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Influence of the type of halogen substituent on *in vivo* and *in vitro* phase II metabolism of 2-fluoro-4-halophenol metabolites formed from 3-halo-fluorobenzenes

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1. The influence of a change in the type of halogen substituent on phase II metabolism of 2-fluoro-4-halophenol metabolites formed from 3-halo-fluorobenzenes was studied *in vivo* and *in vitro* using ^{19}F nmr and spectroscopic assays.

2. The ratio of sulphation to glucuronidation of 2-fluoro-4-halophenol metabolites formed from 3-halofluorobenzenes decreased from 48 to 13 to 6 when the halogen substituent varied from fluorine to chlorine to bromine.

3. When the 2-fluoro-4-halophenols themselves were administered to the rats, the ratio of sulphation to glucuronidation was not affected by the type of halogen substituent at C4 and at a constant value of 0·6, i.e. significantly lower.

4. Kinetic data for P450 catalysed hydroxylation of the 3-halo-fluorobenzenes and for sulphation and glucuronidation of their 2-fluoro-4-halophenol metabolites were obtained from *in vitro* microsomal and cytosolic incubations. These data demonstrate that the effects of varying the halogen substituent on phase II metabolism of the 2-fluoro-4-halophenol metabolites can be mainly ascribed to an apparently decreased K_m for the glucuronidation of the 2-fluoro-4-halophenols with a change in the halo substituent from fluorine to chlorine to bromine.

5. Results from calculations on electronic and structural characteristics of the three 4-halo-2-fluorophenols demonstrate that the best explanation for the decrease in the apparent K_m of the glucuronidation from 2,4-difluoro- to 4-chloro-2-fluoro- to 4-bromo-2-fluorophenol might be an increase in the hydrophobicity of the phenol. An increase in the hydrophobicity of the phenol would provide an increased possibility for substrate accumulation in the hydrophobic membrane environment of the UDP-glucuronyltransferases, resulting in an apparently decreased K_m.

Introduction

Phenolic metabolites from aromatic xenobiotics are known to be excreted from the mammalian body upon their conjugation with either sulphate or glucuronide moieties. Sulphate conjugation, catalysed by cytosolic sulphotransferases, are known to be reactions characterized by relatively low apparent K_m and low apparent V_{max}, whereas glucuronide conjugation, catalysed by membrane-bound uridine-5′-diphosphate (UDP)-glucuronyltransferases, proceeds with relatively high apparent K_m and high apparent V_{max} characteristics (Evelo *et al.* 1984, Mulder 1984). This remarkable difference in the biochemical characteristics of these two phase II enzymes for the conjugation of phenolic derivatives, as well as the species-specific occurrence of the glucuronyltransferases and sulphotransferases (Koster and Mulder 1982, Timbrell 1982, Mulder 1984, Sipes and Gandolfi 1991), are two

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factors known to influence the outcomes of phase II metabolism of phenolic derivatives. The present study was undertaken to investigate whether the substituent pattern of a phenolic compound is an additional factor influencing the type of phase II reaction, i.e. sulphation or glucuronidation. Special emphasis was on possible influence of a change in the type of halogen substituent on the possibilities for metabolism of a phenolic derivative through phase II metabolism. Model compounds chosen to perform this study were 3-halo-fluorobenzenes and their 2-fluoro-4-halophenol metabolites. These model compounds were chosen for various reasons. First, previous metabolism studies on 1,3-difluorobenzene demonstrated formation of the 2,4-difluorophenol metabolite as a major biotransformation pathway (Rietjens *et al.* 1993). Thus, the P450-catalysed conversion of 3-halo-fluorobenzenes can be expected to result in significant formation of 2-fluoro-4-halophenol metabolites. This implies that this series of model compounds provides the possibility of studying the effect of a change in the type of halogen substituent opposite the hydroxyl moiety on the phase II conjugation of this hydroxyl group. In addition, the presence of the fluorine substituent in all model substrates provides the possibility of characterizing and quantifying metabolite patterns in both urine samples and *in vitro* incubations by ^{19}F nmr. This implies that metabolic products can be identified and quantified by the same technique in a single run, without a need for prior extraction of the metabolites from the biological sample (Malet-Martino 1986, Malet-Martino and Martino 1989, 1992, Rietjens and Vervoort 1989, 1992, Vervoort *et al.* 1990).

Materials and methods

Chemical

2,4-Difluorophenol, 2,6-difluorophenol, 3,5-difluorophenol, 4-chloro-2-fluorophenol, 2-chloro-4-fluorophenol, 4-bromo-2-fluorophenol, 2-bromo-4-fluorophenol, 1,3-difluorobenzene and 4-fluorobenzoic acid were purchased from Fluorochem (Derbyshire, UK). 3-Chloro-fluorobenzene, 3-bromo-fluorobenzene and resorufin were obtained from Janssen Chimica (Beerse, Belgium). Uridine-5′-diphosphoglucuronic acid (UDPGA), adenosine 3′-phosphate-5′-phosphosulphate (PAPS) and bovine serum albumin were from Sigma (St Louis, MO, USA). Lactate-dehydrogenase (LDH) (from hog muscle), pyruvate kinase (PK) (from rabbit muscle), β-glucuronidase (from *Escherichia coli* K12), arylsulphatase/β-glucuronidase (from *Helix pomatia*), phosphoenol pyruvate monosodium salt (PEP), ATP, NADH and NADPH were all from Boehringer (Mannheim, Germany). Triton X-100, ascorbic acid, $MgCl_2$, K_2HPO_4, KH_2PO_4, K_2SO_4 and 2-mercaptoethanol were all purchased from Merck (Darmstadt, Germany).

Preparation of microsomes and cytosol

Microsomes were prepared from the perfused livers of male Wistar rats as described previously (Rietjens and Vervoort 1989). Cytosol was obtained as the supernatant from the first 105 000 *g* ultracentrifuge step performed during preparation of the microsomes. The microsomal and cytosolic protein contents were determined as described by Lowry *et al.* (1951), using bovine serum albumin as the standard. The P450 concentration in the microsomal sample was measured as described by Omura and Sato (1964).

In vivo *benzene and phenol administration to rats*

The desired halogenated fluorobenzenes and fluorophenols were administered dissolved in olive oil to male Wistar rats (400 g) by oral gavage or intraperitoneal injections (as indicated) at 500 μmol/kg body weight. This dose was routinely chosen because it was shown in a previous study to be non-toxic and to result in urine metabolite concentrations that could be easily detected by ^{19}F nmr (Rietjens *et al.* 1993). After dosing, 24-h urine samples were collected, recovery being $>65\%$ of the dose for all halogenated fluorobenzenes and $>95\%$ for all the halogenated fluorophenols.

Analysis of urine samples

Identification and quantification of the urinary metabolites were performed by ^{19}F nmr analysis of the samples. Enzyme hydrolysis of the urinary phenylsulphates and phenylglucuronides was carried out as described previously (Rietjens and Vervoort 1992). Urine samples were diluted with an equal volume of 0·2 M potassium phosphate (pH 7·6) and prepared for ^{19}F nmr measurements as described hereafter.

^{19}F nmr measurements

^{19}F nmr measurements were performed on a Bruker AMX 300 spectrometer as described previously (Rietjens and Vervoort 1989, 1992, Vervoort *et al.* 1990). Between 1500 and 50 000 scans were recorded depending on the signal-to-noise ratio required, and the concentrations of the fluorine-containing compounds. The sample volume was 1·71 ml, containing 100 μl ^2H_2O for locking the magnetic field and 10 μl 8·4 mM 4-fluorobenzoic acid solution, added as internal standard. For determination of the 24-h recovery of the 3-halo-fluorobenzenes, 2,3,5,6-tetrafluorophenol was used as internal standard, because the resonance of 4-fluorobenzoic acid overlapped with the ^{19}F nmr signal of one of the urinary metabolites. The samples were made oxygen free by four cycles of evacuating and filling with argon. The ethyl acetate extracts from the microsomal incubations were analysed using a coaxial insert containing a known amount of the internal standard dissolved in ^2H_2O.

Chemical shifts are reported relative to $CFCl_3$. Concentrations of the various metabolites were calculated by comparison of the integrals of their ^{19}F nmr resonances of the metabolites to the integral of the ^{19}F nmr resonance of the internal standard.

In vitro *P450 catalysed conversion of 3-halo-fluorobenzenes*

P450-dependent conversion of the substrates was studied *in vitro* in incubations containing (final concentrations) 0·1 M potassium phosphate, pH 7·6, 2 μM microsomal P450, 1·0 mM NADPH and 1 mM ascorbic acid to prevent possible autoxidation of hydroxylated reaction products. The desired concentrations of 3-halo-fluorobenzenes were added as 1% (v/v) of a 100 times concentrated cold stock solution in acetone. The reaction was started by addition of the 3-halo-fluorobenzene and NADPH and carried out at 37°C for 10 min. The total reaction volume was 20 ml, and the reaction was carried out in a closed reaction vessel to prevent evaporation of the substrate. The reaction was terminated by addition of 1 ml 12 N HCl and 5 ml ethyl acetate. Upon mixing and centrifugation (10 min at 1200 *g*) the ethyl acetate layer was collected and analysed by ^{19}F nmr as described above. Using a 0·1 mM solution of the three 2-fluoro-4-halophenols in a standard microsomal incubation, their extraction from the incubation into the first ethyl acetate extract was demonstrated to be similar and > 95%. Apparent V_{max} and K_m were determined by fitting the experimental data to the standard Michaelis–Menten equation $v = V_{max}*[S]/(K_m + [S])$ with the KaleidaGraph program, version 2.0.2. (Abelbeck Software).

In vitro *UDP-glucuronyltransferase catalysed conversion of 2-fluoro-4-halophenols*

Kinetic parameters for the microsomal UDP-glucuronyltransferase-catalysed conjugation of the 2-fluoro-4-halophenols were determined by the method described by Mulder and van Doorn (1975) with some minor modifications. In contrast with the original procedure, the present procedure used 0·1 M potassium phosphate, pH 7·6, instead of 0·15 M Tris–HCl, pH 7·4, because the presence of inorganic phosphate appeared to result in a four-fold increase in the activity of the UDP-glucuronyltransferase-catalysed conversion of the 2-fluoro-4-halophenols. The microsomal sample was activated by a five times dilution with a 0·5% (v/v) Triton X-100 solution and stored for at least 20 min on ice before use. The incubations were carried out in 0·1 M potassium phosphate (pH 7·6) containing 0·36 mg microsomal protein/ml, 1 mM ascorbic acid to prevent autoxidation of the phenolic substrates, 5 mM $MgCl_2$, 5 U pyruvate kinase/ml 0·625 U lactate dehydrogenase/ml, 0·2 mM phosphoenol pyruvate and 0·2 mM NADH. The substrate was added as 1% (v/v) from a 100 times concentrated stock solution in a 1 : 1 mixture of ethanol and 0·2 M potassium phosphate, pH 7·6, containing 20 mM ascorbic acid. The final phenol concentrations used ranged from 0·25 to 4·0 mM for 2,4-difluorophenol, from 0·10 to 1·0 mM for 4-chloro-2-fluorophenol and from 0·03 to 0·40 mM for 4-bromo-2-fluorophenol, depending on their apparent K_m. The UDP-glucuronyltransferase activity was determined as the decrease of the NADH concentration in the incubation mixture, monitored by following the absorbance at 340 nm for at least 5 min in a LKB Ultraspec II spectrophotometer at 37°C (ε_{NADH} 340 nm = 6·22 μmol^{-1}.ml.cm^{-1}). Activities were corrected for NADH oxidation observed without UDPGA in the incubation medium. Apparent V_{max} and K_m were determined by fitting the experimental data to the standard Michaelis–Menten equation $v = V_{max}*[S]/(K_m + [S])$ with the KaleidaGraph program, version 2.0.2 (Abelbeck Software).

In vitro *sulphotransferase catalysed conversion of 2-fluoro-4-halophenols*

The *in vitro* sulphation of 2-fluoro-4-halophenols catalysed by cytosolic sulphotransferases was determined in the presence of SO_4^{2-} and ATP for *in situ* synthesis of the cofactor PAPS (3′-phosphoadenosine-5′-phosphosulphate) by the cytosolic enzymes sulphurylase and APS-phosphokinase, or in the presence of added PAPS (Sigma, St Louis, MO, USA). The assays with *in situ* synthesis of PAPS were carried out essentially as described by Lyman and Poland (1983). The cytosolic incubations contained 3·2 mg cytosolic protein/ml, 0·1 M potassium phosphate, pH 7·6, 1 mM ascorbic acid, 1 mM $MgCl_2$, 0·6 mM 2-mercaptoethanol, 6 mM K_2SO_4, and 4 mM ATP. The incubation mixture was preincubated at 37°C for at least 10 min to form the PAPS cofactor. The incubations to which PAPS was added were similar, except they contained no K_2SO_4 and ATP and PAPS was added from a 2 mM stock solution to give the desired final concentrations of 140, 240 and 340 μM. These incubations were preincubated at 37°C for 2 min. After the preincubation period, the arylsulphotransferase-catalysed

reaction was started by the addition of the 2-fluoro-4-halophenol substrates added as 0·5% (v/v) of a 200 times concentrated stock solution in dimethyl sulphoxide. Final concentrations of the 2-fluoro-4-halophenols were 0·1, 0·2, 0·3 or 0·5 mM (as indicated). The reaction was carried out at 37°C and terminated, after 15 min for the *in situ* PAPS assays and after 10 min for the incubations with added PAPS, by freezing the reaction mixture with liquid nitrogen. Upon thawing, the samples were kept at 0°C and immediately analysed by ^{19}F nmr as described above.

Determination of K_i *of 2-fluoro-4-halophenols for the sulphation of resorufin*

To obtain information about the relative affinities of the cytosolic rat liver sulphotransferases for the 2-fluoro-4-halophenols, K_i for the 2-fluoro-4-halophenol-mediated inhibition of the sulphation of resorufin were determined. Resorufin sulphation was measured essentially as described by Beckman (1991). To obtain the cofactor PAPS in the reaction mixture, preincubations were carried out as described above. Incubations contained 0·21 mg cytosolic protein/ml. After at least 10 min at 37°C, the reaction was started by addition of the resorufin, added as 0·5% (v/v) of a concentration range of 0·1–0·8 mM in dimethyl sulphoxide resulting in a final concentration range of 0·5–4·0 μM, and addition of the competitive 2-fluoro-4-halophenol inhibitors, added as 0·5% (v/v) of solutions of 0·025, 0·05, 0·10 and 0·15 mM in dimethyl sulphoxide resulting in 0·125, 0·25, 0·5 and 0·75 μM final concentrations. The reaction rate of the resorufin sulphation was monitored during the first 3 min after addition of the resorufin or resorufin plus inhibitor and measured at 37°C by following the decrease of the resorufin fluoresence in a SPF-500 spectrofluorimeter (SLM Aminco, Urbana IL, USA). The excitation and emission wavelengths were set at 530 and 585 nm respectively, according to the method of Beckmann (1991). K_i's of the competitive 2-fluoro-4-halophenolic substrates were calculated by fitting the data to the equation for competitive inhibition. The regression coefficient of the fits was generally $> 0{\cdot}97$.

Molecular orbital calculations

Molecular orbital calculations were carried out on a Silicon Graphics Iris 4D/85 using Quanta/Charmm (Molecular Simulations, UK). The semi empirical molecular orbital method was used, applying the AM1 Hamiltonian from the AMPAC program. All calculations were carried out with PRECISE criteria. For all calculations the self-consistent field was achieved. Geometries were optimized for all bond lengths, bond angles and torsion angles using the Fletcher–Powell criteria.

Frontier electron densities were calculated from HOMO (highest occupied molecular orbital) and HOMO-1 characteristics using the equation given by Fukui *et al.* (1954).

The outcomes of the *in vacuo* computer calculations were used as an approach to study relative differences within the series of related 2-fluoro-4-halophenols.

Calculation of log $P_{octanol}$

Log $P_{octanol}$ was calculated by the method of Rekker and De Kort (1979) and represents the logarithm of the partition of the compound between octanol and water.

Results

Identification of ^{19}*F nmr resonances of the urinary metabolites*

To identify the ^{19}F nmr resonances of the 2-fluoro-4-halophenylsulphates, 2-fluoro-4-halophenylglucuronides and 2-fluoro-4-halophenols, these 2-fluoro-4-halophenols were orally administered to the rats. The collected 24-h urine samples were analysed by ^{19}F nmr without enzyme pretreatment and after pretreatment with β-glucuronidase or arylsulphatase/β-glucuronidase. The ^{19}F nmr resonances of the glucuronides and sulphate esters could be identified from the disappearance and appearance of ^{19}F nmr resonances upon enzyme hydrolysis. The ^{19}F nmr resonances of the 2-fluoro-4-halophenol metabolites were identified by adding the respective reference compounds. Table 1 gives an example using the urine from a 2,4-difluorophenol-exposed rat. Upon treatment of the urine with β-glucuronidase the resonances at $-121{\cdot}0$ and $-133{\cdot}1$ ppm disappear giving rise to a proportional increase of the resonances at $-126{\cdot}1$ and $-137{\cdot}3$ ppm identified as 2,4-difluorophenol upon addition of this compound to the urine sample. This observation identifies the resonances of 2,4-difluorophenylglucuronide at $-121{\cdot}0$ and $-133{\cdot}1$ ppm. Treatment of the urine with arysulphatase/β-glucuronidase not only results in disappearance of the resonances of the phenylglucuronide, but also of the resonances at $-116{\cdot}5$ and $-129{\cdot}8$ ppm, accompanied by a proportional

Table 1. ^{19}F nmr characteristics of urine samples of a rat orally dosed with 2,4-difluorophenol. Resonances of the 2,4-difluorophenol and the fluoride anion were identified on the basis of added reference compounds. Chemical shift values are presented relative to $CFCl_3$.

Chemical shift (ppm)	Percentage of total fluorine intensity			Identified as
	I	II	III	
−116·5	17·3	19·2	0	2,4-difluorophenylsulphate
−121·0	29·7	0	0	2,4-difluorophenylglucuronide
−123·0	0·8	0·9	0·9	fluoride anion
−126·1	1·7	29·0	48·4	2,4-difluorophenol
−129·8	18·3	20·4	0	2,4-difluorophenylsulphate
−133·1	30·9	0	0	2,4-difluorophenylglucuronide
−137·3	1·7	30·7	50·8	2,4-difluorophenol

I, untreated urine; II, β-glucuronidase treated; and III, arylsulphatase/β-glucuronidase treated urine.

Table 2. Chemical shifts of ^{19}F nmr resonances of identified metabolites from 3-halo-fluorobenzenes and 2-fluoro-4-halophenols in urine 1:1 diluted with 0·2 M potassium phosphate pH 7·6 Chemical shifts are relative to $CFCl_3$.

Compound	Chemical shift (ppm)
Fluoride anion	−123·0
2,4-Difluorophenol	−126·1 (F4), −137·3 (F2)
2,4-Difluorophenylsulphate	−116·5 (F4), −129·8 (F2)
2,4-Difluorophenylglucuronide	−121·0 (F4), −133·1 (F2)
2-Fluoro-4-chlorophenol	−138·5
2-Fluoro-4-chlorophenylsulphate	−131·5
2-Fluoro-4-chlorophenylglucuronide	−134·9
2-Fluoro-4-bromophenol	−138·3
2-Fluoro-4-bromophenylsulphate	−131·4
2-Fluoro-4-bromophenylglucuronide	−134·8

increase of the resonances of 2,4-difluorophenol. This identifies the resonances of 2,4-difluorophenylsulphate at −116·5 and −129·8 ppm. In a similar way, ^{19}F nmr resonances of the various other phenylglucuronide and sulphate metabolites were identified. Table 2 summarizes the various ^{19}F nmr resonances thus characterized.

Ratio of sulphate-to-glucuronide conjugation upon in vivo *metabolism of 2-fluoro-4-halophenols*

Figure 1 presents the ^{19}F nmr spectra of the urine of rats orally dosed with 2,4-difluorophenol, 4-chloro-2-fluorophenol or 4-bromo-2-fluorophenol. Table 3 presents the quantification of the urine metabolite pattern of the 2-fluoro-4-halophenol-dosed rats as determined by ^{19}F nmr analysis. The data presented are the values obtained from two rats. From the results obtained, it follows that the phase II conjugation pattern of the phenols to give their ultimate urine metabolites is similar for all three 2-fluoro-4-halophenols. The glucuronidation is about 1·8 times as important as sulphation. Total urinary recovery was >95% for all three 2-fluoro-4-halophenols.

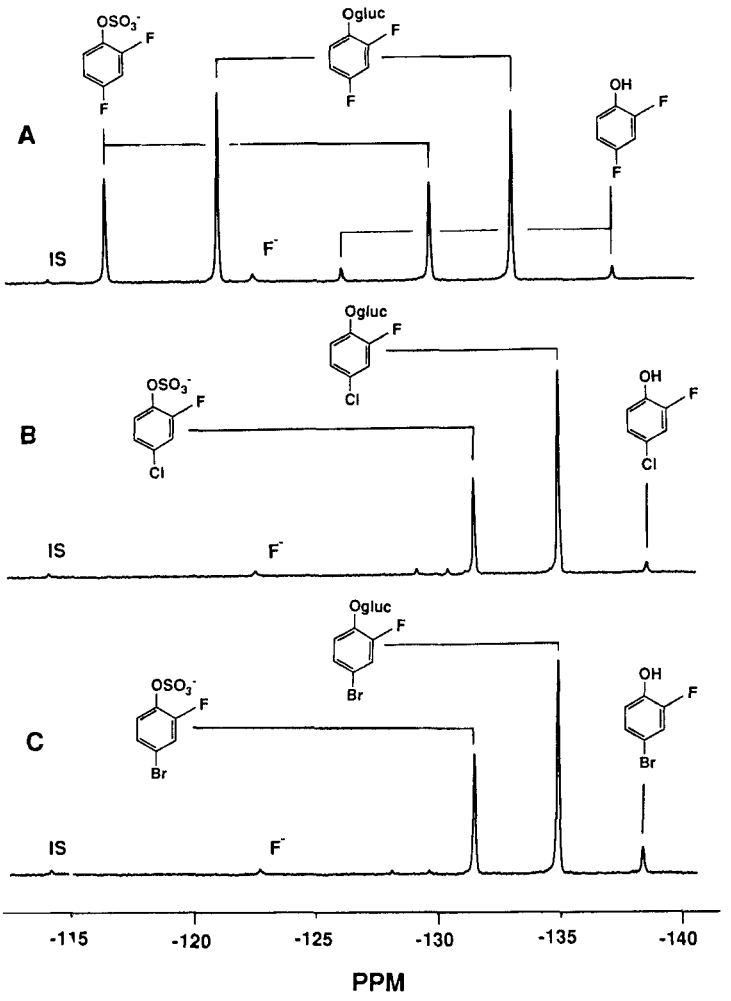

Figure 1. ^{19}F nmr spectra of the urine of rats orally dosed with 2-fluoro-4-halophenols. (A) 2,4-Difluorophenol, (B) 2-fluoro-4-chlorophenol, and (C) 2-fluoro-4-bromophenol. The resonance marked 'IS' is from the internal standard 4-fluorobenzoic acid.

Ratio of sulphation to glucuronidation for 2-fluoro-4-halophenol metabolites formed in vivo *from the corresponding 1-fluoro-3-halobenzenes*

Figure 2 and table 4 demonstrate the results from ^{19}F nmr analysis of 24-h urine samples of rats dosed orally with 1,3-difluorobenzene, 3-chloro-fluorobenzene and 3-bromo-fluorobenzene. Formation of sulphated and glucuronidated 2-fluoro-4-halophenol metabolites is observed for all 3-halo-fluorobenzenes. From these ^{19}F nmr analyses, the relative ratio of sulphated-to-glucuronidated 2-fluoro-4-halophenol metabolites could be derived, and the results are presented in table 4. Comparison of the data presented in table 4 and figure 2 with those obtained upon administration of the 2-fluoro-4-halophenols to the rats (figure 1, table 3) shows an important difference. The ratio of phenylsulphates to phenylglucuronides in rats dosed with the benzenes is 10–80 times higher than the same ratio in the rats dosed with phenols. Furthermore, the results presented in table 4 demonstrate a significant decrease in the ratio of phenylsulphate to phenylglucuronide with the change in the

Table 3. Quantification of the urinary metabolite patterns of a rat orally dosed with 2-fluoro-4-halophenol, as determined by ^{19}F nmr. The data presented ± SEM are the mean values of two rats.

Compound dosed and metabolite	Percentage of total fluorine intensity	Ratio of phenylsulphate to phenylglucuronide	Total recovery (% of dose)
2,4-Difluorophenol			
2,4-Difluorophenol	1·84/4·72		
2,4-Difluorophenylsulphate	34·3/36·4	0·59 ± 0·04	98 ± 2
2,4-Difluorophenylglucuronide	62·6/58·1		
4-Chloro-2-fluorophenol			
4-Chloro-2-fluorophenol	2·27/3·81		
4-Chloro-2-fluorophenylsulphate	36·7/29·8	0·55 ± 0·13	109 ± 29
4-Chloro-2-fluorophenylglucuronide	59·5/63·3		
4-Bromo-2-fluorophenol			
4-Bromo-2-fluorophenol	5·48/7·76		
4-Bromo-2-fluorophenylsulphate	34·5/31·7	0·57 ± 0·20	95 ± 9
4-Bromo-2-fluorophenylglucuronide	58·6/57·8		

C4-halosubstituent of the 2-fluoro-4-halophenol metabolite from fluorine > chlorine > bromine, a phenomenon not observed upon administration of the phenols to the rats (table 3). These results indicate an influence of the type of halogen at C3 in the 3-halo-fluorobenzenes on the phase II metabolism of their 2-fluoro-4-halophenol metabolites.

The results presented in table 5 demonstrate, using 1,3-difluorobenzene and 2,4-difluorophenol as the model compounds, that the marked difference in the ratio of sulphation to glucuronidation for the 4-halo-2-fluorophenol observed upon administration of the benzene or the phenol was also observed when the compounds were administered by intraperitoneal injection instead of oral gavage.

Further experiments were performed to investigate the possible explanations for the decreased phenylsulphate to phenylglucuronide ratio in the 3-halo-fluorobenzene urinary metabolite patterns in further detail.

Microsomal hydroxylation of 3-halo-fluorobenzenes

The decrease in the ratio of sulphation to glucuronidation could originate from an increased *in vivo* steady-state level of the 2-fluoro-4-halophenol metabolites formed from the 3-halo-fluorobenzenes in the order 4-fluoro- < 4-chloro- < 4-bromo-2-fluorophenol. Therefore, the rate of microsomal hydroxylation of the 3-halo-fluorobenzenes was investigated. Product formation was detected by ^{19}F nmr. The results obtained are presented in table 6. Figure 3 A presents a representative ^{19}F nmr spectrum of such an *in vitro* incubation. From the data presented in table 6, it can be concluded that the rate of P450 catalysed hydroxylation of the 3-halo-fluorobenzenes to give the 2-fluoro-4-halophenols shows a tendency to increase with the halogen varying from fluorine < chlorine < bromine, but the apparent V_{max} obtained for the hydroxylation of the three 4-halo-2-fluorophenols did not vary significantly. The K_m for the reaction shows a tendency to decrease going from 3-fluoro- to 3-chloro- to 3-bromo-fluorobenzene, but this effect is also not significant (table 6). Taken collectively, the results presented in table 6 do not point to a possible marked difference to be expected in the steady-state concentration

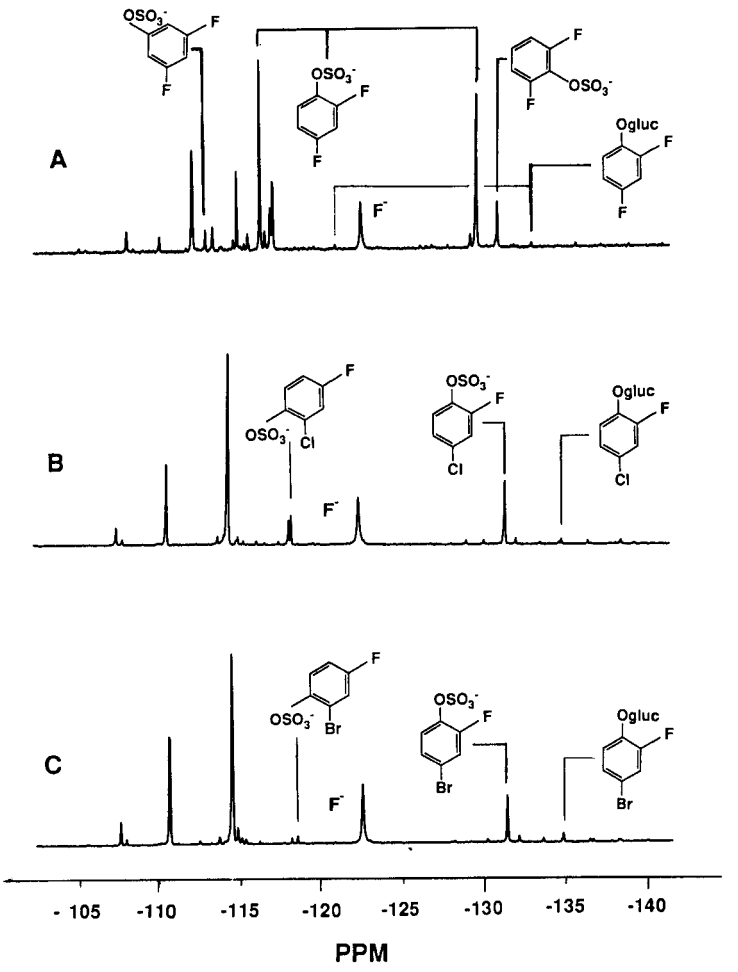

Figure 2. ^{19}F nmr spectra from the urine of rats orally dosed with 3-halo-fluorobenzenes. (A) 1,3-Difluorobenzene, (B) 3-chloro-fluorobenzene, and (C) 3-bromo-fluorobenzene. The major unidentified metabolites can most likely be ascribed to metabolites derived from glutathione conjugation pathways. These type of metabolites have been reported before as important metabolites in the urine of rats exposed to halogenated benzenes (Jollow *et al.* 1974, Jerina and Daly 1974, Monks *et al.* 1982, Zheng and Hanzlik 1991). Because these metabolites would not influence the results and conclusions of the present paper these metabolites were not identified.

of the 2-fluoro-4-halophenol metabolite in the order 1,3 difluorobenzene < 3-chloro-fluorobenzene < 3 bromo-fluorobenzene.

Microsomal glucuronidation of 2-fluoro-4-halophenols

The decrease in the ratio of sulphation to glucuronidation with the 4-halo-substituent from fluorine > chlorine > bromine could also originate from possible differences in the kinetic values for the conversion of the 2-fluoro-4-halophenols by the UDP-glucuronyltransferases. The kinetic apparent K_m and V_{max} for microsomal UDP-glucuronidation of the 2-fluoro-4-halophenols were determined and the results obtained are presented in table 7. From these data it can be concluded that there are significant differences, particularly between the apparent K_m for

Table 4. Quantification of the urinary metabolite patterns of male Wistar rats orally dosed with 3-halo-fluorobenzenes, as determined by ^{19}F nmr. Data presented ± SEM are the mean values of two rats.

Compound dosed and metabolite	Percentage of total fluorine intensity	Ratio of phenylsulphate to phenylglucuronide	Total recovery (% of dose)
1,3-Difluorobenzene			
2,4-Difluorophenol	no*		
2,4-Difluorophenylsulphate	35·9/35·4	47·5 ± 0·3	65 ± 24
2,4-Difluorophenylglucuronide	0·76/0·72		
3-Chloro-fluorobenzene			
4-Chloro-2-fluorophenol	no		
4-Chloro-2-fluorophenylsulphate	12·1/10·2	13·1 ± 1·2	97 ± 4
4-Chloro-2-fluorophenylglucuronide	1·02/0·72		
3-Bromo-fluorobenzene			
4-Bromo-2-fluorophenol	no		
4-Bromo-2-fluorophenylsulphate	7·82/7·58	5·7 ± 1·2	78 ± 7
4-Bromo-2-fluorophenylglucuronide	1·75/1·09		

* Not observed.

Table 5. Urinary phenolic metabolite patterns after the intraperitoneal injection of 200 μmol 1,3-difluorobenzene or 2,4-difluorophenol.

Compound dosed and metabolite	Percentage of total fluorine intensity	Ratio of phenylsulphate to phenylglucuronide	Total recovery (% of dose)
1,3-Difluorobenzene			
2,4-Difluorophenol	no*		
2,4-Difluorophenylsulphate	42·5	34·6	74
2,4-Difluorophenylglucuronide	1·23		
2,4-Difluorophenol			
2,4-Difluorophenol	1·6		
2,4-Difluorophenylsulphate	22·9	0·31	107
2,4-Difluorophenylglucuronide	73·2		

Compounds were administered to male Wistar rats (400 g body weight) and metabolites determined by ^{19}F nmr as described in Materials and methods.

* Not observed.

the UDP-glucuronyltransferase catalysed conjugation of the three 2-fluoro-4-halophenols. When the 4-halo substituent changes from fluorine to chlorine to bromine the apparent K_m decreases. Thus, as long as the phenol concentrations are at sub-saturating levels, the glucuronidation of the 2-fluoro-4-halophenols can be expected to increase with the 4-halosubstituent changing from fluorine < chlorine < bromine.

Cytosolic sulphation of 2-fluoro-4-halophenols

The decrease in the ratio of 2-fluoro-4-halophenol sulphation to glucuronidation with the halo-substituent changing from fluorine to chlorine to bromine could also originate from the differences in the apparent K_m and/or V_{max} of the sulphotransferases for the 2-fluoro-4-halophenols. Measurement of the K_m of the sulphation appeared to be hampered by the low values of these K_m's, i.e., below the detection limit of the ^{19}F nmr measurements (i.e. 1 μM). To obtain information about the relative affinities of the sulphotransferases for the three 2-fluoro-4-halophenols, K_i's

Table 6. Formation of 2-fluoro-4-halophenols from the 3-halo-fluorobenzene substrates by liver microsomes from the male Wistar rat. Data presented are the mean of three experiments ± SEM.

Substrate concentration (mM)	2-Fluoro-4-halophenol formation (nmol. $P450^{-1}.10\,min^{-1}$)		
	1,3 difluorobenzene	3-chloro-fluorobenzene	3-bromo-fluorobenzene
0·5	0·34 ± 0·09	0·39 ± 0·08	0·50 ± 0·05
1·0	0·49 ± 0·11	0·64 ± 0·07	0·75 ± 0·15
2·0	0·81 ± 0·18	0·97 ± 0·24	1·15 ± 0·23
Parameters from kinetic analysis			
V_{max}	1·71	1·95	2·14
K_m	2·27	2·03	1·76
r^*	0·991	0·999	0·997

*r presents the correlation coefficient of the fit of the experimental data to the Michaelis–Menten equation $v = V_{max}*[S]/(K_m + [S])$.

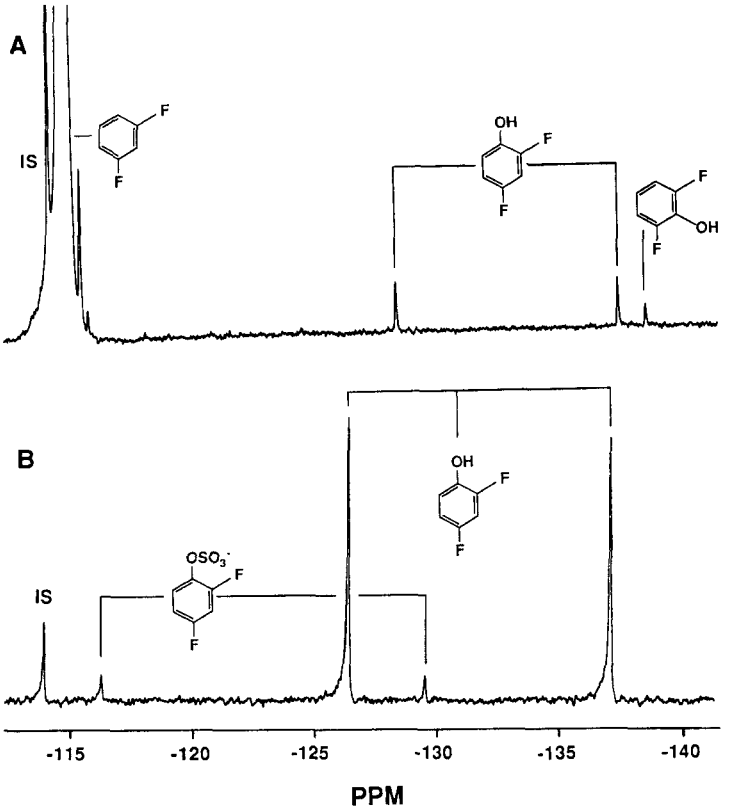

Figure 3. Representative ^{19}F nmr spectra of *in vitro* microsomal and cytosolic incubations. (A) ^{19}F nmr spectrum of the ethyl acetate extract of a microsomal incubation with 2·0 mM 1,3-difluorobenzene, and (B) ^{19}F nmr spectrum of a cytosolic incubation with 0·5 mM 2,4-difluorophenol and 240 μM PAPS. The resonance marked 'IS' is from the internal standard 4-fluorobenzoic acid.

Table 7. Kinetic parameters for the microsomal UDP-glucuronyltransferase catalysed 2-fluoro-4-halophenol conjugation reaction. Microsomal incubations were carried out at 37°C in 0·1 M potassium phosphate (pH 7·6) containing 0·36 mg microsomal protein/ml. Data presented are the mean values ± SEM (n = 6–8).

Substrate	K_m (mM)	V_{max} (μmol.mg^{-1}.min^{-1})	Range [S] used (mM)
2,4-Difluorophenol	0·79 ± 0·07	1·07 ± 0·06	0·25–4·00
4-Chloro-2-fluorophenol	0·18 ± 0·03	1·16 ± 0·07	0·10–1·00
4-Bromo-2-fluorophenol	0·11 ± 0·02	0·86 ± 0·06	0·03–0·40

Table 8. Kinetic parameters for the sulphotransferase catalysed 2-fluoro-4-halophenol conjugation. Rates of conversion were determined by ^{19}F nmr analysis of cytosolic incubations with various concentrations of the fluorophenols and/or the cofactor PAPS, which was either synthesized *in situ* from ATP and sulphate, or added to give the final concentrations as indicated. K_m's were too low to be detected. K_i's presented were for the 2-fluoro-4-halophenol inhibition of resorufin sulphation. The data presented for K_i are mean ± SEM (n = 3). Data presented for the rates of conversion are data from independent measurements (n = 2 for the measurements with *in situ* PAPS synthesis and N = 1 for the measurements with PAPS addition).

[PAPS] (μM)	[Phenol] (mM)	2,4-Difluorophenol	4-Chloro-2-fluorophenol activity (nmol.mg^{-1},min^{-1})	4-Bromo-2-fluorophenol
*In situ**	0·1	1·09/1·17	0·94/1·04	1·05/1·17
In situ	0·2	1·00/1·04	1·03/1·17	1·04/1·14
In situ	0·3	1·00/1·14	1·10/1·18	1·03/1·05
In situ	0·5	0·97/1·08	1·13/1·11	1·10/1·21
140	0·5	1·38	1·35	1·46
240	0·5	1·67	1·45	1·56
340	0·5	2·05	1·73	1·84
K_i(μM)		0·20 ± 0·02	0·10 ± 0·01	0·07 ± 0·01

* *In situ*, means that the cofactor of the reaction, PAPS was synthesized in the incubation from ATP and sulphate by the cytosolic enzymes sulphurylase and APS-phosphokinase.

for the 2-fluoro-4- halophenol-mediated competitive inhibition of the sulphation of resorufin were determined. The results obtained are presented in table 8. The affinity of the sulphotransferases for the three 2-fluoro-4-halophenols, represented by the K_i for competitive inhibition of resorufin sulphation, is high (low K_i); the affinity increases (K_i decreases) with the 4-halo substituent changing from fluorine < chlorine < bromine.

Table 8 also presents results for the rate of sulphation of the three 4-halo-2-fluorophenols under various incubation conditions as determined by ^{19}F nmr. Figure 3 B presents a representative ^{19}F nmr spectrum of a cytosolic sulphotransferase incubation. The data presented in table 8 demonstrate that the rate of conversion of the three 4-halo-2-fluorophenols was always similar. When PAPS was added to the assay instead of derived from *in situ* synthesis in the incubation, the rates of conversion were higher, suggesting that in the *in vitro* system where the PAPS had to be synthesized from ATP and sulphate by sulphurylase and APS-phosphokinase, the rate of sulphation is limited by the biosynthesis of the cofactor, rather than by limitation of the phenol concentration. When this also holds for the *in vivo* situation the *in vivo* rate of conversion of the three 2-fluoro-4-halophenols in sulphate conjugation reactions can be expected to be comparable as well, i.e. determined mainly by the rate of syntheses of the cofactor PAPS.

Table 9. Calculated electronic and structural characteristics of 2,4-difluorophenol (F), 4-chloro-2-fluorophenol (Cl) and 4-bromo-2-fluorophenol (Br).

Parameter	F	Cl	Br
Van der Waals volume (Å^3)	99	105	106
Log $P_{octanol}$	1·95	2·48	2·67
E(HOMO)(eV)*	−9·3	−9·3	−9·3
Dipole moment (Debeye)*	1·5	1·4	1·4
HOMO density on oxygen*	0·13	0·13	0·12
HOMO/HOMO − 1 density on oxygen*	0·25	0·24	0·22
Net charge on oxygen*	−0·23	−0·24	−0·24

* Data presented are means between the two possible orientations of the hydroxyl substituent; in plane with the aromatic ring with the hydrogen orientated either towards or away from the fluorine at C2.

Taken collectively, the results presented in table 8 indicate that a change in the kinetic characteristics of the sulphate conjugation of the 2-fluoro-4-halophenols with a change in the halo substituent, is unlikely to be a major factor underlying the change in the ratio of sulphation to glucuronidation of the 2-fluoro-4-halophenol metabolites of the 3-halo-fluorobenzenes.

Calculated electronic and structural characteristics of the 2-fluoro-4-halophenols

To study whether electronic and/or structural differences between the three 2-fluoro-4-halophenols may provide an insight into factor(s) underlying the marked difference in their apparent K_m for glucuronidation, molecular orbital computer calculations were performed and their log $P_{octanol}$ calculated. Table 9 presents the results obtained. Electronic characteristics presented focus on the oxygen atom of the hydroxyl moiety because for chemical reactivity in the nucleophilic attack on the cofactor of the UDP-glucuronyltransferase reaction, electronic characteristics of this oxygen reaction centre are of importance. The results demonstrate that the net atomic charge on the oxygen as well as the energy and the density of the reactive electrons on the oxygen in the HOMO, parameters that will influence relative differences in reactivity between the phenols for a nucleophilic attack on the cofactor (Fleming 1976), do not show any differences when the halogen substituent in the 2-fluoro-4-halophenol is changed. Furthermore, the dipole moment of the molecule is similar for the three 2-fluoro-4-halophenols. The main differences observed between the three 2-fluoro-4-halophenols are in the relative hydrophobicity of these compounds, i.e. in log $P_{octanol}$, and in their Van der Waals volume. Both parameters increase when the halo substituent in the 2-fluoro-4-halophenol varies from fluorine to chlorine to bromine.

Discussion

In the present study, the effect of variation in the halo substituent on the type of phase II conjugation of 2-fluoro-4-halophenol metabolites, formed from 3-halo-fluorobenzenes, was studied. ^{19}F nmr was used for the identification and quantification of the several hydroxylated and conjugated metabolites in *in vivo* and *in vitro* samples. The ratio of sulphation to glucuronidation observed for the 2-fluoro-4-halophenol metabolites after their *in vivo* formation from the corresponding 3-halo-fluorobenzenes, decreased significantly going from the fluoro to the chloro to the bromo derivative. Thus, the type of halogen substituent opposite the hydroxyl moiety influences the type of phase II conjugation of the phenolic

benzene metabolites. Results from further experiments described here provided insight in the kinetic factors causing this effect. Direct administration of the 2-fluoro-4-halophenols to the rats resulted in urine metabolite patterns with a ratio of phenylsulphates to phenylglucuronides that was similar for the fluoro, chloro and bromo derivatives. This result demonstrates that the differences in the ratio of the two phase II conjugations with the type of halogen is not observed under conditions where the phenol concentrations are relatively high, i.e. possibly saturating for the phase II enzymes involved. From this observation it can be concluded that the influence of the halogen substituent on the type of phase II conjugation of the phenolic metabolites is not likely to result from an influence of the type of halogen substituent in the 2-fluoro-4-halophenol on the V_{max} for its sulphation or glucuronidation.

UDP-glucuronyltransferases are known to have relatively high K_m and high V_{max} compared with arylsulphotransferases, which are known to have low K_m and low V_{max} (Mulder 1984, Koster and Mulder 1982, Evelo *et al.*, 1984). This implies that the effect of the halogen on the phenol conjugation might result from (1) a difference in the steady-state level of the phenolic metabolite, i.e. in the rate of its formation from the benzene by P450, increasing in the order fluorine < chlorine < bromine, or from (2) a decrease in K_m for glucuronidation going from fluorine to chlorine to bromine, or (3) and increase in K_m for sulphation from fluorine to chlorine to bromine. *In vitro* experiments were performed to investigate these latter possible explanations for the decreased 2-fluoro-4-halophenylsulphate to 2-fluoro-4-halophenylglucuronide ratio in the 3-halo-fluorobenzene urinary metabolite patterns. The microsomal hydroxylation of the 3-halo-fluorobenzenes to 2-fluoro-4-halophenol metabolites was demonstrated to show no significant changes in the apparent V_{max} and K_m going from fluorine to chlorine to bromine. This means that, assuming similar body distribution and thus liver concentration of the three 3-halo-fluorobenzenes upon *in vivo* exposure, marked differences in the steady-state concentration of the 2-fluoro-4-halophenol with the halo substituent changing from fluorine to chlorine to bromine are not to be expected. Second, the data for the glucuronidation obtained from microsomal incubations demonstrated a significant decrease in the apparent K_m for the three 2-fluoro-4-halophenols with the halo substituent changing from fluorine to chlorine to bromine. This might be an important factor contributing to the effect of the halo substituent on the decrease in the ratio sulphation to glucuronidation with the type of halo substituent in the phenol metabolite. Third, the decrease in the ratio of sulphation to glucuronidation varying with the C4-halo substituent of the 2-fluoro-4-halophenol from fluorine to chlorine to bromine could originate from a decrease in the affinity of the sulphotransferases reflected by an increase in K_i for the competitive inhibition of resorufin sulphation. From the results obtained, however, it can be concluded that sulphotransferases show K_i's that decrease instead of increase from 4-fluoro- to 4-chloro- to 4-bromo-2-fluorophenol. This means that the decrease in the ratio of sulphation to glucuronidation varying with the C4-halo substituent cannot originate from the differences in affinity of the sulphation enzymes for the three 2-fluoro-4-halophenols. Thus the main effect that might be responsible for the difference in the ratio of sulphation to glucuronidation of the 4-halo-2-fluorophenol metabolites of the benzenes, might be a marked decrease in the apparent K_m for glucuronidation from the fluorine to the chlorine to the bromine analogue. The following kinetic consideration further supports this conclusion. When the actual *in vivo* rate of P450

catalysed hydroxylation of the 3-halofluorobenzenes and, thus, the steady-state concentration of the 4-halo-2-fluorophenols, as well as the rate of sulphation of the 4-halo-2-fluorphenols would be similar, the relative differences in the ratio sulphation to glucuronidation will be caused by the relative differences in the kinetic parameters for the glucuronidation. Although absolute kinetic values obtained in *in vitro* studies cannot be extrapolated as such to the *in vivo* situation (Dutton 1980) one can assume that relative differences observed *in vitro* can be used as a basis to interpret relative differences observed *in vivo*. The ratio of sulphation to glucuronidation for the chlorine and bromine analogue can then be compared with the ratio obtained for the fluorine analogue in the following way. The ratio sulphation to glucuronidation is proportional to the rate of sulphation divided by the rate of glucuronidation ($=v_{sulph}/v_{gluc}$). Substitution of the Michaelis–Menten equation ($v = V_{max}*[S]/(K_m + [S])$ for the rate of glucuronidation (v_{gluc}) and assuming that the rate of sulphation (v_{sulph}) is similar for the various 2-fluoro-4-halophenols, it can be derived that the ratio sulphation to glucuronidation for the chlorine analogue, divided by the ratio obtained for the fluorine analogue will equal;

$$v_{gluc}\,(\text{fluorine})/v_{gluc}\,(\text{chlorine}) = \frac{\{V_{max}*[S]/(K_m + [S])\}_{gluc\ \text{fluorine}}}{\{V_{max}*[S]/(K_m + [S])\}_{gluc\ \text{chlorine}}}.$$

when $[S] \ll K_m$, which might be the case for glucuronidation of the 2-fluoro-4-halophenol formed from the 3-halofluorobenzene *in vivo*, this equation becomes:

$$v_{gluc}\,(\text{fluorine})/v_{gluc}\,(\text{chlorine}) = \frac{\{V_{max}*[S]/K_m\}_{gluc\ \text{fluorine}}}{\{V_{max}*[S]/K_m\}_{gluc\ \text{chlorine}}}.$$

Substitution of the apparent V_{max} and K_m presented in table 7 into this equation, and in a similar equation derived for the bromine analogue, results in a ratio sulphation to glucuronidation for the chlorine and bromine analogue that would be respectively 0·21 and 0·17 times the ratio of sulphation to glucuronidation observed for the fluorine analogue. Thus, based on the ratio of sulphation to glucuronidation of the 2-fluoro-4-halophenol metabolite of 1,3-difluorobenzene of 47·5 (table 4) this calculation would predict a ratio of 10·0 and 8·0 for the chlorine and the bromine analogue. Comparison of these values with the actual values observed, respectively 13·1 and 5·7 (table 4), indicates that on the basis of the relative differences in kinetic parameters for glucuronidation, a major part of the differences in the ratio of sulphation to glucuronidation of the 2-fluoro-4-halophenol metabolites can actually be explained.

Differences between the values thus calculated and the experimentally determined ratios might be due to other factors influencing the ratios to a minor extent. Such factors may include differences between the various 2-fluoro-4-halophenols in the extent of excretion of their glucuronide conjugate into bile, in the extent of hydrolysis of the glucuronide by the gut flora, reabsorption or non-fully justifiable assumptions made in the kinetic calculation.

Additional results of the present paper provide an insight into the main electronic and structural characteristics of the three 2-fluoro-4-halophenols. The main differences observed are an increase in the $\log P_{octanol}$ and in the Van der Waals volume of the 2-fluoro-4-halophenols with the halogen changing from fluorine to chlorine to bromine. An increase in the Van der Waals volume and, as a result, in steric hindrance, would be expected to increase instead of decrease K_m. Therefore it may be concluded that the decrease in the apparent K_m for glucuronidation going

from 2,4-difluoro- to 4-chloro-2-fluoro- to 4-bromo-2-fluorophenol might best be explained by the increased hydrophobicity of the compound. This increased hydrophobicity, reflected by an increased $\log P_{octanol}$, can be expected to result in an increased possibility for accumulation of the phenol in the membrane environment of the UDP-glucuronyltransferases, thus resulting in an increased local concentration and a decreased apparent K_m. The observed correlation ($r = -0{\cdot}98$) between $\log P_{octanol}$ and the apparent K_m for glucuronidation further supports this view.

In conclusion, the results of the present study clearly demonstrate that the halogen substitution pattern can be a factor influencing the type of phase II conjugation of a phenolic benzene metabolite. Our results particularly demonstrate the influence of a halogen opposite the incorporated hydroxyl moiety and indicate that the effects are mainly due to a decreased apparent K_m for glucuronidation with the halogen changing from fluorine to chlorine to bromine. This decrease in the apparent K_m for glucuronidation might best be ascribed to an increase in the hydrophobicity of the substrate resulting in better possibilities for accumulation of the 2-fluoro-4-halophenol in the membrane environment of the glucuronyl transferases, giving rise to an apparently decreased K_m. Together, these effects result in significantly decreased ratios of sulphation to glucuronidation of the 4-hydroxylated metabolites from 3-halofluorobenzenes with the halogen changing from fluorine to chlorine to bromine. Whether similar changes in the ratio of the phase II conjugation reactions will also be observed for hydroxyl moieties incorporated *meta* or *ortho* with respect to the varying halogen substituent, remains a topic for future research.

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