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FORMATION AND METABOLISM OF [^{14}C]DOPAMINE 3-*O*-SULFATE IN DOG, RAT AND GUINEA PIG

ILMAR MERITS

Drug Metabolism Department, Abbott Laboratories, North Chicago, Ill. 60064, U.S.A.

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Abstract—Oral administration of [^{14}C]dopamine to dogs resulted in urinary excretion of predominantly [^{14}C]dopamine 3-*O*-sulfate, while after intravenous administration the labeled drug was metabolized largely by *O*-methylation and deamination pathways. Experiments *in vitro* pinpointed the small intestinal wall of the dog as the site of dopamine sulfate conjugate formation. The small intestines of rat and guinea pig lack this sulfating ability. When trace amounts of [^{14}C]dopamine 3-*O*-sulfate were administered to dog, rat and guinea pig, the compound turned out not to be metabolically inert. In the guinea pig, [^{14}C]dopamine 3-*O*-sulfate was almost completely desulfated and metabolized according to the pattern characteristic to orally administered dopamine in this animal species. In rat, about 40 per cent of the administered [^{14}C]dopamine 3-*O*-sulfate (19·1 μg/kg) was excreted in urine unchanged, whereas a smaller dose (7·4 μg/kg) was totally metabolized according to the pattern characteristic to rat. In dog urine, more than 80 per cent of the radioactive dose (2·5 μg/kg) emerged in urine as unchanged [^{14}C]dopamine 3-*O*-sulfate, the normal metabolism end product of dopamine in dog.

Sulfate conjugates of dopamine have been known to be excretory metabolites in animals and man, although Jenner and Rose [1, 2] suggest the possibility that they may also represent intermediates of metabolism. In our previous study concerning the metabolic fate of orally administered [^{14}C]dopamine [3], dogs excreted the labeled dose largely in the form of urinary [^{14}C]dopamine *O*-sulfate ester, whereas rats and guinea pigs excreted sulfate conjugated dopamine in minor quantities or hardly at all. It was of interest, therefore, to investigate the exact site of formation of dopamine *O*-sulfate in the dog. In addition, it was decided to investigate the metabolic fate of dopamine 3-*O*-sulfate in rat and guinea pig where such metabolite is produced normally only in minor quantities.

MATERIALS AND METHODS

Chemicals. Glass-distilled water was used during this work. Amberlite CG-50 weakly acidic carboxylic type cation exchange resin, 200–400 mesh, Lot ZEC, was obtained from Mallinckrodt. The resin was cycled with hydrochloric acid and sodium hydroxide, and equilibrated with Na-phosphate buffer, pH 6·0, as described by Minard and Grant [4]. AG1-x8 anion exchange resin in the formate form, 200–400 mesh, from Bio-Rad Laboratories, was soaked in 4 M formic acid and thoroughly washed with water before it was filled into the columns. Formic acid, 88%, analytical reagent, was obtained from Mallinckrodt. Silica gel Q1F Quanta/Gram plates, 5 × 20 cm, were purchased from Quantum, Inc. Ketodase, brand of β-glucuronidase, containing 5000 units/ml, was purchased from Warner Chilcott's General Diagnostics Division. Glusulase preparation from Endo Laboratories, Inc. contained 158,700 units glucuronidase and 52,400 units sulfatase/ml. Aquasol scintillator solution was obtained from New England Nuclear. Methanol–toluene-based scintillator (Formula B) and toluene–cellusolve scintillator solution (Formula A) were purchased from Burdock & Jackson Labs. Adenosine-5′-triphosphate (ATP) disodium salt was obtained from P-L Biochemicals, Inc. Dopamine·HBr, 99% pure, was purchased from Aldrich Chemical Co., Inc. All other reagents were analytical grade.

Synthesis of dopamine 3- *and* 4-*O-sulfates.* This synthesis was accomplished according to the scaled-up procedure of Jenner and Rose [1]. The products were characterized by i.r. and NMR spectra and by elemental analysis.

Animals. Female purebred beagle dogs (7·1–7·5 kg) were dosed by stomach tube. In addition, a 440-g male albino guinea pig and a 216-g male white Sprague–Dawley rat received [^{14}C]dopamine 3-*O*-sulfate by gavage. A 915-g male guinea pig and a 487-g male white rat were given the [^{14}C]dopamine 3-*O*-sulfate dose intravenously. All species were fasted for 18 hr prior to experimentation. Six hr after the dosage the animals were fed Gaines dog food and Purina Laboratory Chow. The injections were given into the saphenous vein (dog), the tail vein (rat), and the penis vein (guinea pig).

Formation in vivo of [^{14}C]*dopamine* 3-*O-sulfate.* 3,4-Dihydroxyphenylethylamine·HBr [1-^{14}C] ([^{14}C]dopamine·HBr, 9·28 mCi/m-mole) labeled at the α-carbon of the phenethyl moiety was obtained from New England Nuclear. It was diluted with an equal amount of unlabeled dopamine·HBr thus resulting in a specific radioactivity of 4·64 mCi/m-mole. The drug was administered in the form of a freshly prepared saline solution at a dose of 0·15 mg/kg orally to dog No. 1 and intravenously to dog No. 2. Urine, voided up to 24 hr after dosing, was collected and the radioactive urinary metabolites were separated by column chromatography on AG1 (formate) resin. Eight days later the dogs were switched: now [^{14}C]dopamine was given intravenously to dog No.

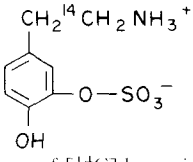

Fig. 1. Structure of [^{14}C]dopamine 3-*O*-sulfate.

1 and orally to dog No. 2 and again 0- to 24-hr urine samples were analyzed on AG1 anion-exchange resin columns.

The fractions containing radioactive dopamine sulfate (Fig. 2) were pooled and freeze-dried. The combined freeze-dried material was dissolved in 25 ml saline. Its radiochemical purity (>90 per cent) was determined by thin-layer chromatography in three solvent systems and by hydrolysis with Glusulase followed by column chromatographic analyses. This material was co-chromatographed on 0·8 × 50 cm AG1-x8 (200–400 mesh, in acetate form) columns with authentic unlabeled dopamine 3-*O*-sulfate and dopamine 4-*O*-sulfate samples as described by Bodnaryk and Brunet [5], the eluate being monitored at 275 nm in a Zeiss spectrophotometer and 0·3-ml aliquots of the 7·5-ml fractions being radioassayed in 10 ml of toluene–cellusolve scintillator solution with a Packard Tri-Carb scintillation spectrometer.

The [^{14}C]dopamine 3-*O*-sulfate solution in saline was stored at −16° and thawed immediately before administration to rats, guinea pigs and dog.

Preparation of crude extract of small intestine. The high-speed supernatant of dog, rat and guinea pig small intestine extract was used as a source of the sulfate-transferring enzymes. A beagle dog was sacrificed by intravenous injection of KCl solution; a rat and a guinea pig were killed by a blow on the head. The small intestines were quickly excised, chilled and thoroughly washed with cold water. Sections of 1–3 cm were cut out of the small intestine at every 5–10 cm interval. The sections were blotted and cut with scissors into small pieces in a beaker placed on ice. Five g of the material was homogenized in 20 ml of 0·25 M sucrose solution with the aid of a motor-driven ground-glass homogenizer (Kontes Glass Co.) rotating at 650 rev/min, the suspension being forced past the pestle ten times during 2 min while the homogenizer was submerged into ice. The homogenate was then centrifuged at 100,000 g_{av} for 1·5 hr in a Spinco model L preparative ultracentrifuge using a No. 50 Ti-rotor. The clear supernatant was carefully removed, stored overnight at −20° and used the next day.

Formation of [^{14}C]dopamine 3-O-sulfate ester in vitro. A one-ml portion of the small intestine high-speed supernatant was incubated in a total volume of 10 ml containing 100 μmoles $MgCl_2$, 20 μmoles Na_2SO_4, 50 μmoles K_2HPO_4, 150 μmoles ATP and 0·2 μmole [^{14}C]dopamine·HBr (9·28 μCi/m-mole). All components were adjusted to pH 7·4 with 0·1 N NaOH before mixing. After incubation for 1·5 hr at 37°, the solution was poured on an AG1 (formate) column and the radioactive compounds were eluted with a formic acid gradient as described below.

Preparation of urine samples for analysis. Ascorbic acid and Na_2EDTA were added to the urine samples (both 0·5 mg/ml) to protect the metabolites against oxidation and the samples were kept frozen before they were analyzed. Since most of the ^{14}C was excreted in the urine within 24 hr, the 0- to 24-hr urine samples were used for the determination of the metabolic patterns on AG1 (formate) columns as described below. For the hydrolysis of conjugated metabolites, 15 ml urine was adjusted to pH 6 with 1 M acetic acid or Na-acetate solution and divided into three equal parts. To one part was added 1 ml Ketodase, to the other 0·3 ml Glusulase; the third part served as a control. The samples were incubated at 37° for 15 hr and chromatographed side-by-side on AG1 (formate) columns as described below.

AG1-x8 anion exchange resin columns. Separation of radioactive urinary metabolites was achieved with 0·8 × 50 cm columns filled with AG1-x8 resin in the formate form [3]. In general, 5–10 ml of sample/column was applied; the samples were washed with water and the metabolites were eluted with a gradient of formic acid. Usually four columns were operated simultaneously side-by-side using the same gradient generating system: the constant volume mixer contained 500 ml water and the reservoir 3000 ml of 4 M formic acid. The gradient was pumped by a Desaga multi-channel peristaltic pump through the columns yielding 7·5-ml fractions in 5 min. The shape of the gradient concentration curve was determined by measuring the refractive indices of the fractions with a Bausch and Lomb refractometer. Finally, 0·3-ml aliquots of fractions containing the metabolites of [^{14}C]dopamine were mixed with 10 ml of toluene cellusolve scintillator solution and assayed for radioactivity. From the fractions containing the metabolites of [^{14}C]dopamine 3-*O*-sulfate, 3-ml aliquots were mixed with 10 ml Aquasol and the resulting gels were radioassayed. The AG1 columns could be regenerated by passing through them about 100 ml of 30% (v/v) formic acid containing 10% (w/v) of Na-formate followed by 300–500 ml of distilled water.

Amberlite CG-50 cation exchange resin columns. The effluents of the AG1 (formate) anion exchange resins were then passed through 0·8 × 50 cm Amberlite CG-50 columns. Usually four columns were operated simultaneously side-by-side with a Desaga pump yielding 7·5-ml fractions in 5 min. Glucuronic acid conjugates and deaminated metabolites emerged in the first 15 fractions which comprised the effluent and water wash. The free dopamine was eluted with 1% boric acid solution [4, 6]. Finally, after 80 fractions had been obtained, the *O*-methylated catecholamines were eluted with 1 N hydrochloric acid in about 20 fractions. Three-ml aliquots of the fractions were mixed with 10 ml Aquasol scintillation liquid and assayed for radioactivity.

Calculations. Conjugated and free forms of the compounds were determined by difference based on the assumption that Ketodase hydrolyzes only glucuronic acid conjugates and Glusulase hydrolyzes both glucuronic acid and sulfuric acid conjugates.

Thin-layer chromatography. The identities of the major radioactive peaks, obtained on AG1 (formate) columns, were further confirmed by thin-layer chromatography in four solvent systems. For this the radioactive fractions of the formic acid eluate were lyophilized, dissolved in 0·5 to 1 ml methanol and aliquots of the methanol solution were applied on Silica

Table 1. Thin-layer chromatography R_f values for some dopamine metabolites in four solvent systems

	Solvent systems			
	No. 1 Chloroform 80 Methanol 18 Water 2	No. 2 Chloroform 80 Methanol 17 Acetic acid 3	No. 3 Benzene 95 Acetic acid 5	No. 4 Acetic acid 20 Water 20 *n*-Butanol 80
3,4-Dihydroxyphenylacetic acid	0·35	0·55	0·0	0·90
3,4-Dihydroxymandelic acid	0·10	0·15	0·0	0·50
Homovanillic acid	0·65	0·75	0·35	
Vanillylmandelic acid	0·20	0·25	0·10	0·60
3-Hydroxyphenylacetic acid	0·35	0·75	0·15	0·85
3-Methoxyphenylacetic acid	0·72	0·92	0·60	0·85
Dopamine sulfate	0·10	0·15	0·0	
N-acetyl dopamine	0·55	0·60	0·0	
3,4-Dihydroxyphenetol	0·55	0·65	0·0	0·88
Homovanillyl alcohol	0·70	0·85	0·35	0·90
3,4-Dihydroxyphenyl glycol	0·25	0·35	0·0	0·75

gel plates. Reference standards were spotted on the same plates and the plates were developed for about 1 hr for the first three solvent systems and for 4 hr for solvent system No. 4. Table 1 gives the R_f values of some standards (known dopamine metabolites) as detected under u.v. light. The radioactive bands on the plates were located by sequentially scraping 0·5-cm sections of Silica gel into scintillation vials containing 10 ml methanol–toluene-based scintillator solution and assayed for radioactivity.

RESULTS

Formation in vivo of [^{14}C]dopamine 3-O-sulfate in the dog. In the first part of this study, the metabolic pathways of dopamine in dog were compared after oral and intravenous administration of the drug. Therefore, only relatively small dosages of the drug (0·15 mg/kg) were used. Nevertheless, the metabolic patterns of these orally administered doses were very similar to the patterns obtained earlier in another study where one hundred times higher doses were administered orally to dogs [3].

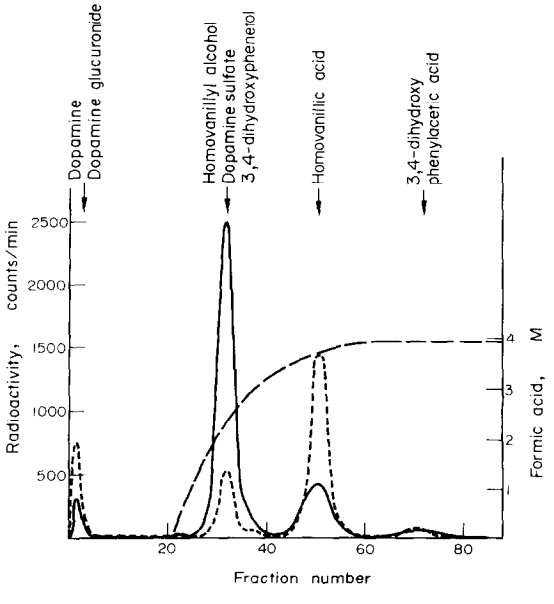

Fig. 2. Separation of dog urinary metabolites of [^{14}C]dopamine on AG1 (formate) column. Formic acid molarity is shown thus. ——. The profile of radioactivity excreted after oral dosage is shown (– – –), and after intravenous dosage (·····). The arrows show the positions of known dopamine metabolites on the chromatogram.

Figure 2 gives the positions of known dopamine metabolites on a typical chromatogram obtained with dog urines after oral and intravenous administration of the labeled drug. Table 2 shows that the difference of dopamine metabolic pathways in the dog depends only on the route of administration of the drug and does not depend on which route was used first in the same dog.

It can be seen that oral administration of dopamine resulted mainly in the sulfation of the drug. Deamination and *O*-methylation, resulting in the production of homovanillic acid, occurred only to a small extent. After intravenous administration of dopamine the deamination and *O*-methylation pathways dominated resulting in considerable production of labeled homovanillic acid, while the sulfate conjugate was a relatively minor urinary metabolite. The labeled dopamine sulfate ester in dog urine after oral or intravenous administration of [^{14}C]dopamine has been identified as [^{14}C]dopamine 3-*O*-sulfate since the radioactive fractions coincided with the added unlabeled dopamine 3-*O*-sulfate marker on AG1 (acetate) column when eluted with a shallow acid gradient according to Bodnaryk and Brunet [5], while the 4-*O*-sulfate isomer was eluted later at higher acetic acid concentration.

The lyophilized radioactive peak material showed very little mobility when subjected to thin-layer chromatography in three solvent systems. Further identification of the labeled dopamine sulfate ester fraction was obtained by enzymatic hydrolysis of the freeze-dried radioactive peak material with Glusulase at pH 6·5 and subsequent rechromatography on AG1 column. Almost all of the applied radioactivity appeared now in the effluent (where free dopamine would emerge) and only less than 5 per cent in the fractions where previously labeled dopamine sulfate ester had been eluted. The effluent containing free

Table 2. Labeled urinary metabolites of orally or intravenously administered [^{14}C]dopamine in dog (0·15 mg/kg)

Metabolite		Percentage of recovered urinary ^{14}C			
		Oral administration		Intravenous administration	
		Dog 1	Dog 2	Dog 1	Dog 2
Dopamine		None	None	0·2	0·2
O-methylated catecholamines		0·2	0·3	3·6	3·0
Dopamine 3-*O*-sulfate		76·1	82·2	22·1	19·8
Homovanillic acid	Free	18·1	13·1	60·6	64·8
	Conjugated	None	0·1	None	None
3,4-Dihydroxyphenylacetic acid	Free	1·5	0·9	4·5	3·3
	Conjugated	2·9	1·2	3·3	2·9
Not identified		1·2	2·2	5·7	6·0
Recovery of radioactivity applied on the column (%)		83	88	87	89
Recovery of radioactive dose in 0- to 24-hr urine (%)		86·2	82·9	90·8	92·7

dopamine was chromatographed further on Amberlite CG-50 column and eluted with boric acid, thus verifying the identity of the liberated dopamine. On the other hand, incubation of freeze-dried dopamine sulfate ester fractions with Ketodase followed by rechromatography on AG1 column did not change the positions of the radioactive fractions on the chromatogram.

Enzymatic formation in vitro *of* [^{14}C]*dopamine 3-O-sulfate.* The experiments *in vivo* show that sulfoconjugation plays a significant role in the elimination of dopamine in the dog. Since the pattern of dopamine metabolism varies with the route of administration and the amount of dopamine 3-*O*-sulfate becomes considerable after oral administration of dopamine to the dog, the possible role of the digestive tract becomes evident. In order to establish the main site of formation of the excreted dopamine sulfate, the high-speed supernatant of dog small intestine extract was used as a source of enzymes responsible for the metabolism of orally administered dopamine. It was found that, indeed, 97–98 per cent of the [^{14}C]dopamine was converted to [^{14}C]dopamine 3-*O*-sulfate when incubated with dog intestinal extract. There was no labeled dopamine sulfate formation when a corresponding high-speed supernatant from dog stomach extract was used for incubation. The high-speed supernatants from small intestine of rat and guinea pig likewise failed to produce [^{14}C]dopamine sulfate ester from labeled dopamine. These data lend support to the view that dopamine is extensively metabolized in the intestinal wall of the dog before it enters into the blood stream, i.e. before it is absorbed.

Metabolic fate of [^{14}C]*dopamine 3-O-sulfate in guinea pig, rat and dog.* The source of labeled dopamine sulfate ester in these experiments was dog urine after the dogs were given [^{14}C]dopamine orally. The fractions of the radioactive peak on AG1 (formate) columns containing [^{14}C]dopamine 3-*O*-sulfate were pooled and lyophilized. Although the material was sufficiently radioactive for many metabolism experiments, the amounts given to the animals were quite low. Thus, tracer amounts in microgram rather than milligram quantities per kg body weight were administered. Table 3 summarizes the results. The data indicate that [^{14}C]dopamine 3-*O*-sulfate was readily absorbed and that the major portion of the absorbed dose was rapidly eliminated in the urine of all species. Contrary to expectation, not all [^{14}C]dopamine 3-*O*-sulfate was eliminated unchanged. The drug had been metabolized to a varying extent in all species and the new labeled metabolites were excreted in urine. Only in dog was there more than 80 per cent of the labeled dose in unchanged form in urine. However, some desulfation of the drug had occurred as judged by the appearance of labeled homovanillic acid, a known metabolite of dopamine in dog urine.

In guinea pig and rat, the administered [^{14}C]dopamine 3-*O*-sulfate had been rather extensively metabolized. Of particular interest is the fact that the new metabolic patterns resembled the patterns of orally administered dopamine metabolism in these species [3]. Thus, in guinea pig, the main urinary metabolite of dopamine was 3,4-dihydroxyphenylacetic acid, comprising 85 per cent of the recovered urinary radioactivity and indicating that the deamination pathway dominated with almost no *O*-methylation and glucuronide formation taking place. In the rat, deamination and *O*-methylation as well as glucuronide conjugation pathways were used, since [^{14}C]dopamine metabolites in urine were its glucuronide conjugate (34 per cent), 3,4-dihydroxyphenylacetic acid (26 per cent) and homovanillic acid (17 per cent) [3].

It can also be seen that intravenous administration caused different metabolic patterns than oral administration of [^{14}C]dopamine 3-*O*-sulfate. It seems that the intravenous route increases *O*-methylation in general as judged by the considerable levels of urinary homovanillic acid in all species.

DISCUSSION

In this study, weak cation exchange resin (Amberlite CG-50) columns [4, 6] have been routinely used

Table 3. Labeled urinary metabolites of orally or intravenously administered [^{14}C]dopamine 3-*O*-sulfate in guinea pig, rat and dog

	Percentage of recovered urinary ^{14}C				
	Oral administration			Intravenous administration	
Metabolite	Guinea pig (21·9 μg/kg)	Rat (19·1 μg/kg)	Dog (2·5 μg/kg)	Guinea pig (10·4 μg/kg)	Rat (7·4 μg/kg)
Glucuronides of *O*-methylated catecholamines	None	8·4	4·2	3·3	18·1
Dopamine glucuronide	0·9	8·4			
Homovanillyl alcohol	None	None	None	3·4	6·0
Dopamine 3-*O*-sulfate	3·0	41·3	81·4	1·0	1·3
3,4-Dihydroxyphenetol	21·4	None	None	23·3	3·2
Homovanillic acid	2·2	11·4	11·1	30·4	52·8
3,4-Dihydroxyphenylacetic acid	72·5	26·3	0·3	34·1	14·6
Not identified	None	4·2	3·0	4·5	4·0
Recovery of radioactivity applied on the column (%)	105	87·4	93·3	83·6	76·7
Recovery of radioactive dose in 0- to 24-hr urine (%)	72·1	86·7	67·0	82·5	89·2

for separation and identification of AG1 (formate) column effluents, both before and after the conjugates had been hydrolyzed with Glusulase at pH 6·0. In our earlier work [3], we attempted to hydrolyze dopamine sulfate in dog urine with Glusulase at pH 5·0, which resulted sometimes in incomplete hydrolysis. Incomplete hydrolysis of sulfate conjugate of dopamine by Glusulase in plasma of humans and dogs at pH 5·0 has been observed also by other workers [7]. These difficulties can be avoided when the hydrolysis is carried out at higher pH values (between 5·5 and 6·5).

Sulfoconjugation is a known pathway in dopamine metabolism for several animal species. In dog [3] and in human [2, 8] dopamine is excreted in urine predominantly as the ester of sulfuric acid after oral administration of dopamine or L-dopa.

Metabolic inactivation of related compounds of catecholamine character appears also to take place in dog and man predominantly by sulfate conjugation, with a much smaller degree of *O*-methylation, when the drugs are given orally, while after intravenous administration these compounds are metabolized mainly by *O*-methylation. Thus, isoprenaline (isoproterenol) is largely metabolized in dog [9] and in human [10] by sulfate conjugation after an oral dose, while *O*-methylation appears to assume a more important role in the metabolism of intravenous isoprenaline. Jenner and Rose [2] believe that, since *O*-sulfation and *O*-methylation appear to occur predominately on the same OH-group, they may represent competing processes.

Contrary to the findings of Jenner and Rose [2], our results indicate that dopamine 3-*O*-sulfate is not metabolically inert. It appears to undergo appreciable desulfation when administered orally or intravenously to guinea pig, rat and dog resulting in dopamine formation which is then metabolized according to the pattern characteristic to the particular animal species.

Unfortunately, the doses of dopamine 3-*O*-sulfate which were administered to the rats by Jenner and Rose are not known to us. In our work, very low doses of dopamine 3-*O*-sulfate were administered to the animals. Critical evaluation of the data in Table 3 would suggest that there appears to be a certain threshold which determines how the dopamine 3-*O*-sulfate is handled by each species. For example, in the dog where the dopamine metabolite occurs normally, 80 per cent of the dopamine 3-*O*-sulfate is readily eliminated unchanged at as low a dose as 2·5 μg/kg. In the rat, which normally does not metabolize dopamine by sulfoconjugation to a large extent, a small dose (7·4 μg/kg) results in extensive metabolism, but a larger dose (19·1 μg/kg) causes 41 per cent of the compound to be eliminated in unmetabolized form. Finally, the guinea pig, which uses hardly any sulfoconjugation in dopamine metabolism, does not eliminate the foreign compound, dopamine 3-*O*-sulfate, without prior extensive metabolism.

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