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Determination of Peripheral Catechol *O*-methyltransferase (COMT) Activity *in vivo* using [2-^{14}C]-3',4'-Dihydroxyacetophenone

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Abstract □ For the determination of peripheral COMT activity, we synthesized [2-^{14}C]-3',4'-dihydroxyacetophenone([^{14}C]-DHAP), a model substrate closely related to catecholamines, which cannot be attacked by monoamine oxidase. After i.v.-injection of [^{14}C]-DHAP in living animals, only 3',4'-dihydroxy-acetophenone (3',4'-DHAP) and 3'-methoxy-4'-hydroxyacetophenone (3'-MHAP) were detected in blood by thin layer radio chromatography. It could be speculated that 3',4'-DHAP was primarily *O*-methylated by COMT, followed by subsequent conjugations. The concentration of 3',4'-DHAP, a substrate for COMT, in blood at 5 min after injection of [^{14}C]-DHAP, were similar in all animals. The rate of 3'-MHAP formation can be therefore used as an indicator for peripheral COMT activity. The velocity of methylation in 15 min after i.v.-administration of [^{14}C]-DHAP was 0.28 μg/ml·min. From these results, 3',4'-DHAP was shown to be used as an appropriate substrate to determine the COMT activity *in vivo*.

Keywords □ COMT activity *in vivo*, [2-^{14}C]-3',4'-dihydroxyacetophenone, Metabolism, Rat.

There have been continuing and increasing interests in catecholamine metabolism, because of its etiological importance of parkinsonism, mania and depression[1-3].

Catechol *O*-methyltransferase (COMT) (E.C.2.1.1.6) are important enzymes in the metabolism of catechols and have been found in animals and in human tissues[4,5]: they catalyse the transfer of the methyl group of S-adenosyl-L-methionine to compounds with a catechol structure[6].

Many investigators reported the presence of COMT activity in blood[7-9]. However, the study of catecholamine metabolism in this tissue drawed little attention, although blood is very easily available from a great number of animal and human subjects. This may be due to the difficulty in determining the peripheral COMT activity using biogenic amines as a substrate, since the blood system containing one or several enzymatic systems is able to degrade these amines[10]. To search an appropriate substrate for the determination of peripheral COMT activity, we synthesized radio labelled [^{14}C]-DHAP, a model substrate closely related to catecholamines, which cannot be attacked by monoamine oxidase (MAO) (E.C.1.4.3.4). [^{14}C]-DHAP was synthesized from catechol and [^{14}C]-acetylchloride according to the methods of Howton *et al.*[11] and Schneider *et al.*[12]. In this paper results are presented on the enzymatic degradation of [^{14}C]-DHAP and peripheral COMT-activity in rats.

MATERIALS AND METHODS

Animals

Female wistar rats (Hagemann, Hannover) weighing 180 to 200 g were housed in groups of 5 animals at constant room temperature (22° ± 1℃) and relative humidity (50± 5%) with tap water and food (Altromin-1324, Altrogge) provided ad libitum and a 12 h light-dark cycle.

Chemicals

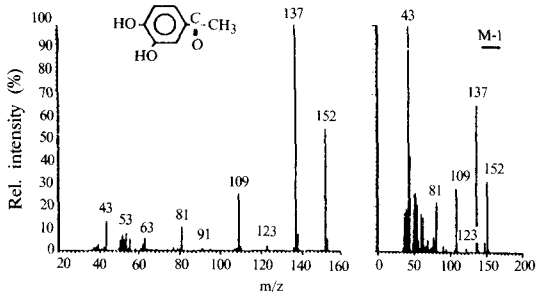

Fig. 1. Mass spectrum of authentic 3′,4′-DHAP and M-1.

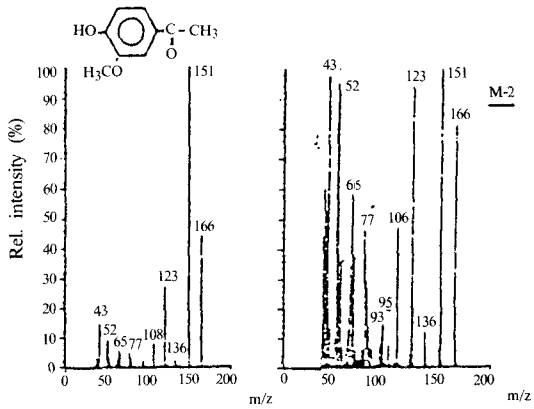

Fig. 2. Mass spectrum of authentic 3′-MHAP and M-2.

Chloralhydrate, toluene, ethylacetate, dioxane, TLC-plate (Kieselgel 60) were purchased from E, Merck, Darmstadt and were of analytical grade. Heparine Na (150,000 IE/g. usp XIX) was obtained from Feinbiochemica. 3′,4′-Dihydroxyacetophenone and 3′-hydroxy-4′-methoxyacetophenone were gifts from Dipl. Chemiker H. Ch. Frohberg. Org. Chem. Institut fuer FU Berlin. 4′-Hydroxy-3′-methoxyacetophenone from EGA-Chemie. β-Glucuronidase (100,000 U/m*l* Glucuronidase activity, 170 U/m*l* Sulfatase activity) was purchased from Sigma and Picofluor 30 from New England Nuclear (NEN).

Synthesis of [2-^{14}C]-3′,4′-dihydroxyacetophenone

A mixture of 1.4 g (12.7 mmole) catechol and 5.1 g (38.2 mmole) aluminum chloride in carbon disulfide (15 m*l*) was stirred at room temp. for 15 min., and then kept at 40℃ for 1 hr. Acetylchloride (1 m*l*= 1100 mg, 14.0 mmole) was added dropwise to the mixture. The acetylchloride contained 1 mCi (^{14}C)-acetylchloride. The reaction mixture was heated at 70℃ until the carbon disulfide was distilled off, then kept at 140℃ for 3.5 hr. The brownish residue obtained was cooled and suspended in 40 m*l* of 3M-HCl, then extracted with EtOAc. The organic layer was washed with water. Removal of solvent gave 0.56 g of the 3′,4′-dihydroxyacetophenone (29% yield) which was recrystallized from benzene afforded colorless needles (Specific activity : 1.33 mCi/mg).

Intravenous injection of [2-^{14}C]-3′,4′-dihydroxyacetophenone

Wistar female rats, weighing 180-200 g were used for the experiment. The animals were lightly anesthetized with an intraperitoneal injection of chloralhydrate (300 mg/kg). After narcosis, the polyethylene catheter inserted into the left femoral vein was used for the injection of drugs and collecting the blood samples. [^{14}C]-DHAP, 10 mg (13.3 mCi) was administered through the catheter and 0.2 m*l* of blood sample was collected at 5, 15, 30, 60 min after administration of drugs.

Sample preparation

For the metabolic studies, 200 μ*l* blood samples were transferred to the test tubes containing 800 μ*l* distilled water. The mixture was extracted with 4 m*l* of toluene/ethylacetate (80 : 20 : V/V) for 20 min at 60 rpm/min using a mechanical shaker. The mixture was then centrifuged at 3000 rpm. 2 m*l* of the organic phase were dried after addition of 50 μ*l* of tracer solution of 3′,4′-DHAP, 3′-MHAP (1 mg/m*l* ethanol). The residue was dissolved in 50 μ*l* of ethanol solution, the tubes were shaken in a test tube mixer for 30 sec, and sonicated for 3 min. 50 μ*l* of redissolved plasma extracts were applied to a TLC-Plate. The TLC-Plate was developed in an unlined glass tank, with a solvent mixture consisting of toluene/dioxane/acetic acid (80 : 20 : 3, V/V) over a distance of 13 cm and finally air-dried. The chromatogram was scanned with a TLC radioscanner.

RESULTS AND DISCUSSION

Separation and identification of 3′,4′-DHAP (M-1) and 3′-MHAP (M-2)

3′,4′-DHAP and its metabolites in blood after application of [^{14}C]-DHAP were separated and identified with authentic samples of 3′,4′-DHAP and 3′-MHAP using thin layer radio chromatographic and mass spectrometric methods. Thin layer radio chro-

Table I. Rf-values 3,4-Dihydroxyacetophenone and other related substances

TLC-systems Substances	Toluene/ Dioxan/ ACOH 80 : 20 : 3	*n*-Hexan/ EtOAc 4 : 3	*n*-BuOH AcOH/ H_2O 80 : 10 : 10	*i*-PrOH/ AcOH/ H_2O 80 : 10 : 10	*i*-PrOH/ Conc. NH_3/ H_2O 80 : 10 : 10	*i*-PrOH/ EtOAc/ NH_3 60 : 30 : 10	$CHCl_3$EtOH/ Ethylamin 70% 80 : 15 : 10
3,4-Dihydroxy acetophenone	0.18	0.43	0.45	0.49	0.36	0.12	0.06
3-Methoxy, 4-hydrdoxy-acetophenone	0.37	0.49	0.45	0.51	0.28	0.25	0.4
4-Methoxy, 3-hydroxy-acetophenone	0.34	0.41	0.45	0.51	0.37	0.53	0.78
3,4-Dimethoxy-acetophenone	0.42	0.48	0.42	0.48	0.43	0.73	0.57
3,4-Dihydroxymandelic acid	–	–	0.26	0.38	–	–	–
Homovanillinic-acid	–	0.08	0.44	0.50	0.18	–	–
2,4,5-Trihydroxy-phenylethylamin	–	–	0.23	0.35	–	–	–
3,4-Dihydroxyphenylalanin (DOPA)	–	–	0.17	0.22	–	–	–
Methyldopa	–	–	0.21	0.29	–	–	–

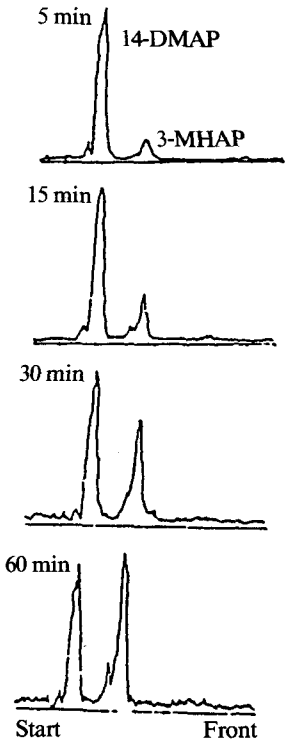

Fig. 3. Radiochromatogram of blood samples after i.v.-administration of [2-^{14}C]-3',4'-dihydroxyacetophenone in rat.

matography (Fig. 3) detected two radio active peaks with Rf-values of 0.18 (M-1) and 0.37 (M-2) in blood sample from rat given [^{14}C]-DHAP. Simultaneous separation of M-1 and M-2 from plasma extracts, which was reproducible and reliable, was made possible with the thin layer chromatographic plates using toluene/dioxane/acetic acid (80 : 20 : 3) as a developing system. From the results of Table I, the M-1 and M-2 were identified to be 3',4'-DHAP and 3'-MHAP, respectively, as interpreted by the patterns of the mass spectra of authentic 3'-4'-DHAP, M-1 and 3'-MHAP, M-2 (Fig. 1, 2).

Table II. Extraction yields and recovery of C-14-3',4'-DHAP and C-14-3-MHAP in percent

	Recovery (%) C-14-3',4'-DHAP	C-14-3'-MHAP
1 Extraction	70	76
2 Extraction	25	22
3 Extraction	3.7	1.5

Recovery of [^{14}C]-DHAP and [^{14}C]-MHAP

Extraction yields and recovery in percent of [^{14}C]-DHAP, [^{14}C]-MHAP in the course of whole procedure were estimated at 2 different concentration (5000, 25000 cpm) with spiked plasma (see Method). As shown in Table II, the recovery of added [^{14}C]-DHAP, [^{14}C]-MHAP were 70, 76% each at first extraction and almost 100% recovery were obtained after third extraction step.

Table III. Absolute concentration and percent of 3',4'-DHAP and 3'-MHAP in blood after application of [^{14}C]-DHAP

		cpm	percent (%)	μg/ml
5 min	3,4-DHAP	19098	86	6.5
	3-MHAP	2988	14	1.0
15 min	3,4-DHAP	10997	79	3.7
	3-MHAP	3003	21	1.0
30 min	3,4-DHAP	5461	56	1.9
	3-MHAP	4359	44	1.5
60 min	3,4-DHAP	1930	46	0.7
	3-MHAP	2306	54	0.8

Metabolism of [^{14}C]-DHAP

As shown in Fig. 3, two radioactive peaks with Rf-values of 0.18 and 0.37 were found to be associated with 3',4'-DHAP and 3'-MHAP in the blood of 5, 15, 30, 60 min after administration of drugs.

It could be speculated that 3',4'-DHAP was primarily *O*-methylated by COMT followed by subsequent conjugations in blood.

In Table III, the results of absolute concentrations and percent of 3',4'-DHAP and 3'-MHAP in blood at 5, 15, 30, 60 min after application of [^{14}C]-DHAP, were described. As shown in Table III, the absolute concentration of [^{14}C]-DHAP in blood at 5 min after the application of substrate was 6.1-7.1 μg/ml (6.5 ± 0.6 μg/ml n=10). Concentrations of 3',4'-DHAP in blood at 5 min after application of [^{14}C]-DHAP, were similar in all animals. The rate of 3'-MHAP formation is therefore an indicator for the peripheral COMT activity. The velocity of methylation in 15 min after i.v.-administration of [^{14}C]-DHAP was 0.28 μg/ml · min.

Four mechanisms for the generation of metabolites from [^{14}C]-DHAP would be proposed:

(1) *O*-methylation by COMT

(2) Conjugation with either glucuronic acid or sulfate

(3) Reduction of a keto group

(4) Oxygenation of a methyl group

In this study, only the metabolities of *O*-methylation and conjugation were identified. The reduction of keto group did not occur; we reduced c[illegible] 3',4'-DHAP and 3'-MHAP with $NaBH_4$ a[illegible] reactions products were applied to a TLC-Plate. After developing a plate, following results (Table IV) were obtained. After application of [^{14}C]-DHAP the peaks obtained after reduction could not be found in blood. This indicates that 3'-4'-DHAP was not reduced by reductase; it seems to be a bad substrate for this enzyme.

Table IV. Rf-values of 3',4'-DHAP and 3'-MHAP before and after reduction with $NaBH_4$

	R_f values		
	Before reduction	After reduction	
3',4'-DHAP	0.18	0.016	2 points
		0.075	
3'-MHAP	0.37	0.24	

Presence of oxygenation of methyl group could not be proved in this study without an appropriate reference material. However, absense of any other peaks in the TLC result imply that the oxygenation reaction is not a significant route of 3',4'-DHAP metabolism in rat.

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