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Regioselective sulfonation of dopamine by SULT1A3 in vitro provides a molecular explanation for the preponderance of dopamine-3-O-sulfate in human blood circulation

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ARTICLE INFO

Article history:
Received 6 February 2007
Accepted 3 May 2007

Keywords:
Dopamine-3-O-sulfate
Dopamine-4-O-sulfate
SULT1A3
Regioselectivity
Enzyme kinetics
HPLC

ABSTRACT

SULT1A3 is an enzyme that catalyzes the sulfonation of many endogenous and exogenous phenols and catechols. The most important endogenous substrate is dopamine (DA), which is often used as a probe substrate for SULT1A3. We developed a new method for analyzing the SULT1A3 reaction products by high-performance liquid chromatography (HPLC) with electrochemical detection. The sulfonate donor 3′-phosphoadenosine-5′-phosphosulfate (PAPS), DA and the two dopamine sulfates, DA-3-O-sulfate and DA-4-O-sulfate, can be separated within 3 min. This enables quantitation of the sulfates without radioactive PAPS or the precipitation of unreacted PAPS. Both sulfates were synthesized as reference substances and characterized by ^{1}H and ^{13}C nuclear magnetic resonance (NMR), mass spectrometry (MS) and tandem mass spectrometry (MS/MS). The purity of the dopamine sulfates was estimated by HPLC using a diode array detector. We determined the enzyme kinetic parameters for formation of DA-3-O-sulfate and DA-4-O-sulfate using purified recombinant human SULT1A3. The reactions followed Michaelis–Menten kinetics up to 50 μM DA concentration, and strong substrate inhibition was observed at higher concentrations. The apparent K_m values for sulfonation at both hydroxy groups were similar (2.21 ± 0.764 and 2.59 ± 1.06 μM for DA-4-O-sulfate and DA-3-O-sulfate, respectively), but the V_{max} was approximately six times higher for the formation of the 3-O-sulfate (344 ± 139 nmol/min/mg protein) than the 4-O-sulfate (45.4 ± 16.5 nmol/min/mg protein). These results are in accordance with the observation that DA-3-O-sulfate is more abundant in human blood than DA-4-O-sulfate and that in the crystal structure of SULT1A3 with dopamine bound to the active site, the 3-hydroxy group is aligned to form hydrogen bonds with catalytic residues of the enzyme.

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Abbreviations: DA, dopamine; DA-3S, dopamine-3-O-sulfate; DA-4S, dopamine-4-O-sulfate; SULT, sulfotransferase; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PAP, 3′-phosphoadenosine 5′-phosphate; MS/MS, tandem mass spectrometry; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance

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doi:10.1016/j.bcp.2007.05.003

1. Introduction

In human blood circulation endogenous catecholamines exist predominantly in the sulfated form; for example, dopamine sulfate accounts for more than 90% of all dopamine [1]. It is believed that the vast majority of circulating dopamine sulfate originates in the upper gastrointestinal tract, and indeed that is the main site of expression of the enzyme responsible for its formation [2]. Sulfonation (Fig. 1) is the most important metabolic pathway that interferes with the binding of dopamine to its receptors. Dopamine-3-*O*-sulfate (DA-3S) predominates in human plasma, with concentrations about 10-fold higher than those of the regioisomer dopamine-4-*O*-sulfate (DA-4S) [3]. The origins of this preponderance for DA-3S have not been determined, although there has been speculation about the contribution of the specificity of transport proteins and/or arylsulfatases. It has also been proposed to depend on the regiospecificity of the metabolizing enzyme(s) for the 3-hydroxy group of dopamine.

Sulfonation of phenolic hydroxyl groups is catalyzed by members of the SULT1 family of cytosolic sulfotransferases (SULT). There are at least six SULT1 enzymes functionally expressed in humans, belonging to four subfamilies: SULT1A, SULT1B, SULT1C and SULT1E [4]. SULT1A3 is the only isoform showing high selectivity and catalytic efficiency for dopamine, and indeed a gene coding for SULT1A3 only exists in humans and other very close primate relatives such as chimpanzees and gorillas. According to X-ray crystallographic studies, SULT1A3 is also the only SULT having a carboxylate side chain in the active site capable of forming an ion pair with the protonated amino ethyl side chain of dopamine or related amines [5–8]. The crystal structure of SULT1A3 has been resolved in complex with 3′-phosphoadenosine 5′-phosphate (PAP) [7], with lithium sulfate [6] and with PAP and dopamine [8]. In this latter structure, the 3-hydroxy group of dopamine is aligned to form hydrogen bonds with residues His108 and Lys106 within the active site, whereas the 4-hydroxy group cannot easily form these bonds. Only the 3-hydroxy group is in line with the sulfate group of 3′-phosphoadenosine-5′-phosphosulfate (PAPS). It has been suggested, however, that dopamine fits in the active site enabling the sulfonation of either hydroxyl group because of the flexibility of the loops around the active site that may undergo conformational change upon ligand binding [6,7,9]. However, these findings have not been supported by an enzyme kinetic analysis of dopamine sulfonation in vitro, as the available assays measure only the sum of the 3-*O*-sulfate and 4-*O*-sulfate and are unable to resolve the two isomers. The most widely used barium precipitation SULT assay method originally described by Foldes and Meek [10], and other methods based on the TLC separation of sulfates, utilize [^{35}S]-labelled PAPS from which the radioactivity is transferred to the substrate along with the sulfonate group. Several ion pairing high-performance liquid chromatography (HPLC) methods for separating dopamine sulfates have been developed, but they have not been used for enzyme kinetic studies [11–15].

The aim of this study was to assess the regioselectivity of dopamine sulfonation in vitro by determining the enzyme kinetic parameters for the formation of the two dopamine sulfates by purified recombinant human SULT1A3. To achieve this, we synthesized and characterized the two dopamine sulfate isomers and developed a rapid and sensitive HPLC method for analyzing dopamine 3-*O*-sulfate and 4-*O*-sulfate produced following incubation with PAPS and SULT1A3.

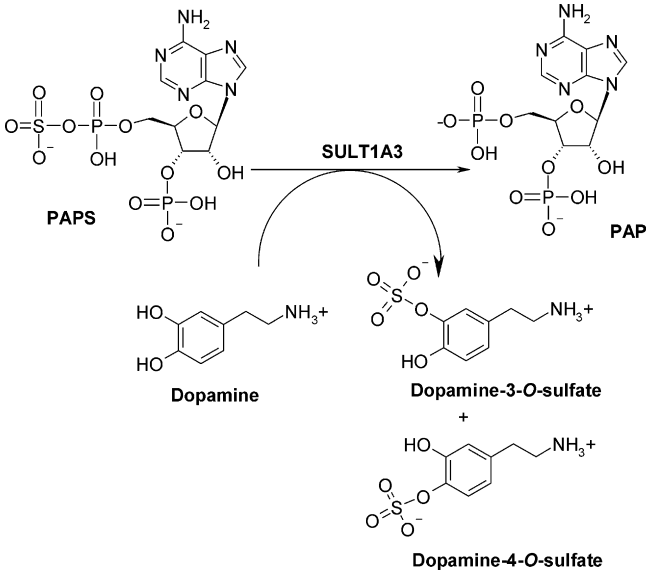

Fig. 1 – Reactions catalyzed by SULT1A3.

2. Materials and methods

2.1. Materials

The ampicillin sodium salt, isopropyl β-D-thiogalactopyranoside, Bradford reagent, dopamine hydrochloride, $ZnSO_4$ and $Ba(OH)_2$ were from Sigma. The PAP^{35}S and Emulsifier Safe scintillation fluid were from PerkinElmer Life and Analytical Sciences (Boston, MA, US) and the sodium dihydrogen phosphate dihydrate and disodium hydrogen phosphate were from Fluka (Germany). The other reagents used were acrylamide (Flowgen, UK), TEMED (National Diagnostics, UK), ammonium persulfate (Bio-Rad Laboratories, Hercules, CA, US), Luria Agar (Gibco BRL Life Technologies, UK), Luria Broth base (Invitrogen Life Technologies, UK), methanol (J.T. Baker, Deventer, Holland) and Idranal® III (Riedel-de Haën, Seelze, Germany). PAPS (99% purity) was purchased from H. Glatt (German Institute for Human Nutrition, Potsdam, Germany), and all other reagents were from WVR International (UK).

2.2. Synthesis and characterization of dopamine sulfates

Synthesis of the dopamine sulfates was based on the methods of Strobel et al. [15] and Jain et al. [16]. Dopamine hydrochloride (2.50 g, 13.18 mmol) was added to concentrated H_2SO_4 (7.5 ml) in small amounts over the course of 30 min at 0 °C. After a further 30 min of stirring, the reaction mixture was poured over crushed ice (50 g) while being vigorously stirred. The resulting cloudy solution of dopamine sulfates was applied to a column of Dowex 50W 1X8, 200–400 mesh (H^+

form) using distilled water as eluent. Fractions having same R_f (relative to front) values on silica coated TLC plates were combined and evaporated to dryness in vacuo. TLC plates were developed using butanol:acetic acid:water 4:1:5 as eluent and products were spotted with Pauly's reagent, which stained both DA-sulfates to yellow. Different fractions were identified with nuclear magnetic resonance (NMR) and the fraction containing DA-4S was purified by dissolving in hot distilled water and cooled to 4 °C overnight. The resulting crystallized white residue was separated by suction and dried in a desiccator. The crystallization procedure was repeated once to produce pure dopamine-4-O-sulfate (53 mg, 1.7% yield) as white crystals. All fractions (including the filtrates from the DA-4S crystallizations) containing dopamine sulfates from the above column were combined, evaporated to a small volume, and applied to another column of Dowex 1X8, 200–400 mesh (CH_3COOH form) using a gradient of 0–1 M acetic acid as eluent. Fractions having same R_f values on silica coated TLC plates were combined again and evaporated to dryness in vacuo. TLC plates were developed using butanol:acetic acid:water 4:1:5 as eluent and products were spotted with Pauly's reagent. Different fractions were identified with NMR and fractions containing DA-4S or DA-3S were combined separately evaporated to dryness in vacuo. White residues were dissolved in hot distilled water and cooled to 4 °C overnight. Crystallized white residues were separated by suction and dried in a desiccator to yield DA-3S and additional DA-4S.

The synthesis products were characterized by ^1H and ^{13}C NMR, MS and MS/MS, and the purity of the dopamine sulfates was estimated by HPLC using a diode array detector (Agilent Technologies). The NMR experiments were carried out in DMSO-d_6 at a temperature of 298K with a Varian Unity 300 MHz spectrometer. Chemical shifts are reported in ppm relative to tetramethylsilane (TMS).

The mass spectra were measured with a quadrupole time-of-flight mass spectrometer, the Micromass Q-TOF Micro (Micromass, Manchester, UK), equipped with a LockSpray dual-electrospray ionization source. The source conditions were as follows: capillary voltage 3000 V, sample cone voltage 23 V, extraction cone voltage 2 V, collision energy 10–30 V (MS/MS experiments), temperature 100 °C and desolvation temperature 300 °C. A mass range of 80–300 Da was acquired in the continuum mode. Nitrogen produced by a High Purity Nitrogen Generator (Peak Scientific, Inchinnan, Scotland) was used as desolvation gas (600 l/h) and as cone gas (60 l/h), and argon (99.95%) as the collision gas with a pressure of 1.4 bar. Accurate masses of the metabolites were measured by using internal lock mass calibration, and data were processed with Masslynx 4.0.

2.3. HPLC method and equipment

For the analysis of the sulfotransferase enzyme assay samples, a Shimadzu solvent delivery module LC-10ATvp, column oven CTO-10ASvp and auto injector SIL-10ADvp with sample cooler 10ADvp were used in concert with a Synergi Polar-RP ether-linked phenyl column (75 mm × 4.6 mm, Phenomenex). The mobile phase comprised 25 mM phosphate buffer (pH 3) and 0.1 mM ethylenediamine tetra-acetic acid in water purified by Milli-Q Plus equipment (Millipore, Molsheim, France). The flow rate was 1 ml/min, and the column oven was set at 35 °C. For the detection of dopamine sulfates, a Coulochem II® Multi-Electrode Detector with Model 5011A Analytical Cell (ESA Biosciences, Inc., MA, US) was used with electrode potentials of 200 and 400 mV in the coulometric and amperometric cells, respectively. Only the signal from the amperometric cell was recorded. For the purity analysis of the synthesis products DA-3S and DA-4S, Agilent 1100 series HPLC equipment with a diode array detector (Agilent Technologies, Waldbronn, Germany) was used. The wavelengths used were 210, 220, 230, 250, 270, 280 and 300 nm. The other conditions were the same as for the enzyme kinetic studies.

2.4. Expression and purification of recombinant SULT1A3

The human SULT1A3 cDNA was expressed in *E. coli* and purified as described previously [5,17]. Briefly, the SULT1A3 was purified from the *E. coli* cell-free extract by ammonium sulfate precipitation and two steps of chromatographic separation. First, the protein was applied to a HiTrap Q HP column (Amersham Biosciences), and second, to a 3′,5′-ADP agarose affinity column. The fractions eluted were tested using a SDS-PAGE and SULT enzyme assay using the barium precipitation method of Foldes and Meek [10]. The most active and pure protein fractions were pooled and dialyzed overnight between the purification steps and desalted using a PD-10 column (Amersham Biosciences) at the end of the procedure. The final composition of the buffer used to store the protein was 50 mM Tris/HCl buffer (pH 8) with 1 mM 2-mercaptoethanol. Protein concentration was estimated by the method outlined by Bradford [18] using bovine serum albumin (Perbio Science, UK) as standard.

2.5. Enzyme assay

The assays were carried out in 10 mM sodium phosphate buffer, pH 6.8, in a final volume of 160 μl, and the reactions were started with the addition of the enzyme. The reaction mixtures contained 0.5–1000 μM dopamine and 10 μM PAPS and were incubated at 37 °C for 10 min. All samples were prepared in duplicate. The conditions were chosen such that the reactions were within the linear range for the incubation time and enzyme concentration, and the reactions were stopped with 160 μl of chilled methanol. Control samples were incubated in the absence of dopamine, PAPS or enzyme to verify that no peaks eluted in HPLC chromatograms at the same time as dopamine sulfates. To some control samples, the enzyme was added after the addition of methanol to verify that the enzyme was properly deactivated under these conditions. The reaction mixtures were then frozen at −70 °C and lyophilized. The dried samples were dissolved in 50 μl of HPLC mobile phase and centrifuged for 5 min at 13,200 rpm after which 20 μl of supernatant were injected into the HPLC system. The enzyme kinetic parameters were determined by fitting the observed values of the reaction rate to the Michaelis–Menten equation by non-linear least squares regression method using SigmaPlot 9.0 with Enzyme Kinetics 1.1 (SPSS, Chicago, IL, US).

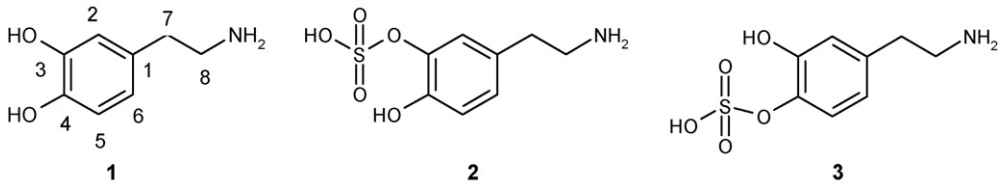

Fig. 2 – Structures of dopamine (1), dopamine-3-O-sulfate (2) and dopamine-4-O-sulfate (3).

3. Results

3.1. Characterization of dopamine sulfates by MS and MS/MS

The mass spectra of DA-3S and DA-4S showed an abundant protonated molecule (*m*/*z* 234) and a sodium adduct ion (*m*/*z* 256) in positive ion mode, and an abundant deprotonated molecule (*m*/*z* 232) in negative ion mode. The accurate masses for the protonated species of DA-3S and DA-4S were 234.0426 and 234.0436, respectively, and those for the deprotonated molecules 232.0280 and 232.0280, respectively. In MS/MS mode, the protonated molecules of DA-3S and DA-4S produced abundant product ions at *m*/*z* 217 and 137, respectively, and deprotonated molecular ions at *m*/*z* 152, 122 and 80, respectively.

3.2. Characterization of dopamine sulfates by NMR

Structures of dopamine, dopamine-3-O-sulfate and dopamine-4-O-sulfate are presented in Fig. 2 and ^{1}H and ^{13}C NMR data of dopamine sulfates are provided in next section. Clear deshielding effect of sulfate ester group in dopamine sulfates was observed in ^{1}H and ^{13}C NMR spectra. The NMR spectra were identical to the ones reported by Strobel et al. [15].

3.2.1. Dopamine-3-O-sulfate

^{1}H NMR (300 MHz, DMSO-d_6) δ 2.73 (t, J = 8.0 Hz, 2H, H-7), 2.98 (t, J = 8.0 Hz, 2H, H-8), 6.78 (d, J = 8.1 Hz, 1H, H-5), 6.84 (dd, J = 8.1, 2.0 Hz, 1H, H-6), 7.07 (d, J = 2.1 Hz, 1H, H-2).
^{13}C NMR (75 MHz, DMSO-d_6) δ 32.2 C-7, 40.3 C-8, 117.3 C-5, 123.2 C-2, 124.9 C-6, 128.0 C-1, 140.9 C-3, 148.0 C-4.

3.2.2. Dopamine-4-O-sulfate

^{1}H NMR (300 MHz, DMSO-d_6) δ 2.73 (t, J = 7.8 Hz, 2H, H-7), 3.00 (t, J = 7.7 Hz, 2H, H-8), 6.64 (dd, J = 8.4, 2.4 Hz, 1H, H-6), 6.74 (d, J = 2.4 Hz, 1H, H-2), 7.07 (d, J = 8.1 Hz, 1H, H-5).
^{13}C NMR (75 MHz, DMSO-d_6) δ 32.6 C-7, 40.3 C-8, 117.5 C-2, 119.6 C-6, 123.3 C-5, 133.9 C-1, 139.8 C-4, 149.2 C-3.

3.3. Yield and purity of dopamine sulfates

The yields from the dopamine sulfate synthesis process were 94 mg (3.1%) and 96 mg (3.1%) for DA-4S and DA-3S, respectively. The purity of the synthesis products was estimated by HPLC using a diode array detector, and the resulting chromatograms showed over 99% purity for both regioisomers. The impurities were not identified.

3.4. HPLC method for separation and quantification of dopamine sulfates

The HPLC method was validated by determining resolution, the limit of quantitation, linearity and the repeatability of retention times and peak areas (Table 1). The resolution between DA-4S and DA-3S was 1.15, and was sufficient for accurate and reproducible separation of dopamine sulfates (Fig. 3). The relative standard deviations (R.S.D.) of the chromatographic peak areas of DA-4S and DA-3S (including sample preparation) were 14.6 and 5.55%, respectively. The R.S.D. of the retention times within one day were 0.243 and 0.366%, and between days were 2.02 and 2.15% for DA-4S and DA-3S, respectively. The limit of quantitation was 33.3 nM for both sulfates. A chromatogram of a sample incubated with the lowest concentration of dopamine (0.5 μM) is presented as an example in Fig. 3. Linearity was determined using six concentrations between 33.3 and 3.2 μM, and R^2 was 0.994 for DA-4S and 0.997 for DA-3S.

3.5. Enzyme kinetics of sulfonation

The rate of sulfonation was measured as a function of dopamine concentration and the kinetic parameters were determined by fitting the Michaelis–Menten equation by a

Table 1 – Validation parameters for the HPLC method

	DA-4S	DA-3S
Resolution (mean ± S.D.)	1.15 ± 0.084	
Retention time (min)	2.44	2.65
Retention times R.S.D.% within one day	0.243	0.366
Retention times R.S.D.% between days	2.02	2.15
Calibration curve (weighted 1/y)	y = 27018x − 579.33	y = 24769x − 387.97
R^2	0.994	0.997
Signal repeatability R.S.D.% within one day (sample preparation included)	14.6	5.55
Limit of quantitation (nM)	33.3	33.3

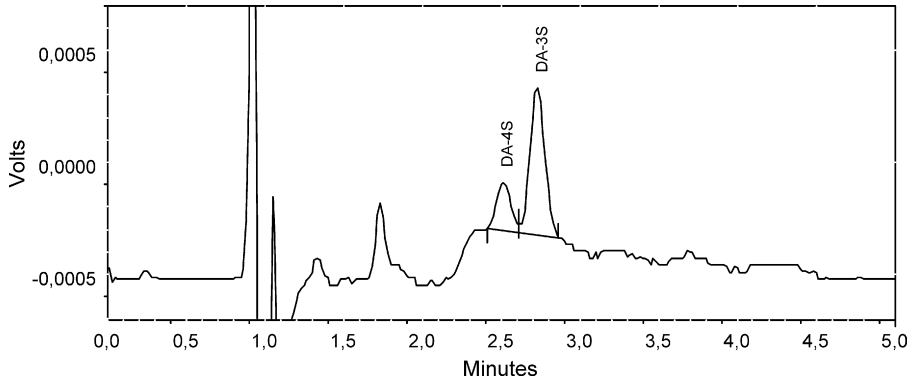

Fig. 3 – The separation of dopamine and its sulfates by HPLC with electrochemical detection. The sample was incubated in the presence of 0.5 μM dopamine.

non-linear least-squares regression using a weighting factor 1/y to the observed reaction rate values between 0.5 and 30–60 μM dopamine where substrate inhibition was not significant. The reaction followed Michaelis–Menten kinetics at low concentrations, but strong substrate inhibition was observed at high dopamine concentrations (Fig. 4).

Apparent K_m values for the formation of DA-3S and DA-4S were almost identical at 2.59 and 2.21 μM, respectively, but the V_{max} for the formation of DA-3S (344 nmol/min/mg) was more than seven times higher than that for DA-4S (45.4 nmol/min/mg) (Table 2). The observed substrate inhibition at higher dopamine concentrations varied between the days for both sulfates. Fig. 4 shows examples of the curves describing the initial reaction rates plotted as a function of dopamine concentration for both sulfates.

4. Discussion

The synthesis presented here was based on methods previously reported by Jain et al. [16] and Strobel et al. [15].

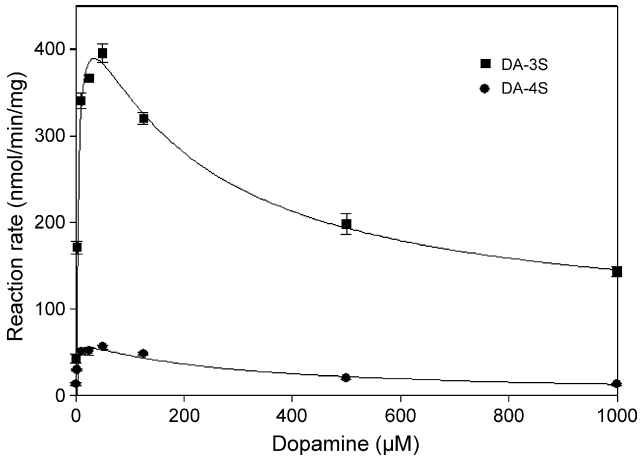

Fig. 4 – Formation rates of DA-3S and DA-4S as a function of dopamine concentration. All the samples were prepared in duplicate, and the lines are the best fits to substrate inhibition equation presented by Gamage et al. [22] $(v = V_\infty[S](K_1 + [S])/(K_2K_3 + [S]K_3 + [S]^2)$**.**

Dopamine sulfates have often been synthesized by a method reported by Jenner and Rose [19], but Kienzl and Eichinger [14] claimed it is likely that preparation of DA-3S and DA-4S according to this method leads to contamination of DA-3S and DA-4S with dopamine hydrogen sulfate. Therefore, we chose the methods of Jain et al. [16] and Strobel et al. [15] as the basis of our synthesis. The aim of the synthesis was to produce pure dopamine sulfate standards for development of the HPLC method and the quantitation of the sulfates in assays incubated with purified recombinant SULT1A3. Although the yields were low, 3.1% for both dopamine sulfates, the final amounts produced (94–96 mg) were sufficient for our experiments. The methods used for the purification of the standard compounds provided full separation of DA-3S and DA-4S and therefore over 99% purity determined by HPLC-UV. The structures of the products were characterized by mass spectrometry (MS), high-resolution MS (HRMS), tandem mass spectrometry (MS/MS), and ^1H and ^{13}C NMR.

The abundant protonated and deprotonated molecules recorded by positive and negative ion electrospray mass spectrometry, respectively, indicate the correct molecular weights of the synthesized dopamine sulfates. The accurate masses of the protonated molecules of DA-3S and DA-4S deviated from the calculated ones by −1.0 and −0.1 mDa, respectively. The respective deviations for deprotonated molecules were 0.6 and 1.5 mDa. These accuracies indicate the correct elemental composition of the standard compounds.

The product ion spectra of protonated (*m/z* 234) and deprotonated (*m/z* 232) molecules of DA-3S and DA-4S showed

Table 2 – Enzyme kinetic parameters for the formation of dopamine sulfates by purified recombinant human SULT1A3

	DA-4S	DA-3S
K_m (μM)	2.21 ± 0.764	2.59 ± 1.06
V_{max} (nmol/min/mg)	45.4 ± 16.5	344 ± 139
V_{max}/K_m (ml/min/mg)	20.8 ± 6.19	134 ± 39.4
Substrate inhibition (%)	40–80	35–85
$V_{max(3S)}/V_{max(4S)}$	7.46 ± 1.41	
$K_{m(S3)}/K_{m(4S)}$	1.23 ± 0.582	

Values are mean ± S.D. ($n = 4$).

Table 3 – ^{1}H NMR chemical shifts of dopamine and dopamine sulfates

Compound	Proton number				
	H-2	H-5	H-6	H-7	H-8
DA-3S	7.07	6.78	6.84	2.73	2.98
DA-4S	6.74	7.07	6.64	2.73	3.00
Dopamine	6.66	6.71	6.49	2.74	2.92

diagnostic product ions $[M+H-NH_3-SO_3]^+$ (*m*/*z* 137), $[M-H-SO_3]^-$ (*m*/*z* 152) and $[SO_3]^-$ (*m*/*z* 80), clearly indicating the presence of the sulfate moiety. However, the spectra showed no difference between DA-3S and DA-4S, and the determination of the site of the sulfonation was not possible by mass spectrometry. The NMR spectra were identical to the ones reported by Strobel et al. [15]. In the DA-3S ^{1}H NMR spectra, the H-2 proton is shifted downfield compared to dopamine due to the deshielding effect of the 3-sulfate ester group (Table 3). Similarly in the DA-4S ^{1}H NMR spectra, the H-5 proton is shifted downfield compared to dopamine due to the deshielding effect of the 4-sulfate ester group. In the DA-3S ^{13}C NMR spectra, C-3 carbon is shifted upfield relative to dopamine due to the deshielding effect of the 3-sulfate ester group (Table 4). Carbons C-2 and C-4 at the *ortho* positions and C-6 at the *para* position are shifted downfield. Similarly in the DA-4S ^{13}C NMR spectra, C-4 carbon is shifted upfield compared to dopamine due to the deshielding effect of the 4-sulfate ester group. Carbons C-3 and C-5 at the *ortho* positions and C-1 at the *para* position are shifted downfield. These results support the correct structures of synthesized DA-3S and DA-4S.

The HPLC method developed here provides a fast, sensitive, reliable and easy method for the separation and analysis of the regioisomers of dopamine sulfate. The ether-linked phenyl stationary phase with the aqueous mobile phase provides enhanced selectivity for polar and aromatic compounds and is therefore optimal for the separation of dopamine sulfates. The separation of dopamine, DA-3S and DA-4S with an acceptable resolution (1.15) was obtained within 5 min. The reproducibility of retention times (R.S.D. $< 2.15\%$) and peak areas of the dopamine sulfates (R.S.D. 5–15%) were good, indicating high stability and reliability of the method. High correlation coefficients ($R^2 > 0.994$) also indicated a good quantitative linearity of the method. The use of electrochemical detection provides high selectivity and sensitivity for the dopamine sulfates, which are efficiently oxidized with an electrode potential of 400 mV. The low limit of quantification (33 nM) allowed incubations with low enough concentrations of dopamine. The method developed here seems superior to previous methods based on the use of ion pairing reagents and C-18 reversed phase columns [11–15,20].

Our results from the enzyme kinetic analysis are in accordance with previous observations that SULT1A3 has selectivity for the 3-*O*-sulfonation of L-dopa [21] and that DA-3S predominates the DA-4S in human blood. Apparent K_m values for the formation of DA-3S and DA-4S were similar, but the V_{max} values differed more than seven-fold, and therefore, the specificity constant determined as V_{max}/K_m was also approximately seven-fold higher for the sulfonation of the 3-hydroxyl group. The values obtained are in good agreement with those published previously using the barium precipitation assay, which showed a K_m of approximately 1 μM and a V_{max} of almost 200 nmol/min/mg, obviously representing the combined formation of DA-3S and DA-4S [17].

As expected, between 35 and 85% substrate inhibition was observed at high dopamine concentrations. Substrate inhibition is a very common feature of SULT enzymes. SULT1A1 has been crystallized with PAP and two *p*-nitrophenol (pNP) molecules in the active site, and this has been proposed to explain the substrate inhibition [22]. Gamage et al. [22] also studied the substrate inhibition with enzyme kinetic methods. They suggested that the enzyme can bind the pNP at site 1 (the active site) or site 2 (additional binding site) and that occupancy of site 1 does not affect the binding of pNP at site 2, although pNP cannot be bound to site 1 if site 2 is occupied. The product pNP-sulfate cannot be released if site 2 is occupied. With high substrate concentration, both sites are occupied, but still some residual activity is observed. Another inhibition mechanism has been proposed for SULT2A1 by dehydroepiandrosterone. It can bind to the active site of SULT2A1 in two different orientations, which may be related to substrate inhibition as well [23]. However, the substrate inhibition of SULT1A3 by dopamine has been suggested to be caused by the binding of two dopamine molecules to the enzyme in a similar way as pNP binds to SULT1A1 [9]. Mn^{2+} averts the substrate inhibition by dopamine, supposedly by interacting with Glu89 and Asp86 and thus displacing the second dopamine molecule from the active site. On the other hand, in the latest SULT1A3 crystal structure, only one dopamine molecule is bound in the active site [8]. In the present study we observed varying substrate inhibition by dopamine, and in some cases the data fitted well in the equation proposed Gamage et al. [22] where the residual enzyme activity is taken into account, which might implicate the same substrate inhibition mechanism as for SULT1A1. In some cases, however, the substrate inhibition seemed to behave differently, and the activity approached zero with high dopamine concentrations. A further mechanism for SULT substrate inhibition is proposed by Gamage et al. [24] for SULT1A1 and 17β-estradiol, involving the formation of a SULT1A1·PAP·17β-estradiol dead-end complex demonstrated by X-ray crystallography.

Table 4 – ^{13}C NMR chemical shifts of dopamine and dopamine sulfates

Compound	Carbon number							
	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
DA-3S	128.0	123.2	140.9	148.0	117.3	124.9	32.2	40.3
DA-4S	133.9	117.5	149.2	139.8	123.3	119.6	32.6	40.3
Dopamine	128.0	115.9	145.3	144.0	116.1	119.2	32.3	40.3

The reason for the different enzyme kinetics observed is not clear. Previously it has been observed that oxidation and reduction affect the function of SULTs in vitro. For rat aryl sulfotransferase IV, the reduced form of the enzyme shows substrate inhibition, but the oxidized form displays normal Michaelis–Menten kinetics [25]. SULT1E1 has also been reported to lose its activity through oxidation [26]. We had the SULT1A3 enzyme in reduced form because of the 2-mercaptoethanol that was present in the enzyme storage buffer. We also observed that placing additional 1 mM 2-mercaptoethanol into the reaction mixtures did not affect the kinetic behavior of the enzyme.

It has been suggested that specificity of transport proteins or arylsulfatases might be the factors influencing the blood circulating levels of DA-3S and DA-4S [27]. In conclusion, the results presented here indicate that SULT1A3 strongly favors the 3-hydroxy group of dopamine over the 4-hydroxy group and may indeed be primarily responsible for the difference between the circulating levels of dopamine sulfates in human blood. Our results are fully consistent with the increasing amount of crystallographic and other data appearing on the mechanism of the sulfonation reaction.

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