Glucuronidation of Entacapone, Nitecapone, Tolcapone, and Some Other Nitrocatechols by Rat Liver Microsomes

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Purpose. Nitrocatechol COMT inhibitors are a new class of bioactive compounds, for which glucuronidation is the most important metabolic pathway. The objective was to characterize the enzyme kinetics of nitrocatechol glucuronidation to improve the understanding and predicting of the pharmacokinetic behavior of this class of compounds.

Methods. The glucuronidation kinetics of seven nitrocatechols and 4-nitrophenol, the reference substrate for phenol UDP-glucuronosyltransferase activity, was measured in liver microsomes from creosote-treated rats and determined by non-linear fitting of the experimental data to the Michaelis-Menten equation. A new method that combined densitometric and radioactivity measurement of the glucuronides separated by HPTLC was developed for the quantification.

Results. Apparent K_m values for the nitrocatechols varied greatly depending on substitution pattern being comparable with 4-nitrophenol (0.11 mM) only in the case of 4-nitrocatechol (0.19 mM). Simple nitrocatechols showed two-fold V_{max} values compared with 4-nitrophenol (68.6 nmol min^{-1} mg^{-1}), while all disubstituted catechols exhibited much lower glucuronidation rate. V_{max}/K_m values were about 10 times higher for monosubstituted catechols compared to disubstituted ones. The kinetic parameters for COMT inhibitors were in the following order: K_m nitecapone >> entacapone > tolcapone; V_{max} nitecapone > entacapone > tolcapone; V_{max}/K_m tolcapone > nitecapone > entacapone.

Conclusions. Nitrocatechols can in principle be good substrates of UGTs. However, substituents may have a remarkable effect on the enzyme kinetic parameters. The different behaviour of nitecapone compared to the other COMT inhibitors may be due to its hydrophilic 5-substituent. The longer elimination half-life of tolcapone *in vivo* compared to entacapone could not be explained by glucuronidation kinetics *in vitro*.

KEY WORDS: nitrocatechols; glucuronidation; rat liver microsomes; Michaelis-Menten kinetics; HPTLC.

INTRODUCTION

Entacapone, tolcapone, and nitecapone are 3-nitrocatecholic inhibitors of catechol O-methyltransferase (COMT, EC 2.1.1.6). Entacapone and tolcapone are undergoing clinical trials as adjuncts to L-dopa/decarboxylase inhibitor treatment of Parkinson's disease. In spite of their catecholic structure the inhibitors are poor substrates of COMT. The major urinary conjugates of entacapone and nitecapone are glucuronides and they are rapidly excreted in humans with the elimination half-lives after oral administration of 0.3, and 0.3–0.45 hours, respectively (1,2). Tolcapone, however, is excreted much more slowly ($t_{1/2}$ = 2.3 hours) (3), even though it shares with entacapone the common structural features responsible for their high inhibitory activity on COMT, namely a nitro-group in position 3 and another strongly electron withdrawing, larger, hydrophobic substituent in position 5 (4).

Drugs containing phenolic hydroxyls usually undergo direct glucuronidation catalysed by UDP-glucuronosyltransferases (UGTs, EC 24.1.17) and are rapidly excreted in the urine. In metabolism of catechols, however, methylation and sulphation compete with glucuronidation for conjugating the two adjacent phenolic hydroxyls. For example, in the case of catecholamines, methylation and sulphation often prevails over glucuronidation in humans (5).

The structure-activity relationships related to the metabolism and elimination kinetics of COMT inhibitors are not understood, although the significance of this information to drug design is obvious. Surprisingly little is known about the structure-activity relationships of competing catechol conjugations in general, considering the great importance these reactions have in the metabolism of catechols and the potential for metabolic interactions of catecholic drugs with neurotransmitters, hormones and other endogenous catechols.

The purpose of this study was to compare the kinetic properties of COMT inhibitors as substrates of UGTs. To characterize the substrate properties of the core structure, the 3-nitrocatechol group, of COMT inhibitors, simple nitrocatechol analogs were included in the study. Rat liver was chosen as the source of the enzyme. The most abundant urinary metabolites of entacapone and nitecapone in rats, like in humans, are glucuronides (6,7) and pre-clinical pharmacokinetic, toxicokinetic, and metabolic studies on COMT inhibitors have mainly used rat as the animal model. UGT activities were compared to those measured using 4-nitrophenol, which is a structurally relevant and generally used standard substrate. A new, cost effective HPTLC method with densitometry was developed and used to measure enzyme kinetics for various substrates.

MATERIALS AND METHODS

Chemicals

3-Nitrocatechol, 3,5-dinitrocatechol, entacapone, entacapone(Z)-isomer, nitecapone, and tolcapone were kindly supplied by Orion-Farmos Pharmaceuticals (Espoo, Finland). 4-Nitrophenol, 4-nitrocatechol, 4-nitrophenyl-β-D-glucuronide, and UDP-glucuronic acid were obtained from Sigma Chemical Company (St. Louis, Missouri, USA) and UDP-glucuronic acid [glucuronyl-^{14}C(U)] from Du Pont (Boston, USA). Acetonitrile was of HPLC grade (Rathburn Chemicals, Scotland, UK) and the other reagents were of analytical grade. The chemical composition of the coal-tar derived creosote oil was analyzed to contain naphthalene and other PAHs in high amounts in a mixture of about a hundred of compounds as reported by Nylund *et al.* (8).

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ABBREVIATIONS: COMT, catechol *O*-methyltransferase; UGT, UDP-glucuronosyltransferase; HPTLC, high performance thin layer chromatography; PAH, polycyclic aromatic hydrocarbon.

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Animals and Treatment

Male Wistar rats (338 ± 20 g, n = 24) were obtained from the Helsinki University Breeding Centre (Finland). The use of the animals was approved by the local Ethical Committee for Animal Studies, and the guidelines of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes were followed. The rats were divided into three subgroups (eight in each group) and treated with creosote oil (200 mg/4 ml olive oil/kg) by gavage 72 h and 48 h before they were killed, or with phenobarbital (80 mg/3 ml saline/kg) intraperitoneally 72 h, 48 h, and 24 h before killed. The control rats were not pretreated. The animals were anesthetized with CO_2 before they were killed and autopsied. The livers were perfused *in situ* with PBS solution, removed onto ice, weighed, and stored at −70°C.

Preparation of Microsomes

Microsomes were prepared as previously described (9) and protein concentrations were determined according to the method of Lowry *et al.* (10) using commercial protein standards. Microsomes from each group of rats (n = 8) were pooled and stored at −18°C until used.

Kinetic Measurements by HPLC

The glucuronidation kinetics of entacapone and tolcapone were determined for liver microsomes from untreated rats and rats pretreated with creosote or phenobarbital for the purpose to use the microsomes of highest activity in further studies. An incubation mixture contained 5 mM $MgCl_2$, 5 mM UDP-glucuronic acid, 0.1 mg microsomal protein, and 0.025–2.5 mM tolcapone or entacapone [added in 2 µl dimethylsulphoxide at which level (final concentration 0.8%) the solvent did not affect the glucuronidation rate] in 250 µl of 50 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 7.4). Glucuronide formation was found to be linear for at least 60 min and microsomal protein concentration up to 1.5 mg/ml and 30 min was chosen as the incubation time. After the incubation at 37°C the reactions were stopped by adding 250 µl cold 1 M trichloroacetic acid. The precipitated proteins were centrifuged, and the supernatants were analyzed by HPLC (Hewlett-Packard, Germany) using an RP-18 column (Hibar 125-4 LiChrosorb 5 µm) as the stationary phase and a mixture of 25 mM NaH_2PO_4 buffer (pH 2.2) and acetonitrile (6:4 v/v for tolcapone and 7:3 v/v for entacapone) as the mobile phase. The flow rate was 1 ml/min and the temperature 35°C. Glucuronide peak areas of tolcapone (RT = 3.0 min) and entacapone (RT = 3.2 min) were monitored at 278 and 305 nm, respectively.

Kinetic Measurements by HPTLC

Pooled liver microsomes from six creosote-treated rats were used as an enzyme source for the study on glucuronidation kinetics of 4-nitrophenol, 4-nitrocatechol, 3-nitrocatechol, 3,5-dinitrocatechol, entacapone, its (Z)-isomer, nitecapone, and tolcapone (see Fig. 1). An incubation mixture contained 5 mM $MgCl_2$, 5 mM UDP-glucuronic acid, 0.015–0.1 mg microsomal protein, Triton-X-100 0.4 mg/mg protein, and a varying concentration of the aglycone in 250 µl of 50 mM Tris-HCl buffer (pH 7.4). Tolcapone was added in 2 µl dimethylsulfoxide because of its low solubility in the buffer used. To quantify the glucuronides formed, 0.1 µCi UDP-[^{14}C]glucuronic acid was added to the samples containing the highest amount of the aglycone in the kinetic series. The linearity of the reaction was tested with respect to time and protein concentration and for each aglycone the lowest protein concentration that the sensitivity of the method allowed was chosen. Incubation time was 15 min and after the incubation at 37°C the reactions were stopped by adding 250 µl of cold acetonitrile. The tubes were kept in an ice-bath for 10 minutes and centrifuged for 10 minutes (14000 rpm) to remove the precipitated proteins.

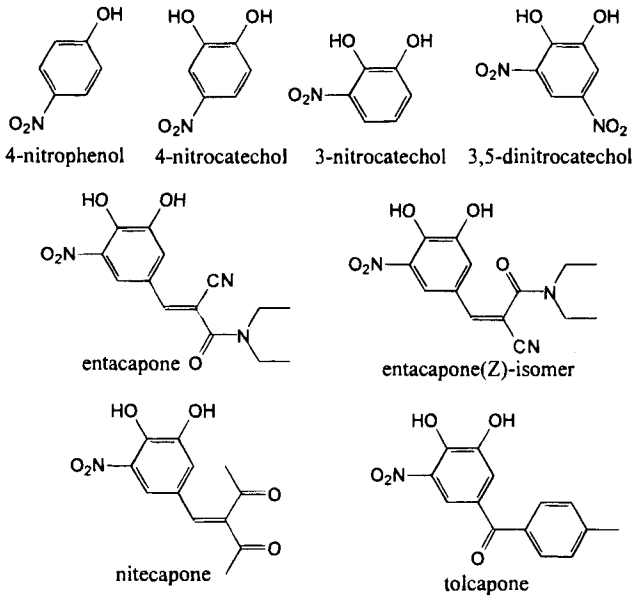

Fig. 1. Structures of the investigated compounds.

Four different volumes (usually between 1 and 12 µl) of the sample containing the radioactively labeled glucuronide were applied to both ends of an HPTLC plate (10 × 10 cm, RP-18W_{254F}, Merck, Darmstadt, Germany) to obtain calibration curves for the quantification of the other samples. Other samples were applied in duplicate (10 or 20 µl each) and three samples could be analyzed on one end of the plate. The Linomat IV spray-on technique (Camag, Muttenz, Switzerland) was utilized in the sample application. The plates were developed in a horizontal chamber (Camag) using a mixture of 50 mM NaH_2PO_4 (pH 2.2) and acetonitrile (6:4 v/v). Development to a distance of 40 mm took 20 min. The air-dried plates were scanned with a Camag TLC Scanner II connected to a computer running the Camag Cats program version 3.17. After the densitometric evaluation three spots containing the radioactively labeled glucuronide were scraped from each HPTLC plate directly into liquid scintillation counting vials, 5 ml of Optiphase HiSafe 2 (FSA Laboratory Supplies, Loughborough, UK) was added, and the radioactivity of the three samples was measured in a liquid scintillation counter (Wallac 1410, Turku, Finland). The amount of the glucuronide was calculated by taking the mean value of these quantifications and correcting it with the background caused by the plain HPTLC plate. Glucuronide concentrations of the scraped standards in the densitometric analysis could now be fed into the Cats program. The glucuronides of the other samples were then quantified by densitometry using

polynomial regression calculated from peak heights. The reliability of the developed method was studied by quantifying the same incubation samples using 4-nitrophenol as the substrate (I) by a validated densitometric method using 4-nitrophenyl-β-D-glucuronide as the reference standard (11) and (II) by our new method combining densitometry and radioactivity measurement (Fig. 3).

Kinetic Data Analysis

The apparent enzyme kinetic parameters K_m and V_{max} were determined by fitting the Michaelis-Menten equation to the data obtained from the HPLC or HPTLC studies. Data analysis was performed using the Leonora Steady-state Enzyme Kinetics program version 1.0 by A. Cornish-Bowden (Cornish-Bowden 1994). For each analysis, the program was allowed to choose the method of fitting and the system of weighing.

RESULTS

Kinetic Method

A new method was developed for quantitation of glucuronides. The method combines densitometric and radioactivity measurement of the glucuronide spots separated by HPTLC analysis. ^{14}C-labeled UDP-glucuronic acid was added only to one sample in each kinetic series in order to calibrate the absolute amount of glucuronide by liquid scintillation counting. The HPTLC method was able to separate all the glucuronides from the respective aglycones and from UDP-glucuronic acid under the same chromatographic conditions. The R_F values of the glucuronides varied between 0.39 and 0.58, while UDP-glucuronic acid moved near to the solvent front (R_F = 0.93) and thus did not interfere with the scraping of the ^{14}C-labeled glucuronides from the plates. A typical densitogram from an incubation sample is shown in Fig. 2. Correlation between the glucuronidation rates of 4-nitrophenol determined using the present method and using a conventional densitometric quantitation based on a reference standard (4-nitrophenyl-β-D-glucuronide) is shown in Fig. 3.

Effect of Induction

At least 13 isoenzymes with different substrate preference are known to contribute to UGT activity in rat liver (12). The effect of two enzyme inducers, creosote and phenobarbital, on UGT activity towards nitrocatechol drugs was studied using entacapone and tolcapone as test substrates to probe for increased activity of relevant isoenzymes. Creosote increased the V_{max} values about twofold and reduced the K_m values almost by half for both entacapone and tolcapone compared to microsomes from untreated rats (Table I). Phenobarbital showed to increase only the glucuronidation rate of tolcapone. Microsomes from the creosote-treated rats were therefore used in further studies.

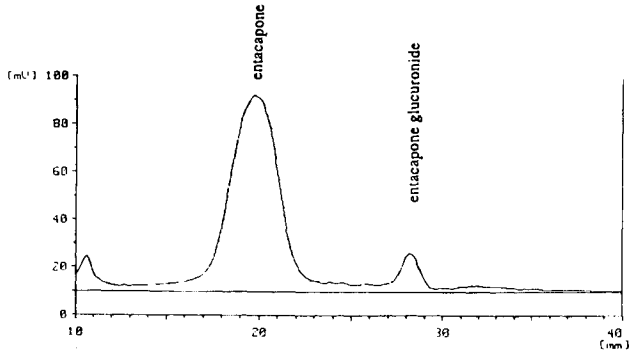

Fig. 2. Densitogram obtained from an incubation sample of entacapone. UDP-glucuronic acid moved near to the solvent front and is not visible in the densitogram.

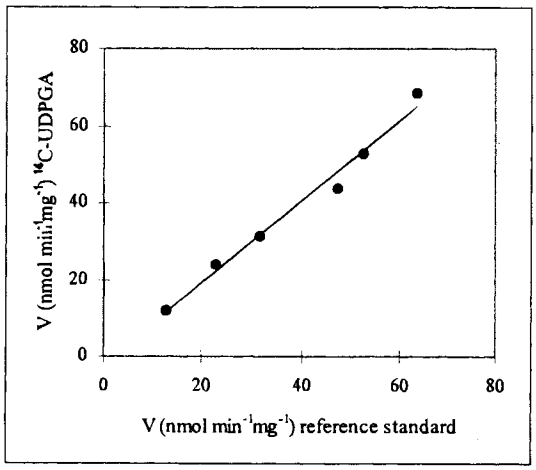

Fig. 3. Glucuronidation rates of different initial concentrations of 4-nitrophenol by rat liver microsomes assayed with the aid of a reference standard of 4-nitrophenyl-β-D-glucuronide and with the aid of UDP-[^{14}C]glucuronic acid, r = 0.9920.

Glucuronidation Kinetics

The kinetics of glucuronidation was studied by measuring the initial velocity of the reaction as a function of aglycone concentration and by non-linear fitting of the data to estimate the apparent Michaelis-Menten parameters K_m, V_{max} and V_{max}/K_m. Glucuronidation of nitrocatechols followed Michaelis-Menten kinetics reasonably well as shown in Fig. 4A for 4-nitrocatechol. Only in the case of nitecapone did the apparent K_m value appear to be so high that the solubility limit of the drug prevented the proper characterization of the kinetic curve (Fig. 4B). Values of apparent kinetic parameters for all substrates are shown in Table II.

Table I. Glucuronidation of Entacapone and Tolcapone by Liver Microsomes from Rats Pretreated with Enzyme Inducers

	Treatment	K_m (mM)	V_{max} (A[a]/min/mg)
Entacapone	Untreated	1.85	4450
	Creosote	1.01	8470
	Phenobarbital	1.50	5420
Tolcapone	Untreated	0.56	4880
	Creosote	0.33	7820
	Phenobarbital	0.65	9650

[a] Glucuronide peak area.

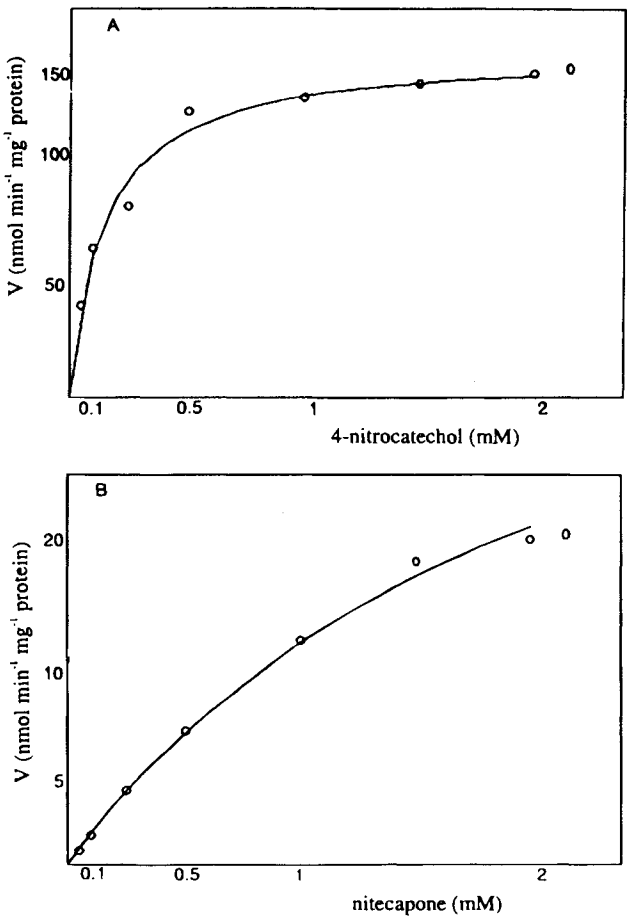

Fig. 4. Michaelis-Menten plots for the glucuronidation of 4-nitrocatechol (A), and nitecapone (B) by liver microsomes from creosote-treated rats.

Monosubstituted nitrocatechols showed about two times higher V_{max} values than 4-nitrophenol. V_{max} values for all disubstituted catechols were lower than for 4-nitrophenol. K_m values varied widely depending on substitution pattern and on the nature of the large substituent of COMT inhibitors. 4-Nitrocatechol had a quite similar K_m as 4-nitrophenol while the apparent K_m for nitecapone may be more than an order of magnitude higher. V_{max}/K_m values, the first order rate constants characterizing the reaction at low substrate concentrations, were approximately one order of magnitude lower for COMT inhibitors than for 4-nitrophenol or monosubstituted nitrocatechols.

Table II. Enzyme Kinetic Parameters for Glucuronidation of Nitrocatechols by Liver Microsomes from Creosote-Treated Rats

Substrate	K_m[a] (mM)	V_{max}[a] (nmol/min/mg)	V_{max}/K_m (ml/min/mg)
4-Nitrophenol	0.11 ± 0.02	68.8 ± 3.8	0.63
4-Nitrocatechol	0.19 ± 0.05	153.0 ± 26.4	0.79
3-Nitrocatechol	0.72 ± 0.11	127.7 ± 15.5	0.18
3,5-Dinitrocatechol	1.04 ± 0.27	36.2 ± 7.7	0.035
Entacapone	0.75 ± 0.27	13.4 ± 2.1	0.018
Entacapone(Z)-isomer	0.40 ± 0.11	15.5 ± 3.4	0.039
Nitecapone	2.40 ± 0.19	52.9 ± 5.9	0.022
Tolcapone	0.29 ± 0.06	11.0 ± 1.5	0.038

[a] Data represent means ± SD from three or four independent experiments.

DISCUSSION

There are no reference standards commercially available for any of the nitrocatechol glucuronides investigated. Therefore in our enzyme kinetic studies we used a radiolabeled co-substrate for quantitation. In our HPTLC method, the ^{14}C-labeled UDP-glucuronic acid was applied only for setting the absolute level of glucuronides to save money and to enable a rapid and reproducible quantitation by densitometric analysis. Only one glucuronide peak for each compound was observed in the densitograms. In principle, two regioisomeric monoglucuronides can be formed from catechols. It is possible that isomeric products, which were not separated by the HPTLC analysis, were formed from some substrates. However, it is known from metabolic studies of entacapone and nitecapone in the rat that one major product, most probably 1-O-glucuronide, is formed (6,7).

UGT isoforms which contribute to the glucuronidation of COMT inhibitors in rat were more clearly induced by creosote than by phenobarbital. Creosote has been found to induce the glucuronidation of 4-nitrophenol about twofold and of a larger planar phenol, 1-hydroxypyrene, about fourfold (9). Induction of the glucuronidation of COMT inhibitors was comparable to that of 4-nitrophenol. The K_m value determined for 4-nitrophenol was somewhat lower than reported earlier (K_m = 0.3–0.4 mM) for creosote induced rat microsomes (9) and in good agreement with earlier studies on partially purified rat liver UGT (K_m = 0.12 ± 0.03) (13) and human liver microsomes (K_m = 0.10 ± 0.04) (14).

The kinetic results with differently substituted nitrocatechols showed that catechols may be better substrates of UGTs than are phenols. A hydroxyl in the *ortho* position of a phenolic hydroxyl obviously did not interfere with the binding of the substrate. Otherwise the substitution pattern seemed to have marked effects on affinity and reactivity of nitrocatechols and probably also on the pattern of isoenzymes that significantly glucuronidate various COMT inhibitors.

The catalytic rate for glucuronidation of all COMT inhibitors (disubstituted catechols) was slower compared to 4-nitrophenol. The fact that 4-nitrophenol is generally used as a model substrate probably reflects the general observation that electron withdrawing substituents increase reactivity of phenols towards glucuronidation. The second electron withdrawing substituent of COMT inhibitors lowers their pKa to 4.5–5 (15), so that they are almost completely ionized at neutral pH. Resonance stabilization of the anion may lower the reactivity for S_N2 type substitution. The literature concerning the electronic effects of the substituents on glucuronidation is rather confusing. Magdalou *et al.* observed a linear relationship between log V_{max} and substituent constant σ using a partly purified pig liver UGT and a positive slope (ρ) for this line (16). The NO_2 group increased the V_{max} a 100-fold. Yin *et al.* reported, however, a negative ρ value and a three-fold decrease in V_{max} for 4-nitrophenol compared to phenol in their study using a partially purified rat liver UGT (13). In human microsomes both electron withdrawing (NO_2) and electron donating (CH_3) hydrophobic substituents at any position of the phenol molecule have been

found to increase the V_{max} (3–5-fold) (14). Hydrophilic basic (NH_2) or acidic (COOH) substituents at any position destroyed the glucuronidation activity completely. These contradictory results can be explained by the fact that in every study different enzyme source have been used. It can be hypothetised that the rate limiting transition state is at a different point on the reaction coordinate depending both on the enzyme form and the substrate structure, and therefore the substituent effect varies.

The order of V_{max} values for COMT inhibitors was nitecapone > entacapone > tolcapone. These results suggest that at saturated or high substrate concentration nitecapone is glucuronidated at a four times higher rate than tolcapone, this is in qualitative agreement with the differences in their biological half lives. On the other hand, the V_{max}/K_m values imply that at low substrate concentration tolcapone may be glucuronidated at a similar or even a slightly higher rate compared to the other COMT inhibitors and thus the longer elimination half-life of it *in vivo* may not be explained by glucuronidation kinetics *in vitro*.

The affinity of UGTs to the nitrocatechol substrates, as reflected by the Michaelis constants, varied in a different way to the catalytic rate. The NO_2 group next to the catecholic hydroxyls may cause steric hindrance and lower the binding affinity to UGTs as seen from the difference displayed in the K_m values between 3- and 4-nitrocatechols. *ortho*-Nitrophenol has also been found to exhibit an over three-fold K_m value compared to *para*-nitrophenol (14). The K_m value of 3,5-dinitrocatechol is even higher than the K_m of 3-nitrocatechol, although the second nitro group in position 5, similarly to that of 4-nitrocatechol, should not cause steric hindrance. The affinity may be lowered by electrostatic effects caused by the lowered pKa of the dinitrocompound. The other disubstituted catechols with larger side chains showed high or low affinity depending mainly on the hydrophobicity of the larger substituent. The most hydrophobic COMT inhibitor, tolcapone, had a similar K_m as 4-nitrocatechol. The order of K_m values for COMT inhibitors was the same as the order of V_{max} values (nitecapone > entacapone > tolcapone).

Although the V_{max} for nitecapone could not be accurately estimated the highest observed rate was already clearly higher than the fitted V_{max} for entacapone or tolcapone (Figure 4B). The V_{max}/K_m value determined as the slopes of the almost linear V versus [S] plots using the first four data points was practically the same (0.018) as obtained by non-linear fitting of the Michaelis-Menten equation. The different behaviour of nitecapone compared to the other COMT inhibitors is obviously due to its hydrophilic 5-substituent. A plausible explanation is that, at higher substrate concentrations, nitecapone is preferentially glucuronidated by very high capacity UGT isoforms for which the COMT inhibitors with a large hydrophobic side chain appear to be poor substrates.

In conclusion, nitrocatechols can be good substrates of UGTs, but the substitution pattern has important effects on affinity and reactivity. Entacapone, tolcapone, and nitecapone, which all have relative big and non-planar side chains were not especially good substrates of UGTs in liver microsomes from untreated, phenobarbital- or creosote-treated rats. For a better understanding of the structure-activity relationships of catechol glucuronidation, further studies should be carried out using different forms of human recombinant isoenzymes, substrates exhibiting more diverse physicochemical properties and kinetic methods capable of assessing the reactivity of each catechol hydroxyl.

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