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STEREOSELECTIVE FORMATION OF FENOTEROL-*PARA*-GLUCURONIDE AND FENOTEROL-*META*-GLUCURONIDE IN RAT HEPATOCYTES AND ENTEROCYTES

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Abstract—The glucuronidation of fenoterol (Berotec®, Partusisten®) in isolated rat hepatocytes and enterocytes was investigated. Two different glucuronides, fenoterol *para*-glucuronide and fenoterol *meta*-glucuronide, were formed in proportions, that were constant over the concentration range investigated (0–1 mM). The fraction of *para*-glucuronide formed was 0.40 ± 0.01 for hepatocytes and 0.54 ± 0.01 for enterocytes. Fenoterol consists of a racemic mixture of *SS'*(+)fenoterol and *RR'*(−)fenoterol. The maximum glucuronidation rate of the (−)enantiomer (V_{max} = 3.6 ± 0.3 nmol/min/mg in hepatic microsomes and 3.4 ± 0.1 nmol/min/mg in intestinal microsomes) is significantly lower than the same values of the (+)isomer (V_{max} = 6.7 ± 0.8 nmol/min/mg in hepatic microsomes and 5.8 ± 0.4 nmol/min/mg in intestinal microsomes). K_m^{app}-values for the (−)enantiomer were lower than for the (+)enantiomer. Similar, but less pronounced, differences in V_{max} were observed in isolated cells: V_{max} = 148 ± 13 and 372 ± 50 pmol/min/mg [(−)fenoterol in hepatocytes and enterocytes], V_{max} = 173 ± 12 and 444 ± 57 pmol/min/mg [(+)fenoterol in hepatocytes and enterocytes]. Calculation of intrinsic metabolic clearance ($Cl_{int} = V_{max}/K_m^{app}$) from the cellular data suggests that the (+)enantiomer may be more efficiently eliminated by liver metabolism *in vivo* than the (−)enantiomer. This can result in stereoselective first-pass metabolism of the fenoterol enantiomers.

The β_2-sympathomimetic fenoterol (Berotec®, Partusisten®) is almost exclusively metabolized by glucuronidation in the rat [1]. In addition to the liver, fenoterol is efficiently glucuronidated in intestinal microsomes and in mucosal cells [2, 3]. After oral administration very substantial intestinal as well as liver first-pass effects are observed as a consequence [1, 4, 5]. The chemical structure of fenoterol (Fig. 1) suggests that at least two different phenolic glucuronides can be formed. Both glucuronides have been identified in rat urine [6] but the contribution of both routes of conjugation to the total metabolism is not known. We, therefore, decided to investigate the formation of FPG‡ and FMG in isolated hepatocytes and enterocytes.

The structure of fenoterol has two asymmetric C-atoms (Fig. 1). One pair of enantiomers, designated as Th1179, is 9–20 times less active than the other pair [7, 8]. The pharmacologically active fenoterol (Th1165) consists of a racemic mixture of *RR'*(−)-fenoterol and *SS'*(+)-fenoterol. In analogy to other adrenergic agonists, the *RR'*(−)enantiomer represents the pharmacologically active component [8]. Usually only plasma levels of the racemate are measured in pharmacokinetic experiments [1, 5, 9] and it is assumed that the data obtained are representative for the active enantiomer. However, stereoselective kinetics have been observed *in vivo* for a number of compounds [10–13]. Relatively small differences in the rate of metabolism of two enantiomers may have important consequences for the oral

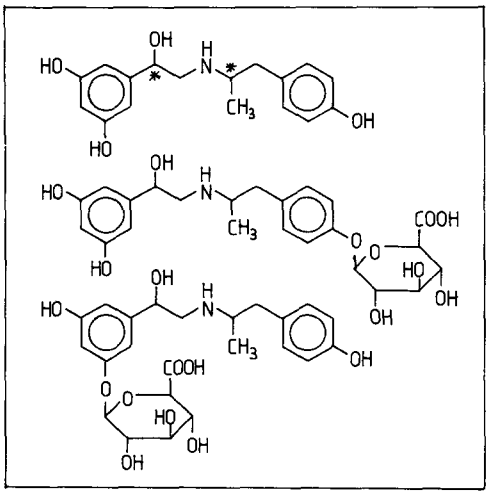

Fig. 1. Structural formulae of fenoterol (top), FPG (middle) and FMG (bottom). The optical active C-atoms are indicated by an asterisk.

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‡ Abbreviations used: AUC, area under the plasma concentration vs time curve; Cl_{int}, intrinsic (metabolic) clearance; (+)F, *SS'*(+)-fenoterol·palmitate; (−)F, *RR'*(−)-fenoterol·palmitate; (±)F, fenoterol·HBr; FMG, fenoterol *meta*-glucuronide; FPG, fenoterol *para*-glucuronide; UDPGT, UDP-glucuronosyltransferase (EC 2.4.1.17).

availability of the enantiomers, especially for high-extraction drugs [11, 14]. It is therefore questionable if the oral availability of the racemate of fenoterol [5] represents the oral availability of the pharmacologically active enantiomer [4]. Because it has been demonstrated that stereoselective glucuronidation plays an important role in the stereoselective kinetics of oxazepam [12] and propranolol [13, 15, 16], it seems appropriate to study the glucuronidation of fenoterol enantiomers in more detail. The results indicate that (+)F is about twice as efficiently glucuronidated as (−)F in hepatic as well as intestinal microsomes. In hepatocytes and enterocytes the difference is less pronounced but statistically significant different maximum glucuronidation rates are observed nevertheless.

MATERIALS AND METHODS

Chemicals. UDP-glucuronic acid (sodium salt) was obtained from Sigma. Brij-58 (polyoxyethyle(20) cetyl ether) was purchased from Janssen Chimica (Beerse, Belgium) and bovine serum albumin from Poviet (Oss, The Netherlands). Racemic fenoterol·HBr (charge no. 411631) was kindly donated by Boehringer-Ingelheim (Alkmaar, The Netherlands). For the synthesis of *SS*(+)-fenoterol palmitate and *RR'*(−)-fenoterol·palmitate 3,5-dibenzyloxyacetophenone was oxidized to 3,5-dibenzyloxyphenylglyoxal. The subsequent reaction with 4-hydroxy-α-methyl-phenethylamine and reduction with sodiumborohydride yielded (1-(3,5-dibenzyloxyphenyl) - 2 - (4 - hydroxy - α - methyl - phenethylamino)-ethanol as a mixture of the two diastereomers (*RR'*, *SS'*/*RS'*, *R'S*) [17]. A solution of 36 g maleic acid in 300 ml ethyl acetate was added to a solution of 148 g of the diastereomers in 1500 ml ethyl acetate. The maleates of the *RR'* and *SS'*-enantiomers crystallized (55 g, Fp. 136° [acetonitrile]). A suspension of the maleates in water was treated with ammonia and the base was extracted with ethyl acetate. A solution of 12.5 g base of the *RR'* and *SS'* enantiomers in a mixture of 70 ml isopropanol and 10 ml water was heated to 80° and 10.5 g (−)0,0'-di-*p*-toluoyl-L-tartaric acid were added. During the first day fraction A crystallized (5.6 g). After standing for several days fraction B was isolated (4.0 g). The compound A was recrystallized from a mixture of 90 ml isopropanol and 10 ml water. The free base was liberated and the *SS'*(+)-1-(3,5-dibenzyloxyphenyl)-2-(4-hydroxy-α-methyl-phenethyl-amino)-ethanol·hydrochloride (3.0 g, Fp. 164°, $[\alpha]_{20}^{D} = +32.8°$ in methanol) was isolated. 2.6 gram of this product was converted into the free base and hydrogenated with Pd/c as a catalyst in 300 ml methanol. After removal of the methanol the base was resolved in acetonitrile and the palmitate of *SS'*(+)-fenoterol (2.1 g, Fp. 50°, $[\alpha]_{20}^{D} = +23.1°$ in methanol) was isolated. The product B was recrystallized from isopropanol and the base liberated. The hydrochloride of *RR'*(−)-1-(3,5-dibenzyloxy-phenyl-2-(4-hydroxy-α-methylphenethylamino)-ethanol (2.5 g, Fp. 163°, $[\alpha]_{20}^{D} = -31.8°$ in methanol) was isolated and hydrogenated in the same manner as described for the product A. The *RR'*(−)-fenoterol·palmitate was isolated in a yield of 0.8 g (Fp. 55–57°, $[\alpha]_{20}^{D} = -23.1°$ in methanol). FPG and FMG (Fig. 1), used to calibrate the HPLC-analysis, were isolated from rat urine using ion-exchange and thin-layer chromatography and characterized by mass spectrometry as described before [6]. All other chemicals were of analytical grade purity and used as supplied.

Animals. Adult male Wistar rats (Cpb:WU)(200–250 g) were obtained from T.N.O. (Zeist, The Netherlands) and allowed free access to water and a commercially available diet (Muracon-1, Trouw, Putten, The Netherlands).

Isolation of hepatocytes, enterocytes and microsomes. Hepatocytes were isolated by collagenase perfusion according to Seglen [18] as modified by Paine *et al.* [19]. Mucosal cells from the small intestine were isolated by vibration in EDTA-containing phosphate buffered saline (90 mM NaCl, 5 mM EDTA-Na_2, 9 mM KH_2PO_4, 34 mM Na_2HPO_4, pH 7.4) as described before [20–22]. Hepatic and intestinal microsomes were prepared from total liver homogenate and isolated enterocytes [20, 23].

Incubation of cells and microsomes. Cells were incubated in polypropylene incubation vessels in a shaking water bath (60 cycles/min) at 37°. Each incubation contained 1.7×10^6 hepatocytes or enterocytes obtained from 0.17 g intestine/ml (2–3 mg cell protein/ml) in a final volume of 1 ml (hepatocytes) or 3 ml (enterocytes) Krebs–Ringer bicarbonate buffer [20] gassed with carbogen gas (95% O_2/5% CO_2, v/v, pH = 7.4 after saturation). After a pre-incubation period (10 min) the reaction was started by adding substrate (0–1 mM final concentration). Cells were incubated for 30 min. Microsomes were incubated in a shaking waterbath (90 cycles/min) at 37° in phosphate-buffer (final concentrations: 9 mM KH_2PO_4, 34 mM $Na_2HPO_4 \cdot 2H_2O$, 0.1 mM EDTA-Na_2, pH 7.4). Each incubation (final volume 0.6 ml) contained 0.2–0.4 mg microsomal protein, 10 mM $MgCl_2$ and 3 mM UDP-glucuronic acid. Microsomes were maximally activated with Brij-58 (1 mg/mg protein). After a pre-incubation period (10 min) the reaction was started by adding substrate (0–200 μM final concentration). Microsomes were incubated for 10 min. All reactions were stopped by adding a threefold volume of 0.5 M ammoniumsulphate (pH 9.3) to the incubation mixture.

Analytical methods. FPG, FMG and fenoterol were extracted using SepPak-C18 cartridges (Waters, Etten-Leur, The Netherlands) [3]. The reaction mixture was poured over the cartridge which had been equilibrated with 5 ml methanol and 5 ml demineralized water. The cartridge was washed with 10 ml of 5 mM ammonium sulphate (pH 9.3) and 0.5 ml demineralized water and FPG, FMG and fenoterol were eluted with 3 ml of a mixture of phosphate buffer (10 mM NaH_2PO_4, brought to pH 2.5 with orthophosphoric acid) and acetonitrile (90/10, v/v). A series of FPG and FMG standards was included in each experiment. Samples (30 μl) were injected on a Hewlett-Packard 1084B-liquid chromatograph and separated as follows: 10RP18-column, 10 mM phosphate buffer pH 2.5/acetonitrile (70/30, v/v) containing 1 mM SDS, flow 1.5 ml/min, detection at 212 nm (Pye Unicam LC-UV detector). Typical retention times are 4.9 (FPG), 6.1 (FMG)

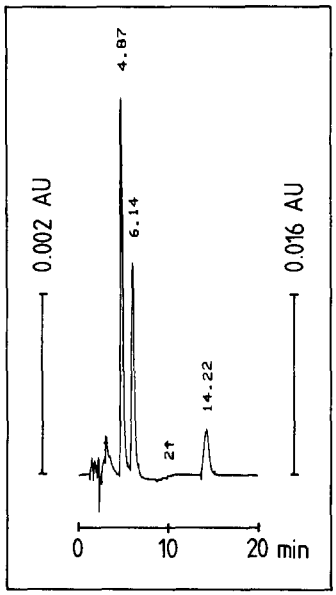

Fig. 2. Separation of FPG (retention time 4.87 min), FMG (retention time 6.14 min) and (±)fenoterol (retention time 14.22 min). Liver microsomes were incubated at 37° in Na/K-phosphate buffer in the presence of Brij-58 (1 mg/mg microsomal protein), 10 mM $MgCl_2$, 3 mM UDP-glucuronic acid and 0.2 mM fenoterol·HBr. After incubation for 10 min the incubate was analyzed as described in Materials and Methods. The sensitivity of the u.v.-detector (212 nm) was changed when 10 min of the chromatogram was elapsed (scales on the left and right, respectively). Calculated concentration of FPG and FMG in the sample are 21.5 and 25.5 μM, respectively.

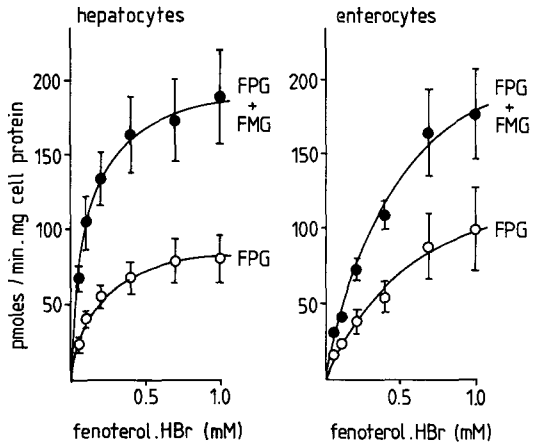

Fig. 3. Formation of FPG (○) and total glucuronide (●) in isolated hepatocytes (left) and enterocytes (right). Cells (2–4 mg protein/ml) were incubated for 30 min in a volume of 1 ml (hepatocytes) or 3 ml (enterocytes) Krebs–Ringer bicarbonate buffer, gassed with 95% O_2, 5% CO_2 (pH 7.4), in the presence of various concentrations fenoterol·HBr. FPG and FMG were quantified as described in Materials and Methods. Data points represent the means ± S.E.M. of duplicate determinations with three batches of cells. When no error bar is given, the S.E.M. was smaller than the symbol used.

and 14.2 (fenoterol) min (Fig. 2). Protein content of the incubates was determined according to Lowry *et al.* [24] using bovine serum albumin as a standard.

Experimental design and statistical analysis. Cell incubations with (±)F were done in duplicate with three batches of cells isolated from one (hepatocytes) or two (enterocytes) rats. Due to the limited amount of fenoterol enantiomers available the conjugation of (+)F and (−)F was estimated in a direct comparison with one batch of hepatocytes, enterocytes, hepatic microsomes and intestinal microsomes each. Duplicate incubations at various concentrations were used and apparent kinetic constants were calculated using a non-linear least-squares fitting programme [25] implemented on an Apple-IIe microcomputer. The proportional S.D. and bisquare weighting options were used.

RESULTS

The formation of FPG and FMG in isolated hepatocytes and enterocytes, incubated with various concentrations of (±)F, is illustrated in Fig. 3. The fraction of FPG formed was constant over the concentration range investigated (0.40 ± 0.01 for hepatocytes and 0.54 ± 0.01 for enterocytes).

The maximum glucuronidation rate of (+)F in hepatic and intestinal microsomes is substantially higher than of (−)F (Table 1). Furthermore, statistically significant differences in affinity (K_m^{app}) and the fraction of FPG formed are observed between (+)F and (−)F in microsomes. Similar, but less pronounced, differences in V_{max}-values and fraction of FPG formed are seen when isolated cells are used (Table 1). The fraction of FPG formed by (±)F in isolated cells can be predicted from V_{max} and fraction-FPG values for each enantiomer. When it is assumed that (+)F and (−)F do not interact with each other during administration of (±)F the fraction of FPG formed during administration of the racemate would be expected to be 0.36 for hepatocytes and 0.60 for enterocytes. These values correspond reasonably well with the observed values (see above).

Because *in vivo* concentrations of fenoterol are usually below K_m^{app}-values intrinsic clearances can be used to predict the *in vivo* metabolism of both enantiomers. Calculation of the Cl_{int} for (+)F and (−)F by isolated cells (Table 1) suggests that (+)F may be more efficiently metabolized *in vivo* by the liver than (−)F. The Cl_{int} for (+)F by hepatocytes is 30% higher than the Cl_{int} for (−)F. As a consequence of the difference in K_m^{app} for (+)F and (−)F the Cl_{int} of (+)F in hepatic and intestinal microsomes is lower than the Cl_{int} of (−)F.

DISCUSSION

Formation of FPG and FMG

FPG and FMG were formed in isolated cells and microsomes in proportions that were constant over the concentration range investigated. The total glucuronidation of fenoterol in isolated enterocytes and in hepatic and intestinal microsomes corresponds with earlier published values [2, 3]. When the three phenolic hydroxyl groups of fenoterol would have an equal change of being glucuronidated, a fraction

Table 1. Glucuronidation of fenoterol enantiomers in hepatocytes, enterocytes, hepatic microsomes and intestinal microsomes

	Enantiomer	V_{max} (nmol/min/mg)	K_m^{app} (μM)	Cl_{int}* (μl/min/mg)	Fraction FPG
Hepatocytes	(+)F	0.173 ± 0.012	90 ± 20	1.92 ± 0.45	0.43 ± 0.02
	(−)F	0.148 ± 0.013‡	100 ± 20	1.48 ± 0.32†	0.28 ± 0.01‡
Enterocytes	(+)F	0.444 ± 0.057	510 ± 100	0.87 ± 0.20	0.66 ± 0.01
	(−)F	0.372 ± 0.050‡	470 ± 100	0.79 ± 0.20	0.52 ± 0.01‡
Hepatic microsomes	(+)F	6.7 ± 0.8	57 ± 15	118 ± 27	0.54 ± 0.01
	(−)F	3.6 ± 0.3‡	26 ± 6‡	138 ± 34	0.33 ± 0.01‡
Intestinal microsomes	(+)F	5.8 ± 0.4	49 ± 7	118 ± 19	0.54 ± 0.01
	(−)F	3.4 ± 0.1‡	19 ± 2‡	179 ± 20‡	0.31 ± 0.01‡

* $Cl_{int} = V_{max}/K_m^{app}$ for concentrations $< K_m^{app}$.

† Significantly different ($P < 0.05$) from same parameter for (+)F.

‡ Significantly different ($P < 0.01$) from same parameter for (+)F.

Cells and microsomes were incubated as described in Materials and Methods. Due to the limited amount of fenoterol enantiomers available the conjugation of (+)F and (−)F was estimated in a direct comparison with one batch of hepatocytes, enterocytes, hepatic microsomes and intestinal microsomes each. Apparent kinetic constants were calculated using a non-linear least-squares fitting programme.

of 0.33 for FPG-formation would be expected. The results demonstrate that this proportion is only obtained when (−)F is incubated with hepatic or intestinal microsomes. When (+)F is used as a substrate formation of FPG is more pronounced. The preferential formation of FPG from (+)F is also observed with isolated cells. Species-specific differences in the formation of morphine 3-glucuronide and morphine 6-glucuronide from morphine enantiomers has recently been found in rat, Rhesus monkey and human liver microsomes [26]. The reason for these differences in fractional formation of different glucuronides undoubtedly must be looked for in the conformation of the substrate [7] and in the active site of the enzyme. Our knowledge of the active site of the UDPGT involved is too limited to try to explain the phenomena observed. It should, however, be remarked that complications may arise from the microsomal nature of the enzyme [15, 16]. More detailed studies require the use of purified enzymes and a strictly characterized microsomal environment [15, 16, 27].

Stereoselective glucuronidation of fenoterol

Several stereoselective metabolic routes have been shown to affect the pharmacokinetics of racemic drugs [10, 11, 13, 28]. Considerable species-specific differences do exist [12, 13, 28]. In rat-liver microsomes or homogenates stereoselective glucuronidation of propranolol [15], oxazepam [12] and morphine [26] has been observed. We now report stereoselective glucuronidation of fenoterol; differences in both V_{max} and K_m^{app} are observed in hepatic as well as intestinal microsomes. Differences in K_m^{app} for the glucuronidation of propranolol enantiomers have been observed in dog and rat liver microsomes [15, 16]. Differences in V_{max} only have been measured in most other cases [12, 16, 26]. In isolated cells the differences in glucuronidation between (+)F and (−)F are less pronounced but nevertheless statistically significant.

From our data it cannot be concluded if mutual interactions of (+)F and (−)F during administration of the racemate will occur. Interactions between propranolol enantiomers have been shown to occur *in vitro* [15, 16, 27] but *in vivo* no interactions between verapamil, warfarin and propranolol enantiomers were observed [10, 11, 14]. Furthermore, the K_m^{app} for the glucuronidation of fenoterol in isolated hepatocytes is 90–100 μM. It is highly unlikely that at concentrations, measured *in vivo* (<2 μM; [1] and [5]) interactions between both isomers will occur.

Differences in intrinsic clearances of (+)F and (−)F were observed. In hepatic as well intestinal microsomes the Cl_{int} of (−)F was higher than the Cl_{int} of (+)F; in hepatocytes and enterocytes the reverse was true (Table 1). It is therefore difficult to decide how intrinsic clearances will differ *in vivo*. We prefer to extrapolate from the cellular data because it is well known that glucuronidation in microsomal preparations is artefactually elevated by detergent activation and by unphysiological UDP-glucuronic acid concentrations (Refs. 29, 30 for review).

A 30% difference in intrinsic clearance of (+)F and (−)F (measured in hepatocytes) was observed in the present study. This result suggests that the pharmacologically active enantiomer (−)F is less efficiently eliminated than the (+)enantiomer. Although the resulting plasma levels of both enantiomers after intravenous administration of the racemate may differ so little that the difference can not be detected by the available radioimmunological methods [9], the small difference in hepatic Cl_{int} may have important consequences for the oral bioavailability of the enantiomers. Because fenoterol is highly extracted during first-pass in the intestinal wall and the liver (98% first-pass effect in the rat) [5] the absolute availability of the active enantiomer (−)F may be higher than the absolute availability of the (+)enantiomer [11, 14]. Stereoselective first-pass metabolism of propranolol has been demonstrated to occur in the dog [14] and the same phenomenon seems to be involved in the pharmacokinetics of verapamil in man [11]. Oral availability, calculated

from plasma racemate AUC-values after intravenous and oral administration of fenoterol [5], will not represent the availability of the active enantiomer in that case. An alternative approach, in which bioavailability of fenoterol is measured on the basis of tachycardia [4] offers an interesting possibility to avoid this problem.

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