 (1H, m), 1.27 (1H, m), 1.30 (1H, m), 1.35 (1H, m), 1.39 (1H, m), 1.45 (1H, m), 1.51 (1H, m), 1.70 (1H, m), 1.88 (1H, m), 1.96 (1H, m), 2.03 (1H, m), 2.16 (1H, m), 2.32 (1H, m), 2.82 (1H, m), 3.47 (1H, m), 3.48 (1H, m), 3.53 (1H, m), 3.66 (1H, t, *J*=8.6 Hz), 3.75 (br d, *J*=*ca.* 9 Hz, 1H), 4.84 (obscured by H_2O), 6.81 (1H, d, *J*=2.2 Hz), 6.81 (1H, dd, *J*=8.6, 2.2 Hz), 7.17 (1H, d, *J*=8.6 Hz). ^{13}C-NMR (125 MHz, CD_3OD) δ: 11.71, 24.07, 27.59, 28.49, 30.70, 30.77, 38.08, 40.43, 44.40, 45.51, 51.38, 73.58, 74.83, 76.98, 77.83, 82.54, 102.90, 115.59, 118.12, 127.20, 135.37, 138.97, 157.12, 175.66.

Estradiol 17-*O*-Glucuronide ^{1}H-NMR (500 MHz, CD_3OD) δ: 0.87 (3H, s), 1.22 (1H, m), 1.30 (1H, m), 1.35 (1H, m), 1.37 (1H, m), 1.43 (2H, m), 1.68 (1H, m), 1.69 (1H, m), 1.86 (1H, m), 2.06 (1H, m), 2.12 (1H, m), 2.14 (1H, m), 2.28 (1H, m), 2.76 (1H, m), 3.23 (1H, dd, *J*=8.8, 7.8 Hz), 3.38 (1H, dd, *J*=9.2, 8.8 Hz), 3.52 (1H, br t, *J*=*ca.* 9 Hz), 3.75 (1H, br d, *J*=*ca.* 10 Hz), 3.78 (1H, t, *J*=8.7 Hz), 4.41 (1H, d, *J*=7.8 Hz), 6.47 (1H, d,

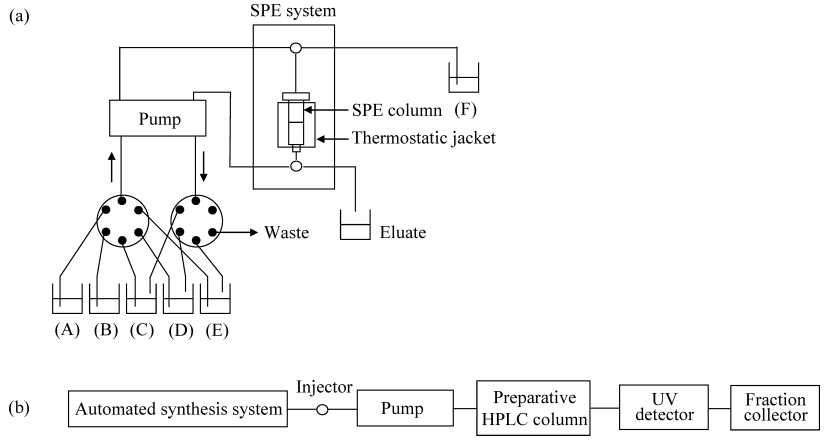

Fig. 1. Schematic Diagram of Automated Synthesis System (a) and Fully Automated Synthesis-Isolation System (b) for Preparation of Glucuronides of Drugs

(A) DPPC solution, (B) water, (C) microsomal solution, (D) substrate solution, (E) coenzyme solution (UDPGA, $MgCl_2$), (F) 70% methanol.

J=2.4 Hz), 6.53 (1H, dd, J=8.4, 2.4 Hz), 7.07 (1H, d, J=8.4 Hz). ^{13}C-NMR (125 MHz, CD_3OD) δ: 12.15, 24.05, 27.66, 28.51, 29.90, 30.76, 38.74, 40.38, 44.65, 45.35, 51.15, 73.32, 75.16, 76.79, 77.73, 90.40, 105.17, 113.78, 116.09, 127.27, 132.65, 138.84, 155.94, 173.00.

Flufenamic Acid 1-*O*-Acylglucuronide ^{1}H-NMR (500 MHz, CD_3OD) δ: 3.57 (2H, m), 3.58 (1H, m), 3.86 (1H, br d, J=*ca.* 8 Hz), 5.77 (1H, d, J=7.1 Hz), 6.88 (1H, br t, J=*ca.* 8 Hz), 7.29 (1H, d, J=8.5 Hz), 7.33 (1H, br d, J=*ca.* 8 Hz), 7.44 (1H, m), 7.49 (1H, m), 7.50 (1H, m), 7.52 (1H, br t, J=*ca.* 9 Hz), 8.13 (1H, dd, J=8.0, 1.4 Hz). ^{13}C-NMR (125 MHz, CD_3OD) δ: 73.41, 73.80, 77.03, 77.88, 95.78, 114.09, 115.78, 118.62, 119.78, 120.52, 125.56 (q, J=270.6 Hz), 125.73, 131.47, 132.88 (q, J=32.8 Hz), 133.39, 135.95, 143.30, 148.18, 168.12, 175.21.

Results and Discussion

Evaluation of Preparation of Microsome-Immobilized SPE Column The amount of DPPC on the SPE column and the amount of immobilized microsomes on the phospholipid-coated SPE column were calculated to be approximately 45 mg and approximately 3 mg as the protein content, respectively. The microsome-immobilized SPE column could be readily prepared by allowing a solution containing the desired ligands to flow through the SPE column.

Automated Synthesis of Mefenamic Acid 1-*O*-Acylglucuronide We synthesized mefenamic acid 1-*O*-acylglucuronide (MFAG) using the automated synthesis system. After the reaction, the eluate from the microsome-immobilized SPE column was injected onto the HPLC system. The peak corresponding to the acylglucuronide was detected around a retention time of 3 min, while the peak of mefenamic acid was detected around a retention time of 11 min (data not shown). The peaks of mefenamic acid and its acylglucuronide could be completely separated under the present HPLC conditions. The product obtained from the fraction solution corresponding to the peak of the acylglucuronide was identified by LC/MS and NMR. Analysis by LC/MS of the product revealed the presence of molecular ions of m/z 242 $[M-176+H]^+$ and m/z 418 $[M+H]^+$, corresponding to the molecular weights of mefenamic acid and its acylglucuronide, respectively.

Figure 2 shows the chemical structure of MFAG. The ^{1}H- and ^{13}C-NMR chemical shifts of synthesized MFAG by the automated synthesis system are given in Experimental. The ^{1}H signals of δ 5.78 (d), δ 3.59 (br t), δ 3.55 (br t), δ 3.60 (br t) and δ 3.95 (d) were assigned to H-1″, H-2″, H-3″, H-4″ and H-5″ of the glucuronic acid moiety, respectively. The H-1″ anomeric proton at 5.78 ppm had a coupling constant of 7.9 Hz, which showed the characteristic diaxial coupling constant between H-1″ and H-2″ in the β-glucuronic acid moiety.[16,17] Also, a long-range ^{1}H–^{13}C correlation, which was observed between δ 5.78 (H-1″) and 168.45 (C-1 of the mefenamic acid moiety) on the HMBC spectrum proved that C-1 of mefenamic acid was linked to C-1″ of β-glucuronic acid through the oxygen atom.[16] As a result, the chemical structure of MFAG synthesized using the automated synthesis system could be completely identified by using LC/MS and NMR.

Evaluation of MFAG Productivity Figure 3 shows the relationship between the enzymatic reaction time and the productivity of MFAG. The productivity of MFAG increased with increasing the flow time of the coenzyme solution, reaching a plateau when the enzymatic reaction time was more than 32 h. As a result, the enzymatic reaction time was set at 32 h for the optimum productivity of glucuronidation.

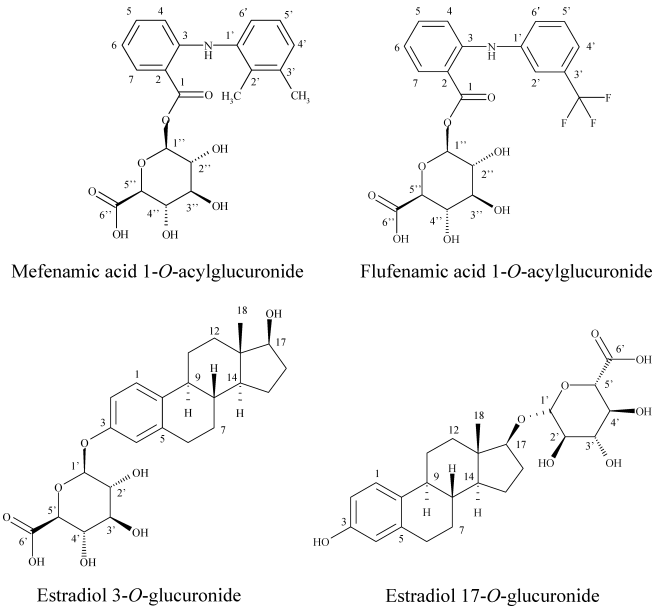

Fig. 2. Chemical Structures of Four Glucuronides Prepared in This Study

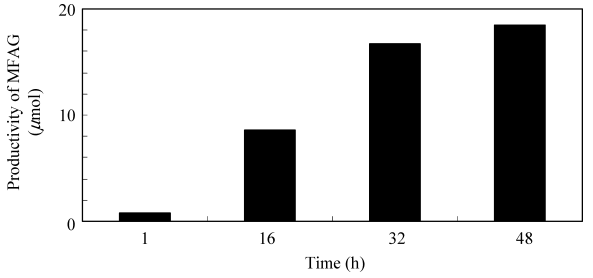

Fig. 3. Relationship between Enzymatic Reaction Time and Productivity of MFAG

UDP-glucuronosyltransferase was proven to be reasonably stable during the reactions as the productivity of MFAG increased proportionally with time up to 32 h. The stability of UDP-glucuronosyltransferase was retained due to the immobilization of microsomes on the phospholipid coating ODS particle, as described previously.[12]

Automated Synthesis of Two Estradiol Ether Glucuronides Using β-CD as Solubilizing Agent Estradiol is practically insoluble in aqueous solutions. We used β-CD as a solubilizing agent for the synthesis of two estradiol ether glucuronides. One hundred micromoles of β-CD were added to 50 ml of 50 mM Tris–hydrochloric acid buffer (pH 7.4) containing 50 μmol of estradiol dissolved in 1.5 ml of methanol. The amount of estradiol dissolved with β-CD was approximately 37-fold larger than that observed without β-CD.

After the reaction, the eluate obtained from the microsome-immobilized SPE column was injected onto the HPLC system, then the peak fractions corresponding to two ether glucuronides were collected, and the solvent of the fractions was evaporated and the residues were lyophilized. The two products were identified by using LC/MS and NMR. The analysis by LC/MS of the two products revealed the presence of the molecular ion of m/z 447 $[M-H]^-$, corresponding to the molecular weight of estradiol glucuronides.

Figure 2 shows the chemical structure of estradiol 3-*O*-

glucuronide (E3G) and estradiol 17-*O*-glucuronide (E17G). The ^{1}H- and ^{13}C-NMR chemical shifts of two synthesized glucuronides using the automated synthesis system are given in Experimental. The ^{1}H signals of E17G of δ 4.41 (d), δ 3.23 (dd), δ 3.38 (dd), δ 3.52 (br t) and δ 3.75 (br d) were assigned to H-1′, H-2′, H-3′, H-4′ and H-5′ of the glucuronic acid moiety, respectively. The H-1′ anomeric proton at 4.41 ppm had a coupling constant of 7.8 Hz, which showed the characteristic diaxial coupling constant between H-1′ and H-2′ in the β-glucuronic acid moiety.[16,17] Also, a long-range ^{1}H–^{13}C correlation which was observed between δ 4.41 (H-1′) and 90.40 (C-17 of the estradiol moiety) on the HMBC spectrum proved that the C-17 of estradiol was linked to C-1′ of β-glucuronic acid through the oxygen atom.[16] Similarly, a long-range ^{1}H–^{13}C correlation of E3G was observed between δ 4.84 (H-1′) and 157.12 (C-3 of the estradiol moiety) through the oxygen atom. As a result, the chemical structures of E3G and E17G synthesized using the automated synthesis system could be completely identified by using LC/MS and NMR.

Evaluation of Productivity of E3G and E17G Figure 4 shows the relationship between the enzymatic reaction time and the productivity of E3G and E17G. The productivity of E3G and E17G increased with increasing the flow time of the coenzyme solution. Twenty three micromoles of estradiol loaded on the microsome-immobilized SPE column almost completely underwent the reaction, when the enzymatic reaction time was set at 24 h.

Comparison of Productivity of Glucuronidation with Free Microsomes The productivities of glucuronides of mefenamic acid and estradiol using the microsome-immobilized SPE column were compared to those observed with free microsomes (batch method) under the same reaction conditions. Figure 5 shows that the productivities of MFAG, E3G and E17G using the microsome-immobilized SPE column were higher than those observed with the free microsomes. It was previously assumed that the difference of the glucuronide productivity between the microsome-immobilized particles and the free microsomes would be caused by the difference of the concentration of substrate in the enzymatic reaction mixture.[12] In the synthesis of glucuronides with free microsomes, the concentration of substrate in the mixture should be lower in order to keep the enzyme activity higher. On the other hand, in the case of the microsome-immobilized SPE column, the glucuronidation could be efficiently effected with excessive substrate, because it was loaded on the SPE column without inhibiting the enzyme activity. As a result, the productivities of MFAG, E3G and E17G with the microsome-immobilized SPE column were higher as compared to those obtained with the same protein content of free microsomes. These findings indicate that the immobilized microsomes on the microsome-immobilized SPE column could efficiently achieve the synthesis of glucuronides, even when there was an excessive substrate.

Fully Automated Synthesis-Isolation of Flufenamic Acid 1-*O*-Acylglucuronide A schematic diagram of the fully automated synthesis-isolation system is depicted in Fig. 1b. The automated synthesis system was directly connected to an automated preparative HPLC system. We prepared the flufenamic acid 1-*O*-acylglucuronide (FFAG) using this fully automated synthesis-isolation system. After the reaction, the eluate obtained from the microsome-immobilized SPE column was automatically injected onto the automated preparative HPLC system, and the peak fraction corresponding to the acylglucuronide was collected using a UV trigger. The solvent of the fraction was evaporated and the residue was lyophilized. The product was identified by using LC/MS and NMR. The analysis by LC/MS of the product revealed the presence of the molecular ion of *m*/*z* 458 $[M+H]^{+}$, corresponding to the molecular weight of FFAG.

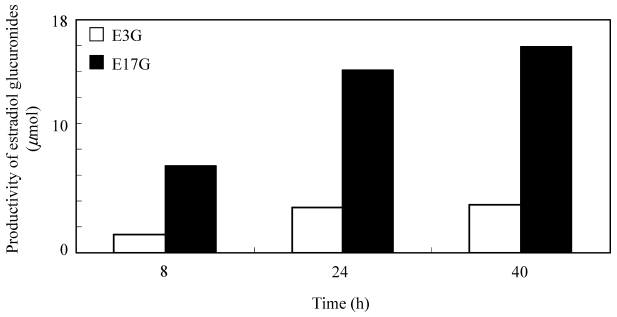

Fig. 4. Relationship between Enzymatic Reaction Time and Productivity of E3G and E17G

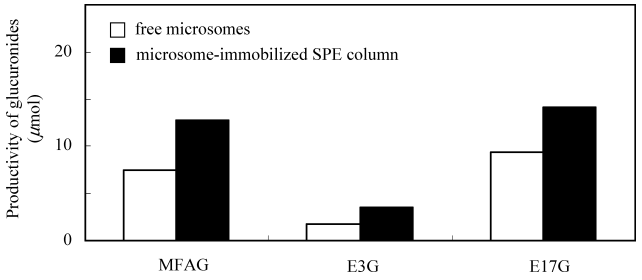

Fig. 5. Comparison of the Productivities of MFAG, E3G and E17G by the Use of Microsome-Immobilized SPE Column and Free Microsomes (Batch Method)

Microsome-immobilized SPE column: mefenamic acid; substrate loaded on the column, 38.5 μmol; estradiol; substrate loaded on the column, 23.0 μmol; immobilized microsomal protein, 3.07 mg. Free microsomes (batch method): mefenamic acid; substrate added to the enzymatic reaction mixture, 38.5 μmol; estradiol; substrate added to the enzymatic reaction mixture, 23.0 μmol; microsomal protein, 3.07 mg. Enzymatic reaction mixture: 5 ml of 50 mM Tris–hydrochloric acid buffer (pH 7.4) containing 50 mM UDPGA and 10 mM magnesium chloride; reaction conditions, 37 °C, 24 h.

Figure 2 shows the chemical structure of FFAG. The ^{1}H- and ^{13}C-NMR chemical shifts of FFAG synthesized by the fully automated synthesis-isolation system are given in Experimental. The ^{1}H signals of δ 5.77 (d), δ 3.58 (m), δ 3.57 (m), δ 3.57 (m) and δ 3.86 (br d) were assigned to H-1″, H-2″, H-3″, H-4″ and H-5″ of the glucuronic acid moiety, respectively. The H-1″ anomeric proton at 5.77 ppm had a coupling constant of 7.1 Hz, which showed the characteristic diaxial coupling constant between H-1″ and H-2″ in the β-glucuronic acid moiety.[16,17] Also, a long-range ^{1}H–^{13}C correlation which was observed between δ 5.77 (H-1″) and 168.12 (C-1 of the flufenamic acid moiety) on the HMBC spectrum proved that C-1 of flufenamic acid was linked to C-1″ of β-glucuronic acid through the oxygen atom.[16] As a result, the chemical structure of FFAG synthesized using the fully automated synthesis-isolation system could be completely identified by using LC/MS and NMR.

The productivity of FFAG was approximately 8.8 μmol using the fully automated synthesis-isolation system (in the presence of 50 mM UDPGA, at 37 °C for 24 h).

Conclusion

We have developed an automated synthesis system for obtaining authentic samples of glucuronides, using a microsome-immobilized SPE column and SPE system. By using the automated synthesis system, the acylglucuronide of mefenamic acid and two ether glucuronides of estradiol could be easily synthesized. Furthermore, estimation of the glucuronidation productivity showed an advantage of the method using the microsome-immobilized SPE column over that using free microsomes. We used β-CD as a solubilizing agent for the synthesis of the glucuronides of estradiol that is practically insoluble in aqueous solutions. The usage of β-CD means that this system could accept a water-insoluble substrate. Furthermore, we coupled the automated synthesis system to an automated preparative HPLC system, an on-line system, to constitute a fully automated system. This allows easy operation to obtain the pure product from the synthetic mixture. We have concluded that the automated synthesis system as well as the fully automated synthesis-isolation system would be helpful in drug development stages for obtaining authentic samples of acylglucuronides, ether glucuronides or other metabolites of drugs that are slightly soluble in aqueous solutions or those hardly synthesized by chemical methods.

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