Glucuronidation of (*R,R*)-Formoterol and (*S,S*)-Formoterol is Catalyzed by cDNA-expressed Human Uridine 5´-Diphospho-Glucuronosyltransferases and Human Liver Microsomes

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Abstract

(*R,R*)-Formoterol, a highly selective, potent and long-acting β_2-adrenoceptor agonist, is primarily metabolized to phenolic (**M1**) and benzylic (**M2**) glucuronides in man. The glucuronidation of formoterol enantiomers with 12 cDNA-expressed BD Supersomes™ Enzymes (Baculovirus-infected insect cells/Insect cell system) and human liver microsomes (HLMs) are reported. Incubations of either (*R,R*)- and (*S,S*)-formoterol with HLMs produced two glucuronide metabolites (**M1** and **M2**). The apparent K_m and V_{max} of the (*R,R*)- and (*S,S*)-formoterol metabolite **M1** in HLMs were 1410 and 864 µM and 788 and 2216 pmol/mg/min, respectively. For (*R,R*)-formoterol, UGT2B17 was the most active cDNA-expressed human UGT isozyme followed by 1A9, 2B7, and 1A1 in decreasing order of activity. UGT1A1 was the most active isozyme studied for (*S,S*)-formoterol glucuronidation. *In vitro*, bilirubin inhibited direct glucuronidation of both (*R,R*)- and (*S,S*)-formoterol (**M1** and **M2**), indicating some involvement of UGT1A1 in the reactions. Despite substantial quantitative and qualitative differences in glucuronidation activity, (*R,R*)- and (*S,S*)-formoterol were found to be substrates for most of the UGT isozymes examined.

Introduction

(*R,R*)-Formoterol (***Figure 1***) is a highly selective, potent, and long-acting β_2-adrenoceptor agonist used clinically as a bronchodilator. Formoterol fumarate is marketed in the US and Europe as the racemic mixture of (*R,R*)- and (*S,S*)-formoterol. Formoterol is primarily metabolized to phenolic (**M1**) and benzylic (**M2**) glucuronides[1,2]. Uridine 5´-diphospho-glucuronosyltransferases (UGTs) comprise a superfamily of membrane-bound enzymes localized in the liver and extra-hepatic tissues (kidney and intestine). These enzymes catalyze the formation of glucuronides of endogenous (*e.g.*, steroids and bilirubin) and xenobiotic (*e.g.*, drugs and carcinogens) chemical compounds containing hydroxyl, amine, sulfhydryl, acetylenic, and carboxylic acid functional groups. Glucuronidation is a major metabolic pathway for the inactivation and excretion of many pharmaceutical products currently in use. UGT enzymes have been categorized into two major families, designated 1A and 2B, based on evolutionary divergence of their gene structures. To date, at least 16 human UGT enzymes have been cloned and sequenced, and several of their cDNA's have been heterologously expressed in a cell system to demonstrate substrate specificity. Like P450s, individual UGTs have unique, yet overlapping substrate specificities[3]. The UGT-mediated metabolism investigated in this report is illustrated in ***Figure 1***.

Methods

Chemicals and Enzymes

Pooled human liver microsomes (HLMs) or microsomes from baculovirus-infected insect cells (BD Supersomes™ Enzymes) were supplied from BD Biosciences (Woburn, MA). Bilirubin, estradiol, trifluoperazine, 7-hydroxy-4-trifluoromethylcoumarin (7-HFC), eugenol, and other chemicals (reagent grade) were obtained from Sigma-Aldrich (St. Louis, MO). Radiolabeled (*R,R*)- and (*S,S*)-formoterol were provided by Sepracor Inc.

Enzyme Assays

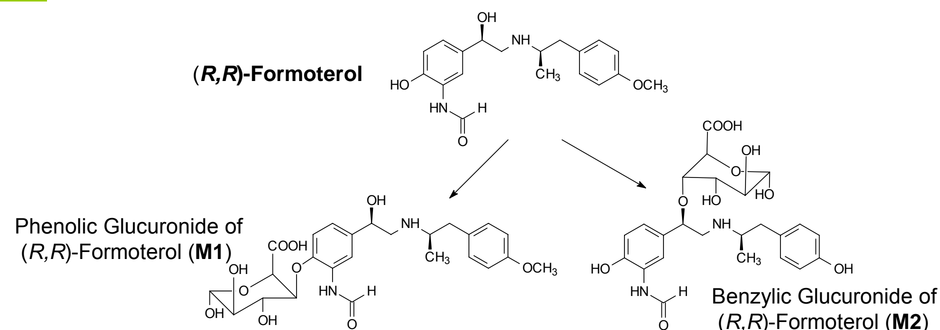
(*R,R*)- and (*S,S*)-Formoterol glucuronidation: The incubation mixture contained 50 mM Tris-HCl buffer (pH 7.5), 2 mM UDPGA, 10 mM $MgCl_2$, 25 µg/mL alamethicin, (*R,R*)- or (*S,S*)-formoterol, and HLMs or UGT protein. The final incubation volume was 0.2 mL. Incubations conducted at 37°C were initiated by the addition of UGTs or HLMs protein and were terminated by the addition of 0.1 mL of acetonitrile containing 6% acetic acid. Protein was precipitated by centrifugation, and a portion of the supernatant (100 µL) was injected onto an HPLC system. The metabolites were separated using a mobile phase of 100 mM ammonium acetate (pH 5.0) and methanol. The response was quantitated using a liquid scintillation detector.

Inhibition of (*R,R*)- and (*S,S*)-Formoterol Glucuronidation by Bilirubin

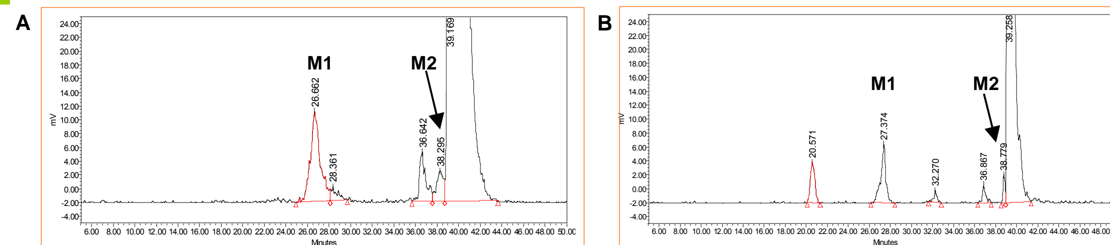
(*R,R*)- and (*S,S*)-Formoterol, individually, and as the (R,R/S,S)-racemic mixture were incubated in the presence or absence of 20 µM bilirubin, and the activity was determined as described above.

Results

1 (*R,R*)-Formoterol Glucuronide Metabolites M1 and M2

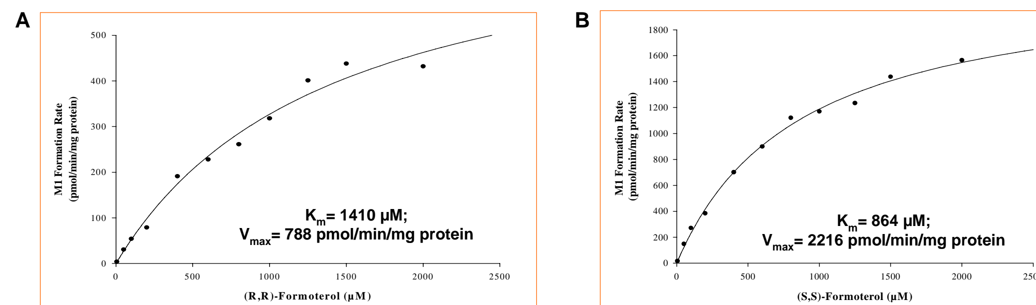


2 HPLC Radiochromatograms of [3H]-(*R,R*)- and [3H]-(*S,S*)-Formoterol Metabolism in HLMs



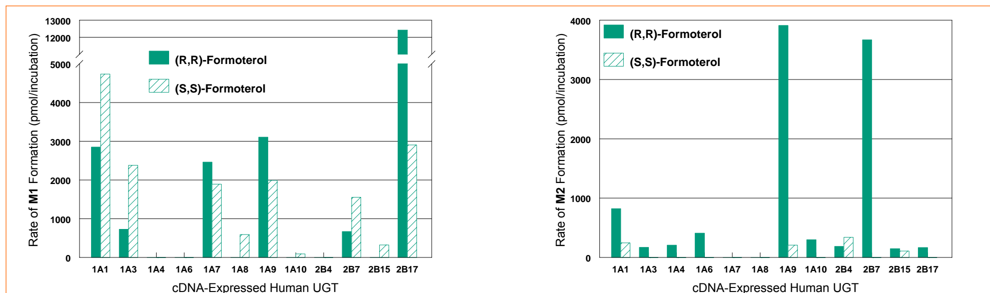
A:[3H]-(*R,R*)- and **B**:(*S,S*)-Formoterol (1.5 mM) were incubated with HLMs (1.2 mg/mL for 120 minutes and 0.6 mg/mL for 30 minutes, respectively). The retention time of metabolites **M1** and **M2** were ~27 and 38 minutes, respectively. The peak eluting at ~20.6 minutes was a contaminant in (*S,S*)-formoterol.

3 Kinetic Analysis of (*R,R*)- and (*S,S*)-Formoterol Glucuronidation in HLMs



Formation of **M1** by **A**: [3H]-(*R,R*)- and **B**: [3H]-(*S,S*)-formoterol by HLMs as a function of substrate concentration.

4 Formation of Formoterol Glucuronides M1 and M2 by cDNA-Expressed Human UGTs



(*R,R*)- and (*S,S*)-Formoterol (2 mM) were incubated with UGTs (1.2 mg protein/mL) in the presence of UDPGA for 180 minutes.

T1

Kinetic Parameter	Formoterol Enantiomer	HLM			UGT1A1			UGT1A9			UGT2B17		
		M1	M2	M1+M2	M1	M2	M1+M2	M1	M2	M1+M2	M1	M2	M1+M2
K_m (µM)	(*R,R*)-	1410	6874	2224	918	750	1009	937	1053	1007	252	4686	284
	(*S,S*)-	864	NC*	1032	1244	2297	1417	1022	2193	1634	184	780	219
V_{max} (pmol/min/ mg protein	(*R,R*)-	788	1340	1607	56	27	86	88	160	247	341	75	369
	(*S,S*)-	2216	NC*	2613	155	36	192	72	25	117	97	58	141

* NC - Parameter not calculated as activity was not saturable.

Table 1A. Kinetic parameters for the metabolism of (*R,R*)- and (*S,S*)-formoterol by HLMs and selected cDNA-expressed human UGTs.

UGT System	Substrate	Intrinsic Clearance* (µL/min/mg protein)		
		M1	M2	M1+ M2
1A1	(*R,R*)-formoterol	0.06	0.04	0.09
	(*S,S*)-formoterol	0.12	0.02	0.14
1A9	(*R,R*)-formoterol	0.09	0.15	0.25
	(*S,S*)-formoterol	0.07	0.01	0.07
2B17	(*R,R*)-formoterol	1.35	0.02	1.30
	(*S,S*)-formoterol	0.53	0.07	0.64
HLMs	(*R,R*)-formoterol	0.56	0.19	0.72
	(*S,S*)-formoterol	2.56	NC†	2.53

*Intrinsic Clearance = V_{max}/K_m; † Not calculable.

Table 1B. *In vitro* intrinsic clearance estimates for metabolites M1 and M2.

T2

Formoterol Enantiomer	Bilirubin (20 µM)	[S] (mM)	Metabolism (pmol/incubation)		Inhibition (%)	
			M1 (Mean)	M2 (Mean)	M1	M2
(*R,R*)-	-	1	4638	1395		
(*R,R*)-	+	1	2937	1019	36.7	26.9
(*R,R*)-	-	2	5854	2457		
(*R,R*)-	+	2	3700	1960	36.8	20.2
(*S,S*)-	-	1	1490	184		
(*S,S*)-	+	1	954	138	35.9	24.9
(*S,S*)-	-	2	1776	398		
(*S,S*)-	+	2	1635	435	6.9	-9.2
(*R,R/S,S*)-	-	2	7551	1197		
(*R,R/S,S*)-	+	2	5408	805	28.4	32.7
(*R,R/S,S*)-	-	4	6177	1056		
(*R,R/S,S*)-	+	4	4415	1347	28.5	-27.6

Table 2A. Bilirubin inhibition of [3H]-(*R,R*)-formoterol, [3H]-(*S,S*)-formoterol and racemic [3H]-formoterol metabolism in HLMs.

Bilirubin (20 µM)	Mean Activity (pmol/incubation)	Inhibition (%)
+	285	63
-	774	0

Table 2B. UGT1A1 Inhibition Positive Control. [3H]-(*S,S*)-formoterol (HLMs: 0.6 mg/mL) and [3H]-(*R,R*)- and [3H]-(*R,R/S,S*)-formoterol (HLMs: 1.2 mg/mL) were incubated in the presence or in the absence of bilirubin for 30 or 120 minutes, respectively. Estradiol (10 µM) was incubated with HLMs (0.5 mg/mL) for 30 minutes.

Discussion

Formoterol is primarily eliminated through direct glucuronidation. The major metabolites **M1** and **M2** are shown in ***Figure 1*** with representative radiochromatograms displayed in ***Figure 2***. Both (*R,R*)- and (*S,S*)-formoterol were metabolized by HLMs to the respective **M1** and **M2** metabolites; formation rates of the major metabolite, **M1,** is shown in ***Figure 3***. The kinetic parameters of the formation of both **M1** and **M2** in HLMs are presented in ***Table 1A***. Intrinsic clearance estimates for **M1** and **M2** in HLMs are shown in ***Table 1B***. In HLMs, (*S,S*)-formoterol appears to be glucuronidated faster than (*R,R*)-formoterol.

Multiple cDNA-expressed UGTs metabolized both (*R,R*)-and (*S,S*)-formoterol enantiomer into **M1** and **M2** glucuronides (***Figure 4***). (*R,R*)-Formoterol forms substantial amounts of both **M1** and **M2** metabolites whereas, (*S,S*)-formoterol formed primarily the **M1** glucuronide. The **M1** metabolite of (*R,R*)-formoterol is formed primarily by UGT2B17 followed by 1A9, 1A1, and 1A7, whereas, **M2** is formed primarily by 1A9 and 2B7. Metabolite **M1** of (*S,S*)-formoterol is formed predominantly by UGT1A1 followed by 2B17, 1A3, 1A7, 1A9, and 2B7. The UGTs 1A, 1A9, and 2B4 are responsible for (S,S)-formoterol **M2** formation, although the amount of this metabolite is minor.

Since UGT1A1, 1A9, and 2B17 were involved in the metabolism of both (*R,R*)- and (*S,S*)-formoterol, the catalytic activity of these isozymes were further characterized. The glucuronidation reactions with these UGT isozymes and HLMs followed normal Michaelis-Menton kinetics as shown in ***Table 1A***. The intrinsic clearance estimates for three cDNA-expressed human enzymes and HLMs are presented in ***Table 1B***.

Bilirubin, a chemical inhibitor and a specific substrate for UGT1A1 inhibited glucuronide formation of (*R,R*)-, (*S,S*)-, and (*R,R/S,S*)-formoterol in HLMs by approximately 30%, confirming some contribution of UGT1A1 in the metabolism (***Table 2A***). The control reaction, UGT1A1-mediated estradiol glucuronidation, was inhibited (63%) by 20 µM bilirubin (***Table 2B***).

Despite substantial qualitative and quantitative differences in the glucuronidation activity, both (*R,R*)- and (*S,S*)-formoterol were found to be substrates for most of the UGT isozymes examined.

Acknowledgement
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References

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