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## Sulfation of indoxyl by human and rat aryl (phenol) sulfotransferases to form indoxyl sulfate

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### SUMMARY

The aim of this study was to identify sulfotransferase (SULT) isoform(s) responsible for the formation of indoxyl sulfate from indoxyl (3-hydroxyindole). Indoxyl was incubated together with the co-substrate 3'-phosphoadenosine 5'-phosphosulfate (PAPS) and either human or rat liver cytosol or recombinant sulfotransferase enzymes. Formation of indoxyl sulfate from indoxyl was measured by HPLC and used for determination of sulfonation rates. Both cytosols sulfonated indoxyl with apparent  $K_m$  values of  $6.8 \pm 0.9 \mu\text{M}$  for human and  $3.2 \pm 0.6 \mu\text{M}$  for rat cytosol. To help identify the isoform(s) of SULT responsible for indoxyl sulfate formation, indoxyl was incubated with human and rat liver cytosols and PAPS in the presence of isoform-specific SULT inhibitors. No inhibition was observed by DHEA, a specific hydroxysteroid sulfotransferase inhibitor, nor by oestrone, an inhibitor of oestrogen sulfotransferase. However, an aryl (phenol) sulfotransferase inhibitor, 2,6-dichloro-4-nitrophenol (DCNP), inhibited the formation of indoxyl sulfate with a  $IC_{50}$  values of  $3.2 \mu\text{M}$  for human and  $1.0 \mu\text{M}$  for rat cytosol indicating that human and rat aryl (phenol) sulfotransferases are responsible for the formation of indoxyl sulfate. When indoxyl was incubated with SULT1A1\*2, a human recombinant aryl SULT, an apparent  $K_m$  value of  $5.6 \pm 1.8 \mu\text{M}$  was obtained. Kinetic studies with human and rat cytosols and human recombinant SULT1A1\*2 gave similar kinetic values indicating that human and rat aryl sulfotransferases efficiently catalyze the formation of indoxyl sulfate, an important uremic toxin metabolite.

### Keywords

Indoxyl; indoxyl sulfate; sulfotransferase; conjugation; metabolism

### INTRODUCTION

Sulfo-conjugation is an important reaction in the metabolism of many drugs, xenobiotics and endogenous substances such as neurotransmitters and hormones (1). These metabolic reactions are catalyzed by sulfotransferases that involve the transfer of a sulfuryl group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to acceptor molecules, thereby producing more water soluble and often less toxic metabolites. Although it is generally true for the sulfation of molecules to aid excretion from the body, sulfonation of certain allylic alcohols and polycyclic aromatic hydrocarbons bearing benzylic alcohol groups and sulfonation of N-hydroxy arylamines may produce metabolites with increased toxicity that cause mutagenic

and carcinogenic responses (2-4). Therefore, sulfate conjugation may act as an important metabolic activation reaction for certain molecules, as well as a detoxication reaction. Indoxyl sulfate, a sulfate conjugate of indoxyl (3-hydroxyindole) is a known circulating uremic toxin promoting the progression of renal failure (5,6), and serum concentration of this metabolite is markedly increased in uremic patients (7). Indoxyl sulfate is mainly produced after bacterial decomposition of dietary tryptophan in intestines (8). The initial product of tryptophan metabolism in intestines is indole, which is hydroxylated to 3-hydroxyindole (indoxyl), and subsequently sulfonated to form indoxyl sulfate (5,9).

Primary observations from different laboratories indicated that sulfotransferase enzymes may be important for the formation of indoxyl sulfate from indoxyl (10,11). The current study provides evidence that sulfotransferases catalyze the formation of indoxyl sulfate and demonstrates the sulfotransferase isoform(s) responsible for the production of this tryptophan metabolite.

## MATERIALS AND METHODS

### Reagents and chemicals

Indoxyl acetate, indoxyl sulfate, 3'-phosphoadenosine 5'-phosphosulfate (PAPS), adenosine 3',5'-diphosphate (PAP), dithiothreitol (DTT), 17 $\beta$ -oestradiol, oestrone, *p*-nitrophenol, *p*-nitrophenol sulfate, and porcine liver esterase were obtained from Sigma Chemical Co. (St Louis, MO, USA). Dehydroepiandrosterone (DHEA) and 2,6-dichloro-4-nitrophenol (DCNP) were from Fluka Chemika (Buchs, AG, Switzerland). Other chemicals were obtained at the highest available grade from commercial sources.

### Liver cytosol and recombinant sulfotransferases

Human liver tissue was obtained from International Institute for the Advancement of Medicine (Exton, PA, USA) under IRB approval. Sprague-Dawley rats (male) were from Charles River (Wilmington, MA, USA) and handled with IACUC approval. Human and rat liver cytosolic fractions (100000g supernatant) were prepared by differential centrifugation in our laboratory as described previously (4). Cytosolic fractions were stored in 1 ml aliquots at  $-80^{\circ}\text{C}$  and, after thawing, were kept at  $4^{\circ}\text{C}$ . Protein concentrations were determined by Biuret assay (Total Protein Reagent; Sigma Diagnostics, St Louis, MO, USA).

Recombinant human sulfotransferases SULT1A1\*2 (thermostable phenol sulfotransferase allozyme), SULT2A1 (hydroxysteroid sulfotransferase), SULT1E1 (oestrogen sulfotransferase) and Sf-9 control cytosolic extract were obtained from Panvera (Madison, WI, USA) as cytosolic extracts from Sf-9 insect cells transfected with a baculovirus containing a corresponding individual human sulfotransferase cDNA. Recombinant rat hydroxysteroid sulfotransferase (STa) was a generous gift of Prof. Michael W. Duffel (University of Iowa, Iowa City, IA, USA).

### Indoxyl (3-hydroxyindole) sulfonation assay

Because of the high spontaneous oxidation rate of indoxyl (10,12), this compound was generated *in situ* from indoxyl acetate by incubation with 3.3 units of porcine liver esterase at pH 7.0 for 2 min immediately prior to use in the sulfotransferase assay. All assays were run under argon atmosphere. The reaction mixture of 60  $\mu\text{l}$  total volume contained 23.3 mM potassium phosphate buffer at pH 7.0, 10.6 mM dithiothreitol, 0.2 mM PAPS, 3.3 units porcine liver esterase, various concentrations of indoxyl acetate in acetone (final concentration of acetone in the assay was no more than 5% v/v). Reactions were pre-incubated for 2 min with porcine liver esterase to form indoxyl from indoxyl acetate. The

sulfonation reaction was initiated by the addition of either human liver cytosol (0.1 mg protein), rat liver cytosol (0.1 mg protein), human SULT1A1\*2 (2.2  $\mu$ g protein), human SULT2A1 (4.5  $\mu$ g protein), human SULT1E1 (3.8  $\mu$ g protein), rat recombinant STa (1  $\mu$ g protein) or Sf-9 control cytosolic extract (6.4  $\mu$ g protein). After incubation for 2-10 min at 37°C, the reaction was terminated by addition of 60  $\mu$ l ice-cold methanol. After centrifugation for 2 min at 12000g, 50  $\mu$ l of the supernatant was injected to HPLC for determination the substrate-dependent formation of indoxyl sulfate.

Indoxyl sulfonation rate was determined by HPLC measuring the amount of indoxyl sulfate formed in the assay. HPLC analysis was carried out on an Alltech Econosphere C18 column (250 mm  $\times$  4.6 mm, 5  $\mu$ m) using isocratic elution with water:methanol (88:12) containing 50 mM  $\text{KH}_2\text{PO}_4$ , 1.0 mM 1-octylamine, and 50 mM  $\text{NH}_4\text{Cl}$  (pH adjusted to 5.45 before addition of methanol). All analysis of reaction mixtures was conducted at a flow rate of 2.0 ml/min, with detection by absorbance at 280 nm with a Hitachi photodiode array detector (Model L-7455). The concentration of the indoxyl sulfate formed in the reaction mixture was determined from a linear standard curve relating peak area to the concentration of indoxyl sulfate.

At least six different concentrations of indoxyl (from indoxyl acetate) were assayed, and these included concentrations both greater and less than the apparent  $K_m$  values. Apparent  $K_m$  and  $V_{max}$  values are presented as  $\pm$  the standard error obtained by non-linear least squares curve fitting (13) of the velocity data to the Michaelis-Menten equation.

#### Determination of SULT activities in cytosolic fractions and with recombinant SULTs

Presence of SULT activities in both human and rat liver cytosolic fractions were determined by using *p*-nitrophenol, DHEA and oestradiol as substrates for aryl, hydroxysteroid, and oestrogen sulfotransferase activities, respectively, by using either a PAPS/PAP conversion assay (14) or a SULT colorimetric method (15). The presence of isoform-specific SULT activities in human and rat recombinant forms were verified using a specific substrate for each recombinant form: *p*-nitrophenol (human SULT1A1\*2), DHEA (human SULT2A1 and rat STa), oestradiol (human SULT1E1) and 1-naphthol (rat AST IV).

#### Inhibition of isoform-specific SULT activities in human and rat cytosolic fractions

Assays of inhibition of indoxyl sulfonation activity in cytosolic fractions were carried out by incubating for 10 min either human or rat liver cytosol with increasing amounts of known isoform-specific SULT inhibitors such as 2,6-dichloro-4-nitrophenol (DCNP) (an aryl SULT inhibitor), DHEA (a hydroxysteroid SULT inhibitor) or oestrone (oestrogen SULT inhibitor) in the presence of indoxyl as a substrate. The formation of indoxyl sulfate after 10 min was determined by the HPLC procedure as described above.  $\text{IC}_{50}$  values for the inhibition of specific SULT activity in the sulfonation of indoxyl, if present, were calculated.

## RESULTS

#### Sulfonation of indoxyl by rat and human liver cytosols

The effect of variation of substrate concentration on indoxyl sulfonating activity of human liver and rat liver cytosols is shown in Fig. 1. Since SULT enzymes generally demonstrate pronounced substrate inhibition (16,17), we used a wide range of substrate concentrations in our experiments before determining  $K_m$  and  $V_{max}$  values for indoxyl sulfonation activity in human and rat cytosols. With the human and rat liver cytosols, substantial substrate inhibition was observed at concentrations above 20  $\mu$ M and 64  $\mu$ M, respectively (Fig. 1). Therefore, we used substrate concentrations below these levels for calculation of apparent  $K_m$  and  $V_{max}$  values. As shown in Fig. 2, seven data points for human cytosol and six data

points for rat cytosol were used to calculate an apparent  $K_m$  of  $6.8 \pm 0.9 \mu\text{M}$  and  $3.2 \pm 0.6 \mu\text{M}$ , respectively, for indoxyl sulfonation activity.

### Effect of isoform-specific SULT inhibitors on indoxyl sulfonating activity with rat and human cytosols

For evaluation of the contribution of various SULT isoforms to the sulfonation of indoxyl, presence of the SULT activity of each isoform was first measured at the optimal concentration for its respective prototype substrate in both human and rat cytosols, i.e.,  $5 \mu\text{M}$  DHEA for human DHEA SULT,  $4 \mu\text{M}$  *p*-nitrophenol for human PST,  $25 \mu\text{M}$  DHEA for rat hydroxysteroid SULT,  $100 \mu\text{M}$  1-naphthol for rat aryl SULT, and  $25 \mu\text{M}$  oestrone for both human and rat oestrogen SULT. After determination of the presence of phenol and DHEA SULT activities in both human and rat cytosols, isoform-specific SULT inhibitors, DCNP, DHEA and oestrone, were used for measurement of aryl (phenol), hydroxysteroid (alcohol) SULT and oestrogen SULT activities on indoxyl sulfonation activity, respectively. Human thermostable phenol SULT (SULT1A1/2) is very sensitive to DCNP inhibition and is inhibited by this compound with an  $\text{IC}_{50}$  value of very low concentration,  $2.3 \mu\text{M}$  (16,18,19). DCNP also inhibits thermolabile phenol SULT (SULT1A3) and DHEA SULT (SULT2A1) activity in human liver although with high  $\text{IC}_{50}$  values, such as 110 and  $85 \mu\text{M}$ , respectively (19). Selective inhibition of rat aryl SULT activity by DCNP has also been reported (20). DHEA is a known competitive inhibitor of DHEA SULT activity in human liver cytosol (21) and hydroxysteroid SULT activity in rat liver cytosol (22), and is used to determine the contribution of this sulfotransferase family towards indoxyl sulfonation activity. Oestrone, a competitive substrate of oestrogen SULT, was used to determine the possible contribution of oestrogen sulfotransferase activity towards indoxyl sulfonation, although oestrogen SULT is expressed at only low levels in the human liver (23). DHEA ( $25$  and  $40 \mu\text{M}$ ) and oestrone ( $25$  and  $40 \mu\text{M}$ ) did not affect the sulfonation of indoxyl measured at the substrate concentration of  $12 \mu\text{M}$ . In contrast, human and rat liver PST indoxyl sulfonation activity was inhibited by DCNP. The concentration of DCNP that inhibited indoxyl sulfonation in human liver cytosol by 50% ( $\text{IC}_{50}$ ) was  $3.2 \mu\text{M}$  for the activity measured with  $12 \mu\text{M}$  indoxyl, while the  $\text{IC}_{50}$  of DCNP inhibition of the same activity in rat liver cytosol was  $1 \mu\text{M}$ .

### Sulfonation of indoxyl by human and rat recombinant aryl (phenol) sulfotransferases

Recombinant human SULTs, SULT1A1\*2 (TS-PST), SULT2A1 (DHEA-SULT) and SULT1E1 (oestrogen SULT) and a recombinant rat SULT, STa (hydroxysteroid SULT) were used to elucidate the responsible sulfotransferase isoforms for the sulfonation of indoxyl. With SULT2A1, SULT1E1 and STa, there was no indoxyl sulfate formed in the enzyme assay with various concentrations of indoxyl. However, SULT1A1\*2 efficiently catalyzed the sulfonation of indoxyl under the assay conditions described in Materials and Methods. As anticipated, there was a pronounced substrate inhibition above  $20 \mu\text{M}$  indoxyl with this recombinant enzyme. Apparent enzyme kinetic determinations, using substrate concentrations below  $20 \mu\text{M}$  with recombinant SULT1A1\*2, allowed saturable kinetics producing an apparent  $K_m$  and  $V_{\text{max}}$  values of  $5.6 \pm 1.8 \mu\text{M}$  and  $1450 \pm 190 \text{ pmol/min/mg}$ , respectively (Fig. 3).

## DISCUSSION

The present study shows that sulfonation of indoxyl to indoxyl sulfate is catalyzed by human and rat liver aryl (phenol) sulfotransferases with apparent  $K_m$ 's of  $6.8 \mu\text{M}$  and  $3.2 \mu\text{M}$ , respectively. The inhibition experiments suggest that the SULT1A1 isoform is responsible for the sulfonation activity in human and rat liver cytosol. This was further established by measurement of indoxyl sulfonation activity with human recombinant SULT1A1\*2. The

apparent  $K_m$  measured for indoxyl sulfonation catalyzed by SULT1A1\*2 (5.6  $\mu\text{M}$ ) was within experimental error of that measured for human liver cytosol. The other major allozyme, SULT1A1\*1, was not available to us, but we expect that it would also catalyze sulfonation of indoxyl. There was no indoxyl sulfonation activity observed with the other sulfotransferase isoforms tested, and no inhibition of indoxyl sulfonation was observed in the presence of isoform-specific inhibitors for these sulfotransferases.

An early study with purified rat liver phenol sulfotransferases, PST I and II, showed that indoxyl served as a substrate for these rat liver phenol SULTs (10). Another study for the metabolism of 3-indolylacetic acid in human skin samples also demonstrated that indoxyl sulfate was one of the metabolites formed from parent molecule (11). These results are in good correlation with the results of our present study indicating that sulfonation of indoxyl is catalyzed by aryl (phenol) sulfotransferases. The apparent  $K_m$  observed with both human and rat liver cytosols (6.8  $\mu\text{M}$  for human and 3.2  $\mu\text{M}$  for rat) and corresponding human recombinant form (5.6  $\mu\text{M}$  for SULT1A1\*2) were similar. Moreover, the binding affinity of indoxyl for SULT1A1\*2 was comparable to the prototype substrate of this enzyme, *p*-nitrophenol (i.e.,  $K_m$  of 4  $\mu\text{M}$ ). This is a good indication that indoxyl serves as a very good substrate for both human and rat aryl (phenol) sulfotransferases.

The conjugation of phenolic type compounds in liver by aryl (phenol) sulfotransferases is generally a major detoxication reaction for the excretion of these compounds. Our observation, that indoxyl is an excellent substrate for sulfonation by both human and rat aryl sulfotransferases, is not surprising because indoxyl is similar chemically and electronically (i.e. planar molecular shape) to phenols. In this case, however, the metabolite indoxyl sulfate causes more toxicity than its metabolic precursor tryptophan. Oral administration of indoxyl sulfate or its precursor, indole, stimulates the progression of chronic renal failure (5,6). Although the mechanism of the toxicity caused by indoxyl sulfate is unknown, it causes damage in proximal tubule cells and stimulates the progression of glomerular sclerosis and tubulointerstitial damage, as shown by histologic studies (24). Indoxyl sulfate also increases the expression of transforming growth factor (TGF)- $\beta$ 1 (25). TGF- $\beta$ 1 is a fibrogenic cytokine which causes increased production of extracellular matrix collagens and is a tissue inhibitor of the metalloproteinase TIMP-1. Increased expression of TGF- $\beta$ 1 leads to tubulointerstitial fibrosis and the consequent decline of renal function (25). Indoxyl sulfate may cause additional toxic effects due to inhibition of drug binding to albumin, causing drug toxicity in uremic patients; about 90% of indoxyl sulfate itself is bound to albumin (26).

Human liver contains at least two aryl sulfotransferase isoforms, thermostable or phenol preferring form (TS-PST, SULT1A1/2) and thermolabile or monoamine preferring form (TL-PST, SULT1A3). SULT1A1 preferentially catalyzes the sulfonation of micromolar concentrations of planar phenols (i.e., *p*-nitrophenol) and is very sensitive to inhibition by DCNP. SULT1A3 preferentially catalyzes the sulfonation of micromolar concentrations of phenolic monoamines and is relatively insensitive to inhibition by DCNP (18,27). Moreover, SULT1A1 is known to be genetically polymorphic which results in expression of SULT1A1 allozymes. Differential expression of these allozymes is thought to be responsible for the wide variation in SULT1A1 activity observed in human populations (14,27,28). Our experimental results based on DCNP inhibition studies and with recombinant SULT1A1\*2 suggest that human polymorphic thermostable aryl SULTs are the only sulfotransferase isoforms involved in the formation of indoxyl sulfate. This determination is important for future elucidation of individual susceptibility of uremic patients to the toxic effects of this metabolite of tryptophan.

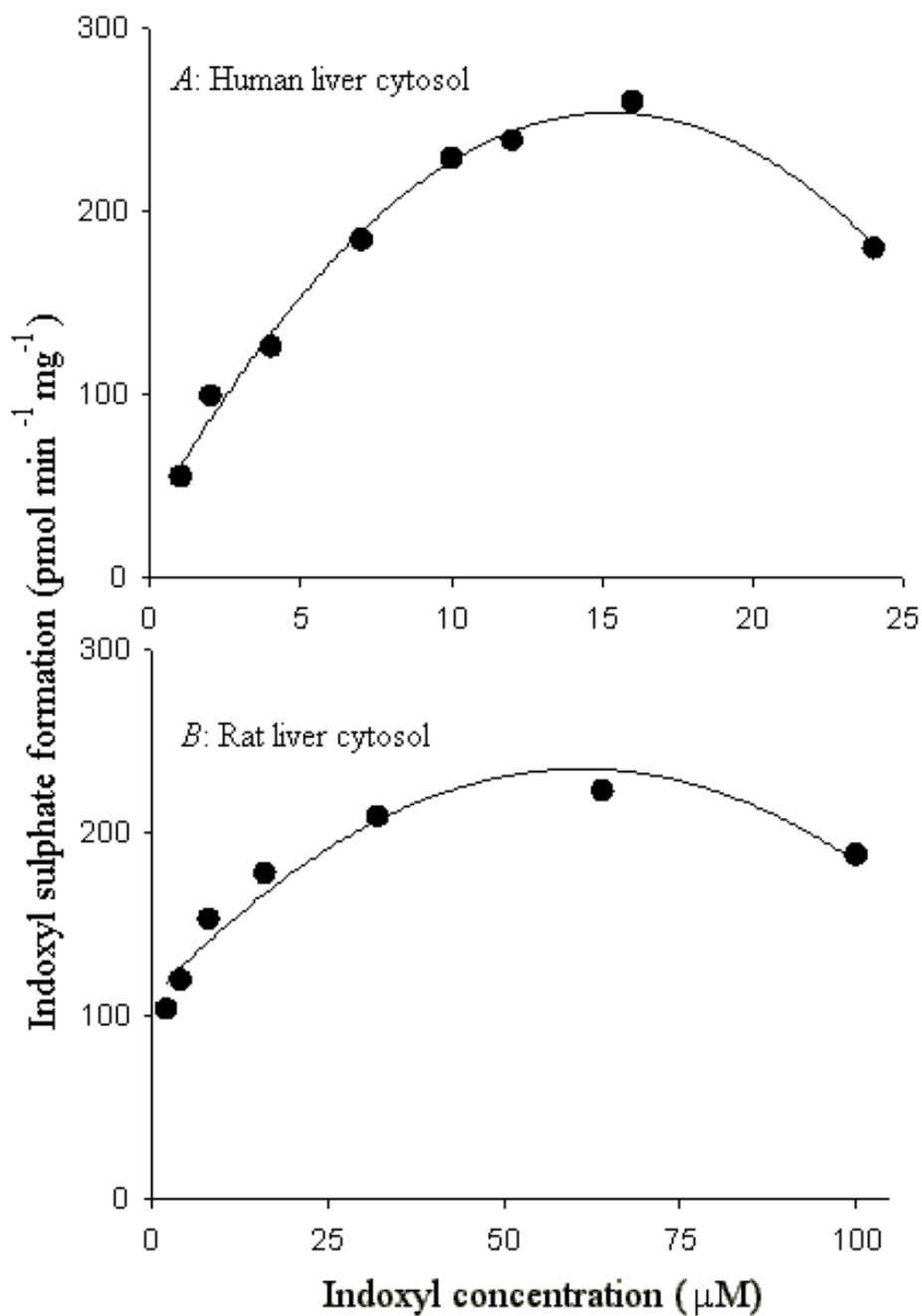
## Acknowledgments

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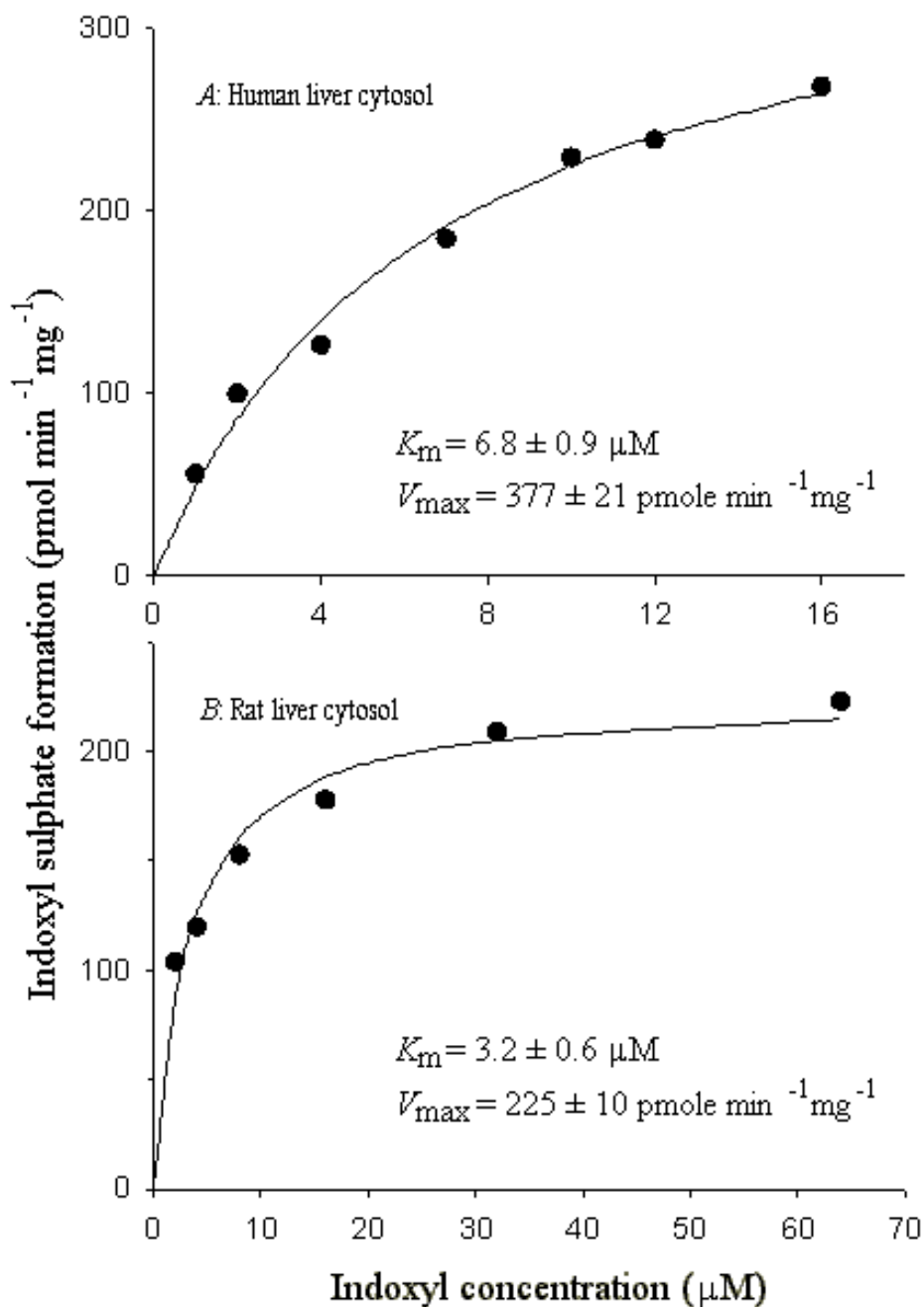
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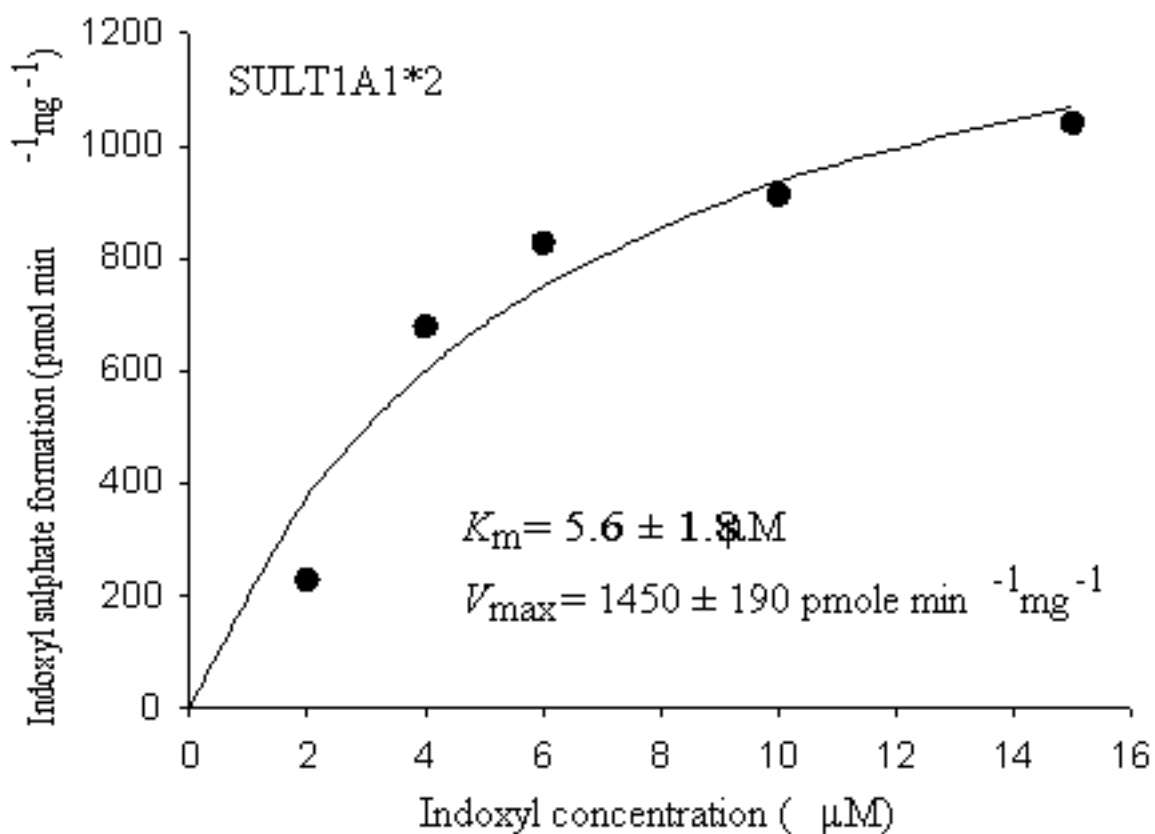


**Fig. 1.** Effect of variation of indoxyl concentration on the sulfonation of indoxyl catalyzed by human (A) and rat (B) liver cytosol. Symbols represent observed values while the line represents a smooth curve indicating substrate inhibition.





**Fig. 2.** Initial velocity for the sulfonation of indoxyl to indoxyl sulfate by human (A) and rat (B) liver cytosol. Symbols represent observed values while the line represents non-linear least squares fitting of the observed data to the Michaelis–Menten equation.



**Fig. 3.**

Initial velocity for the sulfonation of indoxyl to indoxyl sulfate by human recombinant SULT1A1\*2. Symbols represent observed values while the line represents non-linear least squares fitting of the observed data to the Michaelis–Menten equation.