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In vitro sulfonation of 7-hydroxycoumarin derivatives in liver cytosol of human and six animal species

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Abstract

1. Sulfonation is an important high affinity elimination pathway for phenolic compounds.
2. In this study sulfonation of 7-hydroxycoumarin and 13 its derivatives were evaluated in liver cytosols of human and six animal species. 7-hydroxycoumarin and its derivatives are strongly fluorescent, and their sulfate conjugates are nonfluorescent at excitation 405 nm and emission 460 nm. A convenient fluorescence based kinetic assay of sulfonation was established.
3. The sulfonation rate of most of the 7-hydroxycoumarin derivatives was low in liver cytosol of human and pig, whereas it was high with most compounds in dog and intermediate in rat, mouse, rabbit, and sheep. Sulfonation of the 7-hydroxycoumarin derivatives followed Michaelis-Menten kinetics with K<sub>m</sub> values of 0.1 - 12 µM, V<sub>max</sub> of 0.005–1.7 µmol/(min * g protein) and intrinsic clearance (V<sub>max</sub>/K<sub>m</sub>) of 0.004–1.9 L/(min * g cytosolic protein).
4. Fluorescence based measurement of sulfonation of 7-hydroxycoumarin derivatives provides a sensitive and convenient high-throughput assay to determine sulfonation rate in different species and tissues and can be applied to evaluate sulfonation kinetics and inhibition.

Keywords: sulfonation, liver, human, animal, 7-hydroxycoumarin
Introduction

Metabolism of foreign substances in a living organism is essential to avoid their adverse health effects. The substances are transformed in xenobiotic metabolizing reactions of oxidation, reduction, hydrolysis or conjugation, most often to more water soluble and excretable metabolites, but sometimes to reactive toxic metabolites. In conjugation reactions a small endogenous molecule is transferred to a functional group of xenobiotic, which is already present or is created by enzyme catalyzed oxidation, reduction or hydrolysis reactions. Conjugation reactions are eminently important in biotransformation, because they usually inactivate xenobiotics or render reactive metabolites less harmful [Gonzalez et al., 2018, Parkinson et al., 2019].

Sulfonation takes place for phenols, alcohols or primary or secondary amines, whose sulfate conjugates are negatively charged and more water-soluble sulfates or sulfamates. These metabolites are excreted efficiently mostly to urine or sometimes to bile by anionic transporters. Usually coordination of sulfonation to overall metabolism of foreign substances is protecting the body against adverse health effects, but sulfates of hydroxylated amines such as heterocyclic amines can form reactive metabolites with deleterious effects [James & Ambadapadi, 2013, Tibbs et al., 2015].

Cytosolic sulfotransferases (E.C. 2.8.2.) in liver catalyze sulfonation of xenobiotics and endogenous substances such as steroids, bile acids, monoamine neurotransmitters and thyroid hormones by transferring the sulfonate group from the cofactor 3’-phosphoadenylyl sulfate (PAPS). The same compounds may also be glucuronidated by UDP-glucuronosyltransferases. Usually, conjugation of alcohols, phenols and amines by sulfotransferases occurs efficiently at lower concentrations than the corresponding glucuronidation reactions, which are localized in the endoplasmic reticulum and possesses higher conjugation capacity than sulfonation. The function of these two conjugation enzymes is complementary and makes the transformation of alcohols, phenols and amines to excretable conjugates very efficient [Rowland et al., 2013, Suiko et al., 2017, Tibbs et al., 2015].

The rate of sulfonation can be measured by assays based on radiometric, absorbance, fluorescence and HPLC-MS detection. In radiometric assay the sulfur atom of transferred sulfone is labeled with $^{35}$S, which can be measured precisely and at high sensitivity from the isolated metabolite in an endpoint experimental setup [Paul et al., 2012]. The absorbance and fluorescence assays are based on indirect measurement of sulfonation in which the actual
substrates and p-nitrophenol sulfate or 4-methyl-7-hydroxycoumarin sulfate are tightly coupled to the regeneration of PAP to PAPS by aryl sulotransferase IV enzyme. In this coupling reaction sulfates are transformed to absorbing p-nitrophenol or fluorescent 4-methyl-7-hydroxycoumarin. The increase in absorbance or fluorescence can be measured by continuous or endpoint assays; measurement of fluorescence is more sensitive than of absorbance [Chen et al., 2005, Lu et al., 2010]. The decrease of fluorescence of 7-hydroxycoumarin or resorufin during sulfonation has also been measured directly. In HPLC-MS assays substrates and the formed metabolites are separated by HPLC and then detected and analyzed by different kinds of MS approaches [Paul et al., 2012].

We have previously published a simple and convenient method to measure glucuronidation of 7-hydroxycoumarin derivatives in a multiwell plate format [Rahikainen et al., 2013, Juvonen et al., 2018]. We report here several new 7-hydroxycoumarin derivatives to measure sulfonation. These substrates are fluorescent in their parent form and are transformed to nonfluorescent sulfate conjugates by sulfotransferases (Figure 1). The primary aim of this study was to establish fluorescence based assay for sulfonation of the 7-hydroxycoumarin derivatives (Figure 2). The assay was applied to evaluate sulfonation kinetics and compare sulfonation characteristics in liver cytosol of human, rat, mouse, rabbit, dog, pig and sheep.

Figure 1.

Figure 2.

Materials and methods

Chemicals. PAPS, 7-hydroxycoumarin (umbelliferone) (99%), 7-hydroxy-4-trifluoromethylcoumarin (HFC) (99%) were from Sigma-Aldrich (Mannheim, Germany). MgCl₂ were from Riedel-de Haen (Vantaa, Finland). Water was deionized by MilliQ gradient A10. The 7-hydroxycoumarin derivatives (6-methoxy-7-hydroxycoumarin (scopoletin) (3), 3,4-dimethyl-7-hydroxycoumarin (4), 3-ethyl-4-methyl-7-hydroxycoumarin (5), 3-ethyl-4,8-dimethyl-7-hydroxycoumarin (6), 3,4,8-trimethyl-7-hydroxycoumarin (7), 3-(4-methylphenyl)-7-hydroxycoumarin (8), 3-(4-hydroxyphenyl)-7-hydroxycoumarin (9), 3-(4-fluorophenyl)-7-hydroxycoumarin (10), 3-(4-methoxyphenyl)-7-hydroxycoumarin (11), 12 3-(4-dimethylaminophenyl)-7-hydroxycoumarin (12), 3-(4-pyridin-3-yl)-7-hydroxycoumarin (13), 3-(1H-1, 2, 4-tirazol-1-yl)-7-hydroxycoumarin (14) were synthesized using either the Perkin-Ogilialor condensation reaction of von Pechmann condensation reaction described in detail earlier [Timonen et al., 2011, Juvonen et al., 2018b].
Biological samples. The male human liver tissue used in this study was obtained from the Oulu University Hospital (Oulu, Finland) as surplus from organ transplantation surgery. The collection of the surplus tissue was approved by the Ethics Committee of the Medical Faculty of the University of Oulu (January 21, 1986). After surgical excision, the liver samples were immediately transferred to ice, cut into pieces, snap frozen in liquid nitrogen and stored at -80°C until cytosol was prepared. Pig liver samples were from eight month old female pigs used for practicing surgical procedures at the Kuopio University. DBA/2N/Kuo mice (20–25 g) and male Wistar rats (200–300 g) were obtained from the National Laboratory Animal Centre, Kuopio University. Mice were given intraperitoneally 0.1 mL 0.9 % saline or 0.1 mL olive oil/10 g/day for four days, pyrazole (Sigma, 95 % purity) in saline (150 mg/kg) or pregnenolone 16α-carbonitrile (PCN, Sigma, 95 % purity) (5 mg/kg) in oil for four days, or 80 mg/kg phenobarbital intraperitoneally in the first morning and then 40 mg/kg on four consecutive mornings. The mice and rats had unrestricted access to water and standard chow Lactamin R36 (Lactamin). The Ethics Committee for Animal Experiments, University of Kuopio, approved these experiments (01-38, June 1, 2000). Animals were killed 24 h after the last treatment.

male and female Beagle dog necropsy was performed at F. Hoffmann-La Roche Ltd (Nutley, NJ) according to institutional guidelines in compliance with national and regional legislation [Heikkinen et al., 2015]. Female Dutch belted rabbits were 4 – 11 months and weighted 2.7 – 3.8 kg (Licence number: ESAVI/8621/04.10.07/2017). The rabbits were sacrificed by injecting a lethal dose of pentobarbital (Mebunat vet 60 mg/ml, Orion Pharma, Finland; 2 ml/kg) into the marginal ear-vein and the liver samples were immediately transferred to ice, cut into pieces, and stored at -80°C until cytosol was prepared. Adult female sheep liver samples were obtained from Oulu University (Oulu, Finland) and the collection of the liver specimen was approved by the Ethics committee of the University (No ESAVI/3510/04.10.03/2011).

Detailed description of the preparation of liver cytosol samples are described previously [Heikkinen et al., 2015].

**Sulfonation assay.** Reaction mixtures for sulfonation assays contained 100 mM potassium phosphate buffer pH 7.4, 2.5 mM MgCl₂, 10 µM PAPS, 0.05–0.3 g/L cytosol as enzyme source and 10 µM 7-hydroxycoumarin derivative as substrate. In the first experiments three negative control mixtures were tested, namely i) without the substrate, ii) without the cofactor PAPS, or iii) without the enzyme source. In subsequent experiments, the control samples lacked the enzyme source since it gave the highest fluorescence background. Incubations were carried out in a 96 multiwell plate format in 100 µl volume at 37°C. Fluorescence decline of the substrates
was monitored every other minute for 40 min after addition of PAPS, using an excitation filter at 405 nm and detection at 460 nm, in a Victor² 1420 Multilabel counter (PerkinElmer, Life Sciences, Turku, Finland). Fluorescence values were transformed to molarity using the substrates to create standard curves at every time point. Slopes of the decrease in substrate concentration per minute were calculated using linear regression analysis, in which the slope of the linear part of the kinetic assay indicated the sulfonation rate (µM/min). Sulfonation rate was calculated by subtracting the blank value from the full reaction value and then normalizing the sulfonation rate by the protein concentration.

Enzyme kinetic analyses were performed in the same 96 multiwell plate assays, with excitation at 405 nm and detection at 460 nm, using different substrate concentrations (0–10 µM). The reactions proceeded linearly for at least 15 min even at the lowest substrate concentrations. The data was analyzed by the Michaelis-Menten equation \( v = S \cdot V_{\text{max}} / (K_m + S) \), in which \( v \) is the reaction rate at substrate concentration \( S \), \( V_{\text{max}} \) is limiting rate of the reaction and \( K_m \) is the Michaelis constant equal to the substrate concentration, at which the reaction rate is 50 % of \( V_{\text{max}} \).

For multivariate analysis, we did principal component analysis (PCA) using SIMCA 15.0.2 (Umetrics). For analysis of 7-hydroxycoumarin derivatives sulfonation by cytosols of different species, we first normalized the values within samples from the sulfonation of the same substrate, so that all values were divided by the highest value (normalized value = value / max value), due to large variation in sulfonation rate between samples from different species.

**Results**

First, the fluorescence intensity of 7-hydroxycoumarin was measured in cytosols of rabbit liver in the presence of all necessary reagents for sulfonation. Negative control samples lacked cytosol or the cofactor PAPS. Fluorescence decreased in rabbit liver cytosol when all the reagents were present, whereas no decrease occurred in the negative control samples, indicating that the fluorescent 7-hydroxycoumarin was transformed to nonfluorescent sulfate conjugates (Figures 3A and B). Similar decrease in fluorescence occurred for all the 7-hydroxycoumarin derivatives in liver cytosols of different species (data not shown). The decrease in fluorescence was dependent on the amount and cytosol of species used (data not shown). The solvents methanol, ethanol, acetonitrile and dimethylsulfoxide at concentration of up to 10 % (v/v) did not affect the sulfonation rate (Figure 3C). Fluorescence of the 7-hydroxycoumarins standards was changed in a buffer dependent fashion and not depending on the concentrations of 7-
hydroxycoumarin derivatives during the 40-min incubation (Figure 3D). Therefore, a standard line was calculated at every time point and used to transform fluorescence to concentration, which improved accuracy of the results (Figures 3A and B). In the subsequent experiments the amount of cytosol was adjusted so that the reaction took place linearly at least for the first ten minutes at the used substrate concentration. This data indicated that the sulfonation rates of the fluorescent 7-hydroxycoumarin derivatives could be accurately quantitated under standardized assay conditions, using various enzyme sources.

**Figure 3.**

Next, the sulfonation rates of thirteen 7-hydroxycoumarin derivatives at 10 µM concentration by liver cytosol of human, rat, mouse, rabbit, dog, pig and sheep were determined (Figure 4). Sulfonation of the 7-hydroxycoumarin derivatives was catalyzed by cytosol of all species, but the rates varied considerably among species and derivatives. The highest sulfonation rates occurred with coumarin derivatives 8 and 12. The sulfonation rates of all 7-hydroxycoumarin derivatives except 12 were lower in pig and human than the other species, in which sulfonation occurred mostly at high rate. Slow sulfonation rates were observed in rat for 9, in dog for 6, in male mouse for 7-hydroxycoumarin, 4, 5 and 7, in female mouse for 7-hydroxycoumarin and 4, in rabbit for 2, 8, 13 and 14, and in sheep for 2, 3, 13 and 13.

**Figure 4.**

In DBA/2 mice the sulfonation rates of 7-hydroxycoumarin derivatives were mostly similar between males and females (Figure 5). To assess the effect of classical inducers of xenobiotic metabolizing enzymes, the mice were treated with phenobarbital, PCN and pyrazole. Phenobarbital and PCN commonly did not affect sulfonation rate, while pyrazole decreased it for several 7-hydroxycoumarin derivatives, e.g. 4 (Figure 5).

**Figure 5.**

The effect of substrate concentration on sulfonation rate was evaluated. The rates could be analyzed by Michaelis-Menten equation with high statistical significance (mostly $r^2 > 0.9$, Figure S1, Table 1). Michaelis-Menten parameters varied substantially both among species and the substrates. The $K_m$ values varied 13-fold in rat and 220-fold in human, and a 4 to 90-fold variation was observed among the compounds. The $V_{max}$ values varied between 5-fold (rat) and 39-fold (human) among the species and 3 to 118-fold fold among the compounds (Table 1).
Intrinsic clearance ($V_{\text{max}}/K_m$) varied between 17-fold (rat) and 167-fold (sheep) among the species and 15 to 351-fold among the compounds (Figure 6).

**Figure 6.**

Kinetic data was analyzed with the PCA models. PCA model for intrinsic clearance had three components showing large explanation of the variation in the data (cumulative $R^2_X = 0.98$) as well as good predictability of the model (cumulative $Q^2 = 0.86$) (Figure 7). PCA models for $K_m$ and $V_{\text{max}}$ had both four components and good explanation of the variation in the data (Cumulative $R^2_X = 0.88$ and 0.94, respectively), but showed poor predictability $Q^2 = 0.28$ and 0.14, respectively) (Figure 7). The dog had the most efficient sulfonation, as the intrinsic clearance was the highest with compounds 3, 4, 5, 6, 9, 11 and 12. Rat catalyzed also efficiently the sulfonation of several compounds such as 1, 2, 7, 8, 13 and 14. The sulfonation efficiency was intermediate in sheep. Pig exhibited <10 % intrinsic clearance compared with the species with the highest ones. Intrinsic clearance was <10 % for nine compounds in rabbit cytosol and for seven compounds in human cytosol compared to the value of the highest species. $K_m$ values of the compounds in the reactions were >1 µM except for 3 (scopoletin) catalyzed by pig cytosol, whereas the value was <1 µM of many compounds in the reactions by cytosol of other species. The highest intrinsic clearance was obtained for 1 catalyzed by rat cytosol, because the 1’s $K_m$-values was high and its sulfonation $V_{\text{max}}$ high. Close to the highest intrinsic clearance value were obtained for 2 sulfonation catalyzed by cytosol of dog and sheep (>80 %), and for 9 and 6 sulfonation catalyzed by dog cytosol (>70 %). The intrinsic clearance of 12 sulfonation was more similar between species than other 7-hydroxycoumarin derivatives.

**Figure 7.**

**Discussion**

Phenols are typical substrates for sulfonation during metabolism of xenobiotics in mammals [Suiko et al., 2017, Tibbs et al., 2015]. In this study a convenient fluorescence based assay was developed to determine sulfonation rates of phenolic 7-hydroxycoumarin and its 13 derivatives. In the assay fluorescence of the substrates decreased during sulfonation, as the parent compounds are fluorescent and their sulfate conjugates are nonfluorescent at 405 nm excitation and 460 nm emission wavelengths. The sulfonation rate could be precisely quantitated, since the sample acted as its own background at the first time point to which the decrease in fluorescence was compared. Moreover, the substrates themselves acted as standards in every reaction. The sulfonation reaction followed Michaelis-Menten kinetics, indicating lack of
substrate inhibition [James & Ambadapi, 2013, Leyh et al., 2013]. The assay was also so sensitive that the sulfonation rate could be measured at low substrate concentrations with small amounts of enzyme source.

The present method extends the spectrum of technology to determine the rate of sulfonation. As reviewed by Paul et al. [Paul et al., 2012], sulfonation can be measured by various analytical techniques such as radiometric, absorbance, fluorescence or HPLC-MS. The radiometric method based on $^{35}$S labelled PAPS and the MS-based methods are sensitive and are the standard methods to measure sulfonation rate. Their disadvantages are need of special equipment and radioactive reagents. The assay is also laborious and amenable only to endpoint measurements. Previously reported fluorometric assays measured decrease in fluorescence of substrates such as 7-hydroxycoumarin [Leach et al., 1999] and resorufin [Beckman, 1991] or measured coupling of an actual sulfonation substrate and 4-methyl-7-hydroxycoumarin sulfate to regeneration of PAP to PAPS by arylsulfotransferase IV [Chen et al., 2005, Lu et al., 2010].

The advantages of all types of fluorometric sulfonation assays are 1) high sensitivity, 2) applicability from single sample to high throughput format at different types of experimental setups, 3) good repeatability and 4) simplicity and convenience. The extra advantages of the coupling fluorescence sulfonation assay are that it can be used to measure all kinds of substrates and less PAPS is needed. Its disadvantage is that fluorescent 4-methyl-7-hydroxycoumarin is formed during the incubation and it can compete with the actual substrate. In addition, 4-methyl-7-hydroxycoumarin sulfate can inhibit the sulfotransferase enzyme which is measured.

The present study describes a direct, sensitive and convenient sulfonation assay of novel fluorescent 7-hydroxycoumarin derivatives. The assay can be applied to study sulfonation rate and kinetics in different tissues. The reaction follows Michaelis-Menten kinetics, allowing for screening of potential inhibitors of sulfonation in vitro.

Interspecies variation of xenobiotic metabolism is commonly, but inadequately known kinetic factor determining differences in effects of chemicals between species [Reichard 2016]. Marked interspecies variation in sulfonation rates are known to exist. For example, sulfonation activity is particularly low in pig and high in cat [Coughtrie, 2016, Dalgaard, 2015]. Our study, to our best knowledge, is the first to directly compare baseline sulfonation rates in liver cytosol of human, mouse, rat, pig, rabbit, dog and sheep. The results showed that phenolic 7-hydroxycoumarins are sulfonated in all these species, but substantial differences in sulfonation rate and efficiency exists. In dog and rat sulfonation of most of the compounds occurred faster than in other species, while in pig and human sulfonation rate of the compounds was lower in
The substantial variation in sulfonation was illustrated by rates at fixed substrate concentration, and the kinetic parameters $K_m$, $V_{max}$ and $V_{max}/K_m$ varied 2–100 fold (depending on the substrate) among the species. Treatment of mice with inducers of xenobiotic metabolizing enzymes did not affect sulfonation rates.

The human genome contains 14 sulfotransferase genes encoding cytosolic sulfotransferases in 4 gene families [HGNC database; https://www.genenames.org/data/genegroup#!/group/762]. Sulfotransferase genes encoding catalytically active proteins exist also in many other animals, including mouse, rat, rabbit, dog and pig [Blanchard et al., 2004, Gamage et al., 2006]. Sulfotransferases in family sulfotransferase 1 catalyze primarily sulfonation of phenols and sulfotransferase 2 enzymes catalyze primarily sulfonation of steroids including aromatic estrogens [Coughtrie, 2016, James & Ambadapi, 2013]. Therefore the 7-hydroxycoumarin derivatives could be sulfonated by sulfotransferase 1 or 2 enzymes, which are expressed in liver of human [Riches et al., 2009], mouse [Alnouti & Klaassen, 2006, Saeki et al., 1998], rat [Maiti and Chen, 2015, Runge-Morris et al., 1998], pig [Kojima & Degawa, 2014, Moe et al., 2007] and dog [Tsöi & Swedmark, 2005]. Information about sulfotransferase genes in sheep is lacking. There are major interspecies differences in the expressed complement of sulfotransferases, which makes extrapolation of data on xenobiotic sulfonation in animals to humans particularly unreliable. Some of these interspecies differences are further confounded by sex differences that do not occur in humans [Coughtrie, 2016].

At present we cannot ascribe the observed baseline sulfonation to any particular sulfotransferase enzyme in any species. Because sulfonation rates of some 7-hydroxycoumarin derivatives are cluster with each other in PCA and some did not among the different species, sulfonation of these compounds are presumably catalyzed by multiple sulfotransferases. It will be possible in future studies to evaluate substrate selectivity for human sulfotransferases since heterologously expressed human sulfotransferases are commercially available.

**Conclusions**

A convenient fluorescence based sulfonation assay was established for 7-hydroxycoumarin derivatives. Because this assay was sensitive and quantitative, it could be applied to determine sulfonation kinetics in liver cytosol of human, mouse, rat, pig, rabbit, dog and sheep. Sulfonation was fastest in dog showing higher intrinsic clearance ($V_{max}/K_m$) for most of the compounds than the other species. In pig, human and rabbit the intrinsic clearance was less for most of the compounds compared with the other species. The assay can be applied also to other
human and animal tissues to study baseline sulfonation levels. The assay can be also used to study potential inhibitors of sulfonation. Future studies will be directed at assessing which particular sulfotransferase enzymes mediate sulfonation of the 7-hydroxycoumarin derivatives.

Disclosure of interest

The authors report no conflict of interest.

Acknowledgements

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References


Table 1. $K_m$ and $V_{\text{max}}$ values of 7-hydroxycoumarin derivatives sulfonation by cytosol of seven species. The unit of $K_m$ is $\mu$M (95% confidence interval) and $V_{\text{max}}$ is nmol/(min * g protein) (95% confidence interval). Sulfonation rates of 7-hydroxycoumarin derivatives (1 – 14) were determined at 6-12 different concentration intervals 0 – 10 $\mu$M of incubations 0.05 - 0.3 g/L liver cytosol protein, 5 mM MgCl$_2$, 10 $\mu$M PAPS and 0 - 20 $\mu$M 7-hydroxycoumarin derivative in 100 mM phosphate buffer pH 7.4. ND means not determined and no MM that Michaelis-Menten equation was not applicable to the analysis.

<table>
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<th>Species</th>
<th>1</th>
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<th>3</th>
<th>4</th>
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<td>3.5 (0.8-8.3)</td>
<td>24 (0-160)</td>
<td>2.1 (1.5-2.7)</td>
<td>0.51 (0.26-0.79)</td>
<td>0.65 (0.46-0.85)</td>
<td>0.27 (0-0.51)</td>
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<td>4.7 (2.7-6.7)</td>
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$V_{\text{max}}$ (nmol/lin/min * g protein)
Figure captions

Figure 1. Sulfonation of 7-hydroxycoumarin derivatives by sulfotransferases (ST). Fluorescent 7-hydroxycoumarin derivatives are sulfonated to nonfluorescent sulfate conjugates by sulfotransferase enzymes.

Figure 2. Structures of the 7-hydroxycoumarin derivatives. 1 is 7-hydroxycoumarin (umbelliferone), 2 4-trifluoromethoxy-7-hydroxycoumarin, 3 6-methoxy-7-
hydroxycoumarin (scopoletin), 4 3,4-dimethyl-7-hydroxycoumarin, 5 3-ethyl-4-methyl-7-hydroxycoumarin, 6 3-ethyl-4,8-dimethyl-7-hydroxycoumarin, 7 3,4,8-trimethyl-7-hydroxycoumarin, 8 3-(4-methylphenyl)-7-hydroxycoumarin, 9 3-(4-hydroxyphenyl)-7-hydroxycoumarin, 10 3-(4-fluorophenyl)-7-hydroxycoumarin, 11 3-(4-methoxyphenyl)-7-hydroxycoumarin, 12 3-(4-dimethylaminophenyl)-7-hydroxycoumarin, 13 3-(4-pyridin-3-yl)-7-hydroxycoumarin, 14 3-(1H-1, 2, 4-tirazol-1-yl)-7-hydroxycoumarin.

Figure 3. Decrease of 7-hydroxycoumarin fluorescence during sulfonation. Panel A shows decrease of fluorescence and panel B decrease of concentration of 10 µM 7-hydroxycoumarin fluorescence in 100 mM phosphate buffer pH 7.4 in presence of 0.2 g/L rabbit liver cytosol protein, 10 µM PAPS, 5 mM MgCl₂. Panel C shows the effect of solvents on the sulfonation rate of 7-hydroxycoumarin with rabbit cytosol and panel D the effect of 100 mM phosphate or Tris-HCl buffer pH 7.4 on the fluorescence of 7-hydroxycoumarin during the 40 min incubation. Corresponding results were obtained with the other 7-hydroxycoumarin derivatives.
Figure 4. Sulfonation rates of 7-hydroxycoumarin and its 13 derivatives by liver cytosol of different species. The rates were determined in incubations containing 0.1-0.3 g/L liver cytosol protein, 5 mM MgCl₂, 10 µM PAPS and 10 µM 7-hydroxycoumarin derivative in 100 mM phosphate buffer pH 7.4. The activities were determined from the linear phases of the reactions. The number of samples were three for human, four for mice, two for rats and five for pigs, rabbits, dogs and sheep.
**Figure 5.** The effect of pretreatment of mice on sulfonation rate of 7-hydroxycoumarin and its 13 derivatives by liver cytosol. The rates were determined in incubations containing 0.1-0.3 g/L liver cytosol protein, 5 mM MgCl$_2$, 10 µM PAPS and 10 µM 7-hydroxycoumarin derivative in 100 mM phosphate buffer pH 7.4. The activities were determined from the linear phases of the reactions. The samples were pooled from at least ten mice.
Figure 6. Intrinsic clearance of 7-hydroxycoumarin derivatives in liver cytosol of different species. Sulfonation rates of 7-hydroxycoumarin derivatives were determined at different concentrations of incubations 0.05–0.3 g/L liver cytosol protein, 5 mM MgCl₂, 10 µM PAPS and 0–20 µM 7-hydroxycoumarin derivative in 100 mM phosphate buffer pH 7.4. The sulfonation rates were analyzed by Michaelis-Menten equation \( v = \frac{V_{\text{max}} \cdot S}{K_m + S} \). \( K_m \) is the Michaelis-Menten constant, \( V_{\text{max}} \) the limiting rate of the sulfonation reaction and \( v \) is the rate at the substrate concentration S.
Figure 7. Biplot of the principal component analysis model of the Michaelis-Menten kinetic parameters $K_m$, $V_{max}$ and $V_{max}/K_m$ of sulfonation of 7-hydroxycoumarin derivatives in cytosol of human, mouse, rat, dog, rabbit, pig and sheep liver. In the intrinsic clearance ($V_{max}/K_m$) PCA model, the first two components shown explained 68% and 17% of variance in the data, respectively. In the $K_m$ PCA model, the first two components shown explained 36% and 23% of variance in the data, respectively. In the $V_{max}$ PCA model, the first two components shown explained 45% and 27% of variance in the data, respectively. Substrates (black circles) show association with liver cytosols of different species (open hexagons). The Horizontal axis indicates relative magnitude of the indicated parameter and the vertical axis the relative magnitude between the species in the intrinsic clearance ($V_{max}/K_m$), $V_{max}$ or $K_m$ -PCA models.
Figure S1. Michaelis-Menten graphs of sulfonation of 7-hydroxycoumarin derivatives in liver cytosol of different species. Sulfonation rates of 7-hydroxycoumarin derivatives were determined at different concentrations of incubations 0.05–0.3 g/L liver cytosol protein, 5 mM MgCl₂, 10 µM PAPS and 0–20 µM 7-hydroxycoumarin derivative in 100 mM phosphate buffer pH 7.4. The number in parenthesis is correlation coefficient $r^2$-value of nonlinear Michaelis-Menten equation $v = S \cdot V_{\text{max}} / (K_m + S)$, in which $v$ is the sulfonation rate at 7-hydroxycoumarin derivative concentration $S$, $V_{\text{max}}$ is limiting rate and $K_m$ the Michaelis-Menten constant.