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Stereoselective sulphate conjugation of salbutamol in humans: comparison of hepatic, intestinal and platelet activity

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1 The oral bioavailability of the β_2-adrenoceptor agonist salbutamol has been proposed to be stereoselective, presumably due to presystemic sulphate conjugation. In the present study we examined the stereochemistry of the sulphation reaction *in vitro* using human tissue preparations.

2 Sulphation of salbutamol was studied with partially purified hepatic M and P form phenol sulphotransferases (PSTs), 100,000 *g* cytosol of jejunal mucosa and platelet homogenate. The cosubstrate PAP^{35}S was used as the sulphate donor. The acceptor substrate was either (+)-, (−)- or (±)-salbutamol.

3 Sulphation was catalyzed by the M form PST of the liver but not the P form. The sulphation efficiency (V_{max}/K_m) was 11.9-fold greater for the (−)- than for the (+)-enantiomer, due entirely to a lower apparent K_m for (−)-salbutamol, 103 μM, than for (+)-salbutamol, 1394 μM.

4 Sulphation by the jejunal mucosa ($n = 3$) was very similar to that of the M form PST with the efficiency being 9.8-fold greater for the (−)-enantiomer and apparent K_m values 95 μM and 889 μM for (−)- and (+)-salbutamol, respectively.

5 Sulphation by the platelet ($n = 3$) was also very similar to that of the M form PST with the efficiency being 9.9-fold greater for the (−)-enantiomer and apparent K_m values 141 μM and 1190 μM for (−)- and (+)-salbutamol, respectively.

6 The sulphation of racemic (±)-salbutamol by all three preparations behaved as would be predicted from the individual enantiomers alone, if the enantiomers in the racemic mixture are regarded as two different substrates competing for the same binding site.

7 These findings suggest the potential for large enantioselectivity in the oral bioavailability of salbutamol, with lower blood concentrations of the pharmacologically more active (−)-enantiomer.

Keywords salbutamol albuterol stereoselective sulphation sulphate conjugation phenol sulphotransferases human liver human intestine human platelets

Introduction

Salbutamol (albuterol) is a selective β_2-adrenoceptor agonist widely used in the treatment of bronchial asthma (Morgan, 1990). Its pharmacokinetics after oral doses are highly dependent on presystemic metabolism, mainly by sulphate conjugation, which occurs in the liver but may be more important in the intestine (Morgan *et al.*, 1986). Although the structure of the main metabolite of salbutamol has long been known to be the 4′-*O*-sulphate ester (Lin *et al.*, 1977; Morgan *et al.*, 1986), little is known about the sulphoconjugation reaction. Recent evidence from studies of the structurally similar analogue terbutaline had demonstrated that sulphate conjugation of the (+)-enantiomer of this drug occurs with 2-fold higher apparent V_{max} value than that of the more active (−)-enantiomer (Walle & Walle, 1990, 1992), which may explain at least part of the stereoselectivity in the pharmacokinetics of terbutaline following oral doses in man (Borgström *et al.*, 1989). In

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contrast, for salbutamol it has been suggested, based on urinary recoveries of the parent drug enantiomers, that sulphate conjugation of this drug may be preferential for the opposite, (−)-enantiomer by as much as 5- to 10-fold (Tan & Soldin, 1987). In studies using human liver cytosol the sulphation of salbutamol only showed a two-fold preference for the (−)-enantiomer (Pesola & Walle, 1992), along with a substantial enantiomeric interaction. In a subsequent investigation with the human hepatoma cell line Hep G2 as a model system, sulphation showed an 8-fold preference for the (−)-enantiomer (Walle *et al.*, 1993), which appeared to be more in accordance with the only clinical measurements (Tan & Soldin, 1987).

Because of these inconsistent observations and the clinical importance of the stereochemical question, the present study was undertaken to include a comparison of the sulphation of salbutamol by the partially purified M and P forms of phenol sulphotransferases (PSTs) from human liver. As the intestine has been proposed to be of greater importance than the liver for the sulphation of oral doses of this drug (Morgan *et al.*, 1986), studies were undertaken with the cytosol of human jejunum. In addition, we investigated the sulphation of salbutamol by platelet homogenates as a potential model system of the liver and intestine. The sulphation of salbutamol was studied with $PAP^{35}S$ as the radioactive sulphate donor.

Methods

Materials

(±)-Salbutamol was purchased from Sigma (St Louis, MO, USA) and [^{35}S]-3′-phosphoadenosine-5′-phosphosulphate ($PAP^{35}S$) (specific activity 1.0–1.5 Ci mmol^{-1}) from Du Pont/New England Nuclear (Wilmington, DE, USA). (S)-(+)- and (R)-(−)-salbutamol acetate were kindly donated by Glaxo (Dr Barboriak at Research Triangle Park, NC, USA). Human liver (n = 1) was obtained through the Liver Tissue Procurement and Distribution System (University of Minnesota, Minneapolis, MN, USA) and histologically normal jejunum (n = 3) through the National Disease Research Interchange (Philadelphia, PA, USA), non-profit tissue procurement facilities supported by the National Institutes of Health. All tissue samples were from transplant donors and were flash-frozen and stored at −80° C.

Tissue preparations

Hepatic 100,000 *g* cytosol was prepared by homogenization and differential centrifugation (Campbell *et al.*, 1987; Walle & Walle, 1991a). The M and P forms of the PST enzymes were resolved and partially purified from the hepatic cytosol by ion-exchange chromatography on a DEAE-Sepharose column as described previously (Campbell *et al.*, 1987; Walle & Walle, 1992). The M form, also referred to as the thermolabile form, metabolizes monoamines (M), whereas the P form, also referred to as the thermostable form, metabolizes simple phenols (P) (Weinshilboum, 1986). Jejunal mucosal 100,000 *g* cytosol was prepared in a similar fashion as liver cytosol (Sundaram *et al.*, 1989) after scraping off the mucosa with a glass slide. Platelets were isolated from fresh, EDTA-treated blood from healthy volunteers and homogenized for 15 s with a Polytron homogenizer (Anderson & Weinshilboum, 1980; Walle & Walle, 1991b). Protein content was assayed according to Lowry *et al.* (1951).

Incubations

Incubations were carried out for 30 min at 37° C in 33 mM Tris buffer (pH 7.4) with 0.4 μM $PAP^{35}S$ as the sulphate donor, dithiothreitol (8 mM), BSA (0.0625%, w/v) and M or P form PST (1–2 μg protein), jejunal mucosal cytosol (1–3 μg protein) or platelet homogenate (about 4 × 10^6 platelets) (Anderson & Weinshilboum, 1980; Walle & Walle, 1990). As the lowest concentration of salbutamol at which sulphation activity could be determined was 25 μM, a concentration range of 25–1000 μM of (±)-, (+)- and (−)-salbutamol was used in the incubations. Incubates with no substrate were used as controls. All incubations were done in duplicate. Protein and excess $PAP^{35}S$ were precipitated from the incubates with barium acetate/hydroxide and zinc sulphate (Anderson & Weinshilboum, 1980; Foldes & Meek, 1973). After centrifugation aliquots of the supernatant were subjected to liquid scintillation spectrometry. The extent of sulphation of dopamine (20 μM) and *p*-nitrophenol (4 μM) was assayed similarly (Campbell *et al.*, 1987; Walle & Walle, 1992).

The potential loss of salbutamol sulphate in the precipitation procedure was studied using preformed ^{35}S-labelled conjugate. The recovery from the supernatant was 103 ± 1% (mean ± s.e. mean; n = 3) of added product.

Characterization of PST activities in tissue preparations

The liver from which the M and the P form PSTs were isolated as well as the three intestinal tissues were each characterized as normal tissue. This was based on catalytic properties (K_m and V_{max} values) with respect to the prototype substrates dopamine (M form) and *p*-nitrophenol (P form) (Campbell *et al.*, 1987; Sundaram *et al.*, 1989). Characterization of the M and the P forms of hepatic PST after their resolution by ion-exchange chromatography was done as described previously, i.e. based on their catalytic activity (K_m and V_{max} values) towards dopamine and *p*-nitrophenol, respectively, as well as by inhibition of these activities by elevated temperature and dichloronitrophenol (Campbell *et al.*, 1987; Walle & Walle, 1992).

Data analysis

The apparent K_m and V_{max} values for the individual salbutamol enantiomers and the racemate were calculated from double reciprocal plots of velocity *vs* substrate concentration using linear regression. As the assay for the sulphate conjugate of the racemic drug did not distinguish between the enantiomers, the observed

velocity is the sum of two products, giving a single hyperbolic curve. If the enantiomers in the racemic mixture are regarded as two different substrates of identical concentration competing for the same enzyme binding site, the total velocity (V_{total}) will be determined by the following equation (Segel, 1975):

$$V_{total} = \frac{\frac{V_{max(+)}}{K_{m(+)}} + \frac{V_{max(-)}}{K_{m(-)}}}{\frac{1}{\text{enantiomer concentration}} + \frac{1}{K_{m(+)}} + \frac{1}{K_{m(-)}}} \quad \text{(Equation 1)}$$

The experimental data obtained with racemic salbutamol were compared with those predicted by equation (1), using the kinetic constants obtained with the separate enantiomers.

It should be pointed out that the PAPS concentration used was nonsaturating. The apparent K_m values are valid at any PAPS concentration, but the apparent V_{max} values can only be compared with results obtained with identical PAPS concentration (Anderson & Weinshilboum, 1980; Foldes & Meek, 1973).

Results

Kinetics of sulphation by the M and P form hepatic PST

The sulphation of the pure (+)- and (−)-enantiomers as well as of racemic salbutamol by the M form PST isolated from the liver is illustrated in Figure 1a. Hyperbolic curves typical of a single reaction were obtained for all three forms of the drug. The extent of sulphation of (+)-salbutamol was much lower than that of (−)-salbutamol and the sulphation of racemic drug was similar to that of the (−)-enantiomer. The corresponding double reciprocal plots are shown in Figure 1b, being highly linear for all three drug forms. The apparent values of enzyme kinetic parameters calculated from these plots are shown in Table 1. The

Table 1 Apparent enzyme kinetic parameters for the sulphoconjugation of the salbutamol (Salb) enantiomers by human tissues

		K_m (μM)		K_m *ratio*	V_{max} *ratio*	V_{max}/K_m *ratio*
		(+)-Salb	*(−)-Salb*	*(+)-Salb/(−)-Salb*	*(+)-Salb/(−)-Salb*	*(−)-Salb/(+)-Salb*
Hepatic M-form		1394	103	13.5	1.1	11.9
P-form		na[a]	na	na	na	na
Jejunal mucosal						
cytosol	1	821	86	9.5	0.9	10.5
	2	811	82	9.9	1.1	8.9
	3	1036	117	8.9	0.9	10.0
	Mean	889	95	9.4	1.0	9.8
	± s.e. mean	73	11	0.3	0.1	0.5
Platelet						
homogenate	1	1357	124	10.9	1.0	10.9
	2	635	117	5.4	0.6	9.0
	3	1578	182	8.7	0.9	9.7
	Mean	1190	141	8.3	0.8	9.9
	± s.e. mean	285	21	1.6	0.1	0.6

[a]na = no activity.

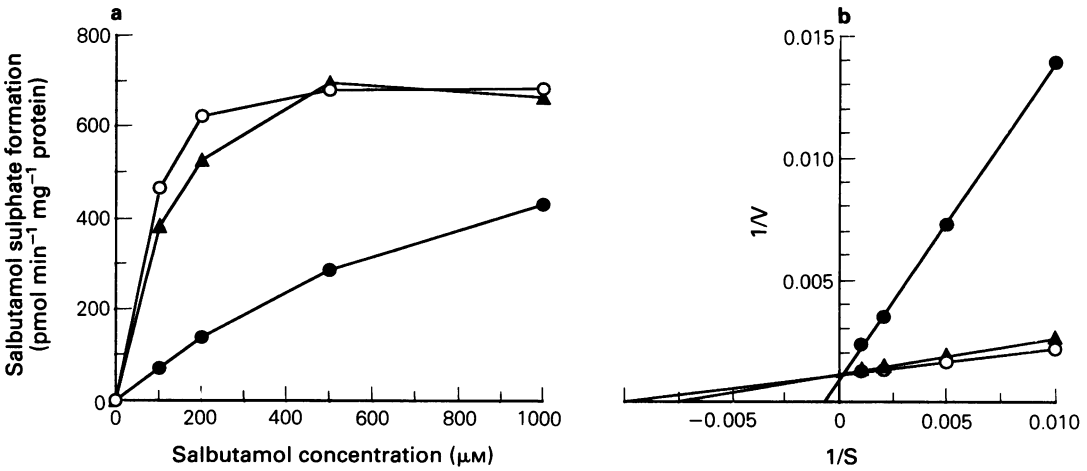

Figure 1 Enantioselective sulphation of (+)- (•), (−)- (○) and (±)-salbutamol (▲) by the M form PST of the human liver using [^{35}S]-labeled PAPS as cosubstrate. a) Velocity *vs* substrate concentration; b) the corresponding double reciprocal plots.

apparent V_{max} values for the enantiomers were virtually identical, whereas the apparent K_m value for (+)-salbutamol (1394 μM) was 13.5 times higher than that for (−)-salbutamol (103 μM). A useful index for *in vivo* extrapolations is the efficiency of the sulphation reaction (V_{max}/K_m), which showed an 11.9-fold preference for (−)- compared with (+)-salbutamol. Racemic salbutamol gave an apparent K_m value (129 μM) very similar to (−)-salbutamol. The apparent V_{max} values were also very similar. The data for the racemic drug agreed with those predicted from the kinetic constants for the individual enantiomers, if the enantiomers in the racemic mixture are regarded as two different substrates competing for the same enzyme binding site. The total velocity for racemic drug is largely determined by the enantiomer with the lowest K_m (see Equation 1), i.e. the (−)-enantiomer.

In contrast to the M form PST, the P form PST showed no activity towards either (+)-, (−)- or (±)-salbutamol over the total concentration range tested. At the same time *p*-nitrophenol showed the extensive sulphation typical of an active P form enzyme (Campbell *et al.*, 1987; Walle & Walle, 1992).

Kinetics of sulphation by the 100,000 g cytosol of the jejunal mucosa

The mean values of the sulphation of the pure (+)- and (−)-enantiomers as well as of racemic salbutamol by the jejunal mucosal cytosol from three donors are shown in Figure 2a. As for the M form PST in Figure 1a typical hyperbolic curves for a single reaction were obtained for all three forms of the drug. Again, the extent of sulphation of (+)-salbutamol was much lower than that for (−)-salbutamol and the sulphation of racemic drug was similar to that of the (−)-enantiomer. The double reciprocal plots were linear and produced the apparent enzyme kinetic parameters listed in Table 1. As for the M form PST, the apparent V_{max} values were similar for (+)- and (−)-salbutamol. The mean apparent K_m value for (+)-salbutamol (889 μM) was 9.4 times higher than that of (−)-salbutamol (95 μM). The sulphation efficiency (V_{max}/K_m) was 9.8 times higher for (−)-salbutamol. Racemic salbutamol gave an apparent K_m value (140 ± 21 μM; mean ± s.e. mean) 1.5 times that of (−)-salbutamol. The apparent V_{max} values were similar.

Kinetics of sulphation by the platelet homogenate

The sulphation of the pure (+)- and (−)-enantiomers as well as of racemic salbutamol by the platelet homogenate from three normal volunteers is shown in Figure 2b. The sulphation profile was virtually identical to that of the M form PST shown in Figure 1a and that of jejunal cytosol shown in Figure 2a. The apparent enzyme kinetic parameters are summarized in Table 1 and show a 9.9-fold greater efficiency (V_{max}/K_m) for the sulphation of (−)- compared to that of (+)-salbutamol, which is largely due to a much lower apparent K_m value for the (−)-enantiomer (141 μM) than for the (+)-enantiomer (1190 μM). Racemic salbutamol gave an apparent K_m value (251 ± 34 μM) 1.8 times that of (−)-salbutamol with similar apparent V_{max} values.

Discussion

In a previous study of the sulphoconjugation of the salbutamol enantiomers by human liver 100,000 *g* cytosol preparations, we found that the reaction was about twice as efficient for the (−)- as for the (+)-enantiomer, due to a lower apparent K_m value for (−)-salbutamol (Pesola & Walle, 1992). However, this stereoselectivity did not explain the much greater, 5- to 10-fold, stereoselectivity implied *in vivo* in man, based on the urinary excretion of the enantiomers of unmetabolized salbutamol (Tan & Soldin, 1987).

In the present study we resolved the liver PST enzymes into their M and P forms by ion-exchange chromatography and examined their catalytic activities towards the sulphation of salbutamol. Only the M form PST demonstrated sulphation of this substrate. The kinetic parameters for the M form showed a more than 10-fold preference for the (−)- compared with the (+)-enantiomer, a selectivity entirely due to an enantiomeric difference in apparent K_m values (103 μM and

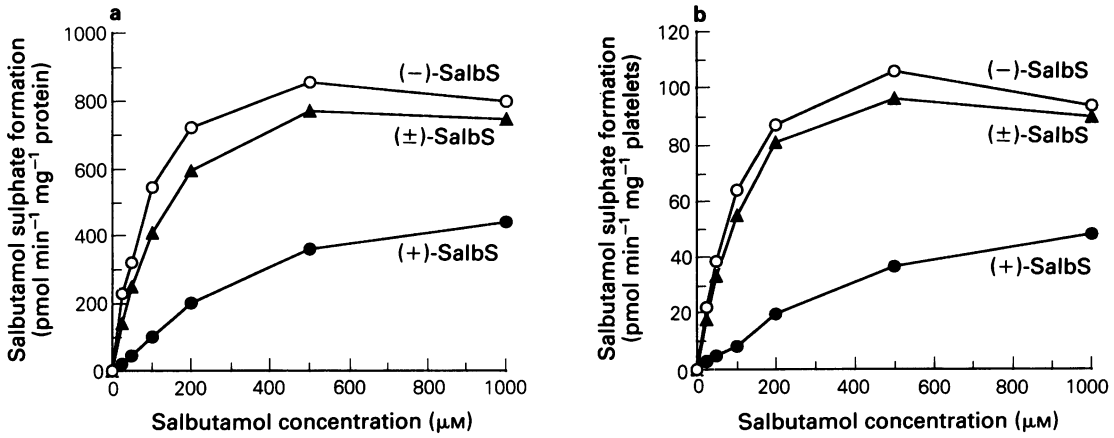

Figure 2 Enantioselective sulphation of (+)-, (−)- and (±)-salbutamol by a) the 100,000 *g* cytosol of human jejunal mucosa and b) homogenate of platelets. Mean values are shown from three donors of jejunal tissue and three normal volunteers for platelets. SalbS = Salbutamol sulphate.

1394 μM for (−)- and (+)-salbutamol, respectively). The fact that salbutamol was not a substrate for the P form PST, at least at substrate concentrations ≤ 10 mM, is consistent with our previous observation on the structural analogue terbutaline (Walle & Walle, 1992).

Indirect evidence strongly supports the hypothesis that oral doses of salbutamol undergo presystemic sulphoconjugation mainly in the intestine (Morgan *et al.*, 1986). We examined this hypothesis by measuring the sulphation of salbutamol by the 100,000 *g* cytosol of the jejunal mucosa from three donors. The mean values produced were almost identical to those generated by the partially purified M form PST, i.e. a 10-fold greater efficiency in the sulphation of the (−)- than the (+)-enantiomer, which was entirely due to a lower apparent K_m value for (−)-salbutamol. These observations are consistent with the notion that jejunal mucosal tissue has a high concentration of the M form PST compared with the P form PST (Sundaram *et al.*, 1989), and that no other PST is involved in the sulphation of salbutamol in this tissue.

As the platelet is an easily accessible tissue, which may be used as a model of other tissues, e.g. intestine and liver, we extended our studies of salbutamol sulphation to this tissue homogenate. The mean data generated were almost identical to those obtained in the experiments with the M form PST and the intestine, with a 10-fold greater efficiency in sulphation of (−)- than of (+)-salbutamol due to a lower apparent K_m value for the (−)-enantiomer. The platelet appears to have similar concentrations of the M and the P forms of PST (Heroux & Roth, 1988; Heroux *et al.*, 1989; Reiter *et al.*, 1983). The data obtained from both platelet and the intestine indicate that the crude cytosol or homogenate preparations of these tissues reflect the sulphoconjugation of salbutamol and probably most β-adrenoceptor agonist drugs (Walle & Walle, 1992) by the M form PST.

The similarity of the apparent enzyme kinetic parameters in Table 1 for the hepatic M form PST, the jejunal cytosol and the platelet homogenate suggests that these reactions are carried out by the same enzyme, i.e. the M form PST. In addition, the human hepatoma cell line Hep G2, investigated because of its potential biosynthetic properties (Walle *et al.*, 1993), also has similar catalytic properties to the human tissue preparations used in the present study, which is probably due to high expression of the M form PST in this cell line (Shwed *et al.*, 1992). These data taken together strongly implicate a high degree of stereoselectivity in the pharmacokinetics of salbutamol *in vivo*, with higher oral bioavailability of the inactive (+)-enantiomer form as proposed by Tan & Soldin (1987).

The marked preference for the sulphation of (−)- relative to (+)-salbutamol is a unique property of this drug among the β-receptor agonists. Structurally similar substrates such as 4-hydroxypropranolol (Walle & Walle, 1991a, 1992), terbutaline (Walle & Walle, 1990, 1992) and isoprenaline (Pesola *et al.*, 1992) all show a preference for the opposite, (+)-enantiomer over the (−)-enantiomer by 2- to 5-fold. Two of these substrates, 4-hydroxypropranolol and terbutaline, show stereoselectivity strictly on the basis of differences in V_{max} values, whereas isoprenaline and salbutamol mainly express stereoselectivity, albeit in opposite directions, in K_m values. The uniqueness of salbutamol as a PST substrate within this class of drugs may be due to the 3′-hydroxymethyl substituent in the binding of this substrate to the enzyme.

The anomalous behaviour of the crude liver cytosol in the sulphation of salbutamol (Pesola & Walle, 1992) as compared with the hepatic M form PST in the present study has no clear explanation, as the experimental conditions as well as one of the livers studied were the same. The apparent K_m values, in particular those of the (−)-enantiomer, were considerably higher in the cytosol than for the M form PST, possibly due to endogenous inhibitors in the crude preparations (Anderson & Weinshilboum, 1979, 1980) or an additional sulphotransferase in the liver cytosol. In addition, the sulphation of racemic salbutamol by liver cytosol showed marked inhibition (Pesola & Walle, 1992). In the present study with the hepatic M form, the jejunal cytosol or the platelet homogenate, the racemate behaved as predicted from the individual enantiomers alone, if the enantiomers in the racemic mixture are regarded as two different substrates competing for the same binding site.

In conclusion, sulphoconjugation of salbutamol by the partially purified M form PST of the human liver, the 100,000 *g* cytosol of human jejunal mucosal tissue and human platelet homogenate showed a uniform pattern with 10-fold greater efficiency for (−)- than for (+)-salbutamol. These data are consistent with the disposition of salbutamol enantiomers *in vivo* in man.

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