Fluorometric Determination of 1,2,3,4-Tetrahydro-6,7-dihydroxyisoquinoline in Biological Materials by HPLC

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In the belief that endogenous 1,2,3,4-tetrahydro-6,7-dihydroxyisoquinoline (DA-Fp) could be a potential marker involved in the etiology of various diseases such as Parkinsonism, we attempted to develop a fluorescence method for DA-Fp. It was synthesized by condensation of dopamine with formaldehyde according to an established method. Periodate was identified by screening from various oxidation reagents as a fluorescence reagent to DA-Fp. Optimal reaction conditions were obtained with 0.25 mM $NaIO_4$ in 0.1 M phosphate buffer (pH 8.0) at 37 °C for 15 min. The fluorescence spectrum of the derivative showed that we had found a new reaction specific for DA-Fp. This reaction was coupled on-line to high performance liquid chromatography (HPLC), which enabled us to achieve a highly sensitive method for determining DA-Fp. A working curve was linear from 2 to 800 pmol of DA-Fp per injection. To determine DA-Fp in biological materials, the pretreatment before HPLC was optimized by hydrolysis of its conjugate and suppression of the artifact with *l*-phenylephrine. Urinary excretion of DA-Fp in men was measured by this new present method. The urinary excretion of endogenous DA-Fp increased in a rabbit given L-DOPA. The DA-Fp concentration was determined in rat brain. The significance of DA-Fp in these biological materials is discussed and evaluated. We conclude that the present method will be useful for studying tetrahydroisoquinolines involved in many diseases.

Key words 1,2,3,4-tetrahydro-6,7-dihydroxyisoquinoline; fluorescence reaction; sodium periodate; HPLC; dopamine; formaldehyde

Tetrahydroisoquinolines (THQs) are condensation products of catecholamines and their analogs with aldehydes following enzymatic or non-enzymatic reaction.[2)] THQs found in body fluids are considered to be associated with some diseases such as diabetes, senility, Parkinsonism, phenylketonuria and alcoholism.[3,4)] It is also well known that they are present as alkaloids in many natural products.[5)] We noticed that a THQ was generated during incubation of epinephrine with ascorbic acid for radioimmunoassay and supposed that this kind of condensation of catecholamines with nascent formaldehyde from ascorbic acid might occur under physiological condition.[6)] Recently, an important report showing that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in narcosed patients gave rise to Parkinson's syndrome suggested that THQs might act as endogenous MTPTs.[7)]

There are several methods to determine THQs by gas chromatography (GC)/mass spectrometry (MS) which are accurate, but inconvenient.[8)] High performance liquid chromatography (HPLC) with an electrochemical detector is also good for practical use.[9)] These methods, however, are not selective enough to discriminate between THQs and various other constituents of physiological samples. During sampling and pretreatment in these methods, no note was taken of the potential artificial condensation of catecholamines with contaminated aldehydes to give rise to THQs. Further, despite the fact that dopamine is an important neurotransmitter, its THQ derivative has not yet been analyzed.

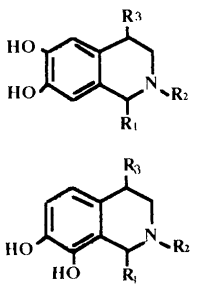
In this paper, we attempted to develop a method to determine 1,2,3,4-tetrahydro-6,7-dihydroxyisoquinoline (DA-Fp, so called norsalsolinol), one of the THQs from dopamine, using HPLC and a new fluorescence reaction. After the synthesis of several THQs, their separation and fluorescence reaction were examined and optimized for biological samples. The structures of the THQs examined in this paper are shown in Table 1. Two types of probable condensation products are classified into type p and o, after the *para* or *ortho* substitution of an aldehyde group on the catechol moiety.

Experimental

Materials *l*-Phenylephrine·HCl and 1,2,3,4-tetrahydro-6,7-dihydroxy-2-methylisoquinoline (En-Fp)·HCl were obtained from Sigma (St Louis). *dl*-Epinephrine, *dl*- and *l*-norepinephrine, and dopamine·HCl were obtained from nacalai tesque (Kyoto). *l*-Epinephrine was obained from Merck (Darmstadt). Sodium metaperiodate was obtained from Kanto Chemical Co. (Tokyo). 1,2,3,4-Tetrahydro-6-hydroxy-2-methylisoquinoline was kindly donated by Dr. Toshihiko Okamoto, Faculty of Pharmaceutical Sciences, The University of Tokyo. Amberlite XAD-4 (nonionic porous polystyrene polymer), obtained from Rohm & Haas (Philadelphia), was ground into fine grains of 150—200 mesh. Boric acid gel was obtained from Aldrich Chemical Co. (Milwaukee). Hitachi gel 3011C (macroreticular carboxylate-anion exchange resin, 10 μm in mean diameter) was kindly donated by Mr. Akira Narita, Nissei Sangyo Co., Tokyo. Alumina, neutral activity, grade 1, was obtained from Woelm

Table 1. Abbreviations and Structures of THQs Studied in this Paper

THQ	R_1	R_2	R_3		Structure
DA-Fp	H	H	H		
En-Fp	H	CH_3	H	type p	
DA-Ap	CH_3	H	H		
NE-Fp	H	H	OH		
E-Fp	H	CH_3	OH		
NE-Fo	H	H	OH	type o	
E-Fo	H	CH_3	OH		



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(Eschwege), washed with 6 N HCl at 100 °C until the aqueous phase became colorless and subsequently with water until the washing was neutral. It was then heated at 115 °C for an hour and stored in a desiccator. All the other chemicals were of reagent grade and commercially available.

Measurement of Chemical Characteristics All melting points were measured on a Yanaco MP-S3 melting point apparatus (Yanagimoto, Kyoto) and were uncorrected. ^{1}H-NMR spectra were recorded with a FMN-FX 100 NMR spctrometer (JEOL, Tokyo). The chemical shifts were expressed in ppm using trimethylsilylpropanesulfonic acid sodium salt as an internal standard. Fluorescence spectra were recorded using a MPF-2A fluorescence spectrophotometer (Hitachi, Tokyo).

Syntheses. 1,2,3,4-Tetrahydro-6,7-dihydroxyisoquinoline (DA-Fp) Hydrochloride One gram dopamine·HCl and 0.5 ml 35% formaldehyde were mixed with 5 ml methanol and heated at 45 °C. The reaction mixture was concentrated to dryness *in vacuo*. The residue was dissolved in a small aliquot of 2 N HCl. The solution was added acetone to give white crystals which were recrystallized with 2 N HCl and acetone. The pure crystals obtained weighed 250 mg; mp 256 °C (dec.) (262 °C reported[10]). ^{1}H-NMR (D_2O) δ: 2.96 (2H, t, J=6.8 Hz, $-CH_2-$), 3.48 (2H, J=6.8 Hz, $-CH_2-$), 4.22 (2H, s, $-CH_2-$), 6.67 (1H, s, Ar-H), 6.71 (1H, s, Ar-H). *Anal.* Calcd for $C_9H_{12}ClNO_2$: C, 53.63; H, 6.00; N, 6.95. Found: C, 53.50; H, 6.02; N, 6.81.

1,2,3,4-Tetrahydro-4,6,7-trihydroxyisoquinoline (NE-Fp) Hydrochloride The synthesis was carried out according to the method described by Sarges.[11] One gram *dl*-norepinephrine·HCl in 1.5 ml water was added with 0.4 ml 35% formaldehyde dropwise. After stirring for 1 h, 10 ml acetone was added and the precipitated solid was recrystallized 7 times with water–acetone. The crystals obtained weighed 180 mg; mp 172 °C (dec.) (172 °C reported[11]). *Anal.* Calcd for $C_9H_{12}ClNO_3\cdot H_2O$: C, 45.86; H, 5.99; N, 5.97. Found: C, 45.80; H, 5.94; N, 5.82. ^{1}H-NMR (D_2O) data were identified to that described by Sarges.[11]

1,2,3,4-Tetrahydro-4,7,8-trihydroxyisoquinoline (NE-Fo) Hydrochloride The synthesis was carried out in a similar manner to 1,2,3,4-tetrahydro-4,6,7-trihydroxy-2-methylisoquinoline (E-Fo) as described in our previous paper.[6] One gram *dl*-norepinephrine·HCl was dissolved in 4 ml 6 N HCl and added to 100 ml 0.1 M tris buffer (pH, 7.0) – 10 mM ascorbic acid solution containing 1 ml 35% formaldehyde. After stirring at room temperature for an hour, 50 g NaCl was added. The solution was passed through a column (1 cm × 20 cm) of Amberlite XAD-4 equilibrated with 20% NaCl and the adsorbed product was eluted under a linear gradient of 20—0% NaCl in 0.01 N HCl. The eluate was monitored by UV detection at 280 nm. The UV-absorbing fractions were pooled and applied to a column of boric acid gel in ten sample volumes. The column was washed with twenty column volumes of water and the product was eluted with 0.025 N HCl. The fractions of UV-absorbing product were pooled and concentrated to dryness *in vacuo*. The residue was recrystallized three times from methanol–ether. The white crystals obtained weighed 110 mg; mp 190 °C (196—198 °C reported[11]). *Anal.* Calcd for $C_9H_{12}ClNO_3$: C, 49.67; H, 5.56; N, 6.44. Found: C, 49.40; H, 5.59; N, 6.33. ^{1}H-NMR (D_2O) data were identified to that described by Sarges.[11]

1,2,3,4-Tetrahydro-6,7-dihydroxy-1-methylisoquinoline (DA-Ap) Hydrochloride Dopamine·HCl (0.5 g) was condensed with acetaldehyde according to the method described by King *et al.*[12] The product was recrystallized three times from methanol–ether. The white crystals obtained weighed 180 mg; mp 231 °C (231 °C reported[12]). *Anal.* Calcd for $C_{10}H_{14}ClNO_2$: C, 55.60; H, 6.54; N, 6.50. Found: C, 55.61; H, 6.58; N, 6.34. ^{1}H-NMR (D_2O) data were identified to that described by King *et al.*[12]

Fluorescence Reaction of DA-Fp with Sodium Periodate 0.1 ml of an aqueous solution of DA-Fp at a given concentration was transferred to a siliconized test-tube. The solution was mixed with 3.9 ml 1 mM $NaIO_4$ in 0.1 M phosphate (pH 8.0) and stood at 37 °C for 15 min. The fluorescence spectrum was measured with the fluorescence spectrophotometer.

Fluorescence Determination of DA-Fp by HPLC A column (500 mm × 2 mm, i.d.) of Hitachi gel 3011C was maintained at 45 °C. The eluent consisted of 0.1 M acetate, 0.03 M disodium hydrogen phosphate and 0.05 M NaCl (pH 4.25) and was pumped through the column at 0.70 ml/min. The inlet pressure of the column was 50—55 kg/cm^2. After emerging from the column, the eluate was mixed with the fluorescence reagent solution, 10 mM $NaIO_4$, at a flow rate of 0.16 ml/min and with another buffer solution (pH 9.2) consisting of 0.5 M disodium hydrogen phosphate and 0.4 M Na_2CO_3 at a flow rate of 0.25 ml/min. The mixed solution was pumped through a reaction coil (20 m × 0.25 mm, i.d.) at 45 °C. The reaction mixture was monitored using a F4T4/BL fluorescence detector (SLM-Aminco, Rochester) with a 7—60 filter (Corning Inc., New York) for excitation and a Wratten 2A filter (Eastman Kodak Co., Rochester) for emission.

Sampling of Biological Materials Urine was collected from the bladder of a Japanese rabbit weighing 3 kg by catheterization and adjusted to pH 1.5 with 6 N HCl. The acidified urine was mixed with *l*-phenylephrine to give a final concentration of 1% and then stored at −20 °C until analysis. Blood was collected from an ear vein of the rabbit. The blood was mixed with one-tenth its volume of a solution of 2% EDTA containing 10% *l*-phenylephrine, centrifuged at 3500 *g* and the plasma stored at −20 °C. More plasma was obtained from the rabbit, after L-DOPA (25 mg/Kg body weight) was injected intravenously into its ear. Human urine was treated and stored in the same way as the rabbit urine. The brain was removed from male Wistar rats weighing 400—500 g immediately after sacrificed. It was frozen on dry ice, weighed (around 2.0 g) and added to fivefold its wet weight of 1% *l*-phenylephrine in 0.4 M $HClO_4$. Then, it was homogenized in a glass homogenizer and centrifuged at 10000 *g* at 4 °C for 30 min. The supernatant was stored at −20 °C.

Pretreatment of Sample To the stored sample (5 ml urine, 2 ml plasma or one brain supernatant) was added 800 pmol of En-Fp as an internal standard in 100 μl 0.1 M HCl. The sample solution was adjusted to pH 1.5 with 6 M HCl and hydrolyzed at 80 °C for 20 min. To the hydrolyzate was added 1.0 ml 10% $Na_2S_2O_5$ and 5 ml 0.5 M Tris–HCl buffer (pH 8.5) containing 0.02 M EDTA. The solution was adjusted to pH 8.5 with 4 M NH_4OH. It was mixed with 100 mg of the washed alumina and stirred for 10 min. The suspension was filtered through a No. 2 filter paper (Toyo Roshi Co., Tokyo) and the residue on the filter paper was washed 3 times with 5 ml water. Then, the analytes on the residue was eluted with 0.5 ml 0.5 M acetic acid. The eluate was analyzed using the HPLC-fluorescence reaction above.

Results

Fluorescence Reaction of DA-Fp There were two ways to find a specific fluorescence reaction of DA-Fp. One is direct oxidation of DA-Fp,[13] and the other is condensation of ethylenediamine with the catechol moiety of DA-Fp as also described for catecholamines.[14]

Firstly, we examined the oxidation reactions using various reagents such as $NaIO_4$, $NaIO_3$, $NaBrO_3$, H_2O_2 and $K_3[Fe(CN)_6]$ at 1 mM concentrations in test-tubes as described in the Experimental section. Only $NaIO_4$ of these compounds showed a strong fluorescence with the emission maximum at 404 nm and an excitation maximum at 362 nm. The other oxidation reagents showed less than one-fiftieth the fluorescence intensity of $NaIO_4$ under conditions such as pH 5, 7 or 9 at 25, 60 or 90 °C. On the other hand, the condensation reaction of ethylenediamine at 25% at 60 °C for 30 min showed a weak fluorescence with an emission maximum at 500 nm and an excitation maximum at 400 nm. The fluorescence intensity with ethylenediamine was one-thirtieth that with $NaIO_4$. Thus, we selected $NaIO_4$ as the best reagent and investigated the quantitative conditions as described below.

As shown in Fig. 1, the fluorescence intensity of the DA-Fp derivative attained a plateau at pH 8.0—8.5. A 0.1 M phosphate buffer was suitable, and a 0.1 M glycine or tris buffer interacted with the fluorescence reagent so that no fluorescence reaction with DA-Fp occurred. A citrate buffer at pH 7 also seemed to interact. The time-courses of the fluorescence reaction were plotted using four solutions at various concentrations of $NaIO_4$, as shown in Fig. 2. A 1-mM solution alone produced the highest stable fluorescence, and so we decided to use 1 mM $NaIO_4$. When 0.5 μM DA-Fp was reacted at pH 8.0 with 1 mM $NaIO_4$, the fluorescence intensity attained a plateau

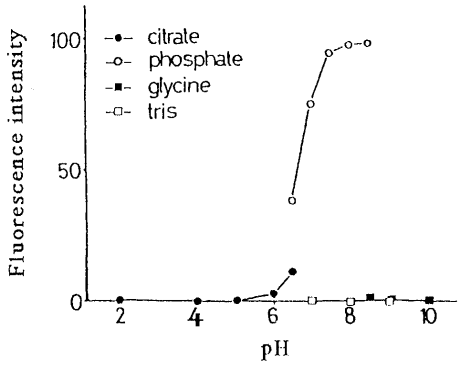

Fig. 1. Dependence of Fluorescence Reaction on the pH of Various Buffers

0.5 μM DA-Fp was reacted with 1 mM $NaIO_4$ at 37 °C for 15 min.

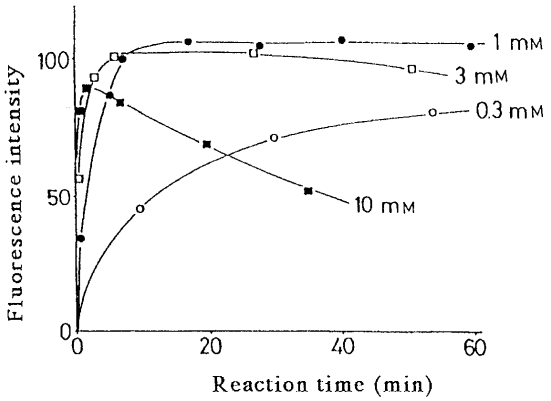

Fig. 2. Time-Courses of Fluorescence Reactions of Various Concentrations of $NaIO_4$

0.5 μM DA-Fp was reacted at pH 8.0 at 37 °C.

Table 2. Relative Fluorescence Intensity (RFI) of Derivatives from THQs and Related Compounds Reacted with $NaIO_4$

Compound	RFI	Compound	RFI
DA-Fp	100	DL-Metanephrine	0.14
En-Fp	18	DL-Normetanephrine	0.14
DA-Ap	6.5	3-Methoxytyramine	0.01
DL-NE-Fp	0.54	DL-Vanilmandelic acid	0.89
DL-E-Fp	0.16	Homovanillic acid	0.09
DL-NE-Fo	0.36	DL-3,4-Dihydroxyphenyl-mandelic acid	0.75
DL-E-Fo	0.01		
Dopamine	0.30	3,4-Dihydroxypheny-acetic acid	0.18
D-/-Norepinephrine	0.27		
D-/-Epinephrine	0.08	1,2,3,4-Tetrahydro-6-hydroxy-2-mehtylisoquinoline	0.01
L-DOPA	0.01		

in 20 min at 18 °C, in 5 min at 37 °C and in 2 min at 45 °C. In a manual reaction, the reaction was normally carried out at 37 °C for 15 min. However, we encountered a problem with the specificity of the fluorescence reaction. Various related compounds reacted with 1 mM $NaIO_4$ under the same conditions as described above as shown in Table 2. Fortunately, the reaction was fairly specific for DA-Fp, although En-Fp and DA-Ap showed weak fluorescence. The other THQs, catecholamines and related compounds did not react significantly. This showed that the fluorescence reaction of DA-Fp was different from the known reaction. Fortunately, a working curve for DA-Fp was linear from 2 pmol to 2 nmol per tube as was that for

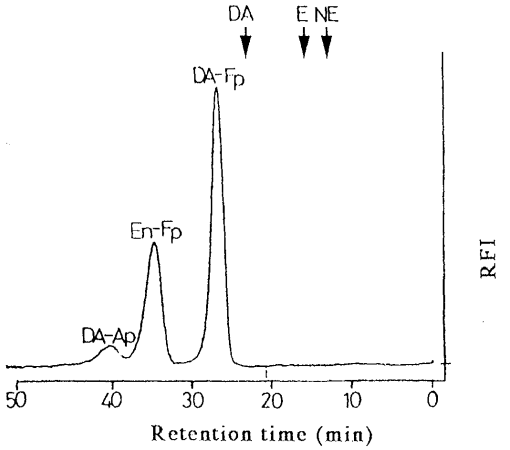

Fig. 3. Chromatogram of a Mixture of 200 pmol Each of DA-Fp, En-Fp and DA-Ap

The arrows show the retention times of the catecholamines.

En-Fp or DA-Ap.

HPLC-on Line Fluorescence Reaction with $NaIO_4$ The manual method was limited to pure samples and many kinds of catecholamines and related compounds occur in biological samples. In order to increase the selectivity of the determination, the fluorescence reaction should be coupled on-line with HPLC. We examined differnt kinds of columns, separation and reaction conditions. Finally, we adopted the system described in the Experimental section. A reversed phase column of octadecyl silica gel (Devolosil 5 or 10, Nomura Chemical Co., Seto, or LS-410, Tosoh, Tokyo), an adsoption column of porous polystyrene polymer beads (Hitachi gel 3010 or 3011, Nissei Sangyo Co., Tokyo) and a strong anion exchange column of porous polystyrene beads (Hitachi gel 3011P, Nissei Sangyo Co.) were found to be unsuitable. The weak anion exchange column of Hitachi gel 3011C showed a good resolution of THQs and catecholamines as shown in Fig. 3. In this system, DA-Fp was well separated from DA-Ap and the internal standard, En-Fp. Dopamine (DA), norepinephrine (NE) and epinephrine (E) were also separated as shown by the arrows. As far as an eluent was concerned, the acetate and phosphate buffer did not interact with $NaIO_4$ and was adopted. At a pH above 4.0, the resolution of DA-Fp from dopamine was more than 1.0. The elution time, however, was too long for practical purposes. In order to shorten it, NaCl was added to the buffer and was more efficient than methanol. The flow rate of the eluent and column temparature were set 0.7 ml/min and 45 °C, taking into consideration the analytical time and stability of the column. Next, the post-column fluorescence reaction was based on the manual method and checked again. The concentration of $NaIO_4$ became 1.5 mM after the reaction coil, when it was pumped at the minimum constant flow rate. The concentration of the mixture ranged from 0.5 to 3.0 mM for the stable derivatizaton of DA-Fp. Its peak height was highest at pH 9.2 with the phosphate-carbonate buffer, and the pH was changed from 8.0 to 9.5 after the mixing coil. When the coil length was changed from 5 to 30 m, the peak height reached a plateau at 20 m and this length was adopted. Thus, the HPLC-on line derivatization system was opti-

mized as described in the Experimental section. A working curve of the peak height ratio of DA-Fp to En-Fp was linear from 2 to 800 pmol DA-Fp injected, as shown in Fig. 4. The minimum detectable amount was 2 pmol (S/N = 5). The repeatability of the peak-height ratio of 200 pmol DA-Fp injected at 2 h intervals was CV = 1.5% (n = 5) within a day. The peak-height was not dependent on the injection volume from 5 to 100 μl, but gradually declined above 100 μl and up to 250 μl, the maximum volume used. The peak-height ratio was constant as expected in principle.

Pretreatment of Sample Although the HPLC-fluorescence derivatization system was highly selective for DA-Fp, it was still essential to clean-up biological samples. As DA-Fp is a type of catecholamine derivative, pretreatment before HPLC followed that of catecholamines in biological samples as described in the Experimental section. It consisted of hydrolysis of conjugated DA-Fp and adsoption of the free DA-Fp on alumina. The peak height of DA-Fp in the urine of the rabbit given L-DOPA attained a plateau after 10 min hydrolysis, as shown for catecholamine conjugates, which suggests the presence of the conjugated form of DA-Fp together with its free form in a ratio about 1 : 1. In addition to these treatments, we developed two means of suppressing the artificial conversion of catecholamines present in the biological samples into THQs. One was the addition of excess *l*-phenylephrine which produced a THQ with concomitant formaldehyde in the sample and reagent solutions as described by Kaito.[15] When a human urine sample was supplemented with 2×10^{-4} M dopamine as the final concentration, and where the artificial production of DA-Fp was 1.2% in yield after incubation at 37 °C for 3 h, the peak height of DA-Fp sharply declined following addition of 0.1 ml 5% *l*-phenylephrine and was comparable with that of the sample without dopamine supplementation following addition of more than 0.5 ml 5% *l*-phenylephrine(data not shown). The other was the addition of 10% $Na_2S_2O_5$ which acted not only as an anti-oxidant, but also suppressed artificial production of DA-Fp from dopamine in the sample. When a urine sample supplemented with 2×10^{-4} M dopamine was pretreated, the peak height of DA-Fp gradually declined depending on the volume of 10% $Na_2S_2O_5$ added. It fell to that of a sample without any supplementation following the addition of 1 ml 10% $Na_2S_2O_5$. Thus, the pretreatment was as described in the Experimental section. By this treatment, recovery curves for added DA-Fp were linear up to 500 pmol added in the urine (one of the chromatograms is shown in Fig. 5), 300 pmol in the plasma and 200 pmol in the brain as shown in Fig. 6. The overall recovery of both DA-Fp and En-Fp was around 60%. No DA-Fp was detected in normal plasma.

Determination in Biological Samples Finally, we were able to analyze the biological samples. As shown in the chromatogram of rabbit urine in Fig. 5, the DA-Fp peak

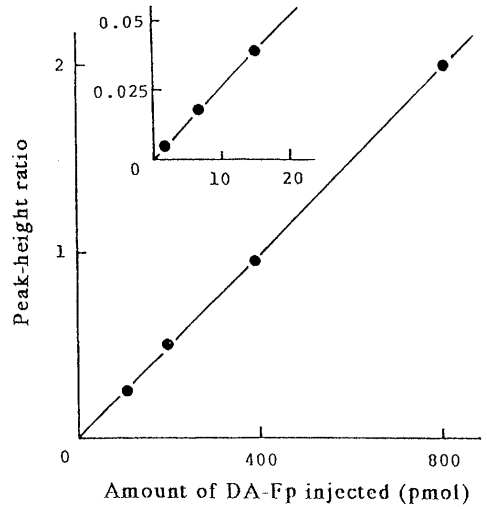

Fig. 4. A Working Curve for DA-Fp Measured by HPLC
The amount of En-Fp injected was 800 pmol.

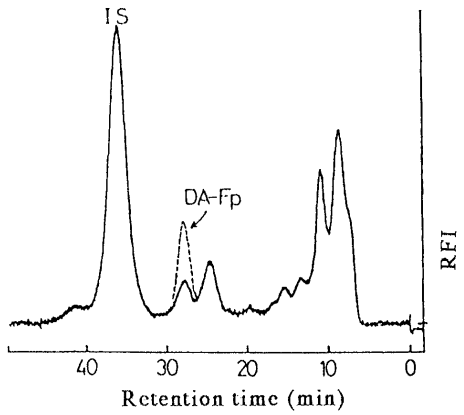

Fig. 5. Chromatogram of Rabbit Urine
The DA-Fp peak increased as shown by the dotted line, when 20 pmol standard DA-Fp was injected with the same urine sample. IS is the internal standard.

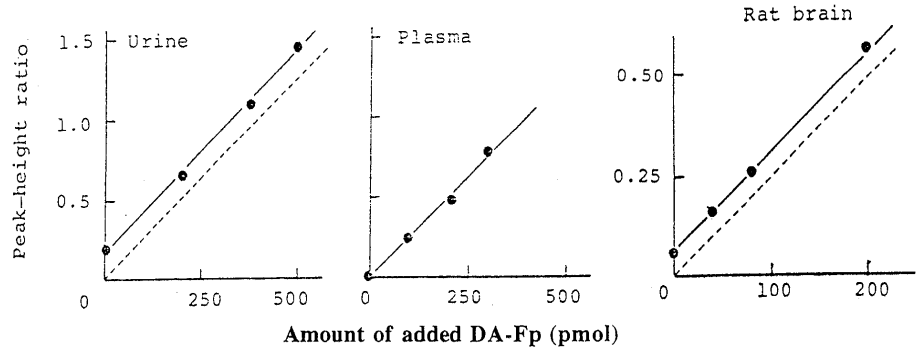

Fig. 6. Working Curves for the Biological Samples Added with DA-Fp
800 pmol of the internal standard and a given amount of DA-Fp was added to 5 ml urine, 2 ml plasma or 2 g rat brain.

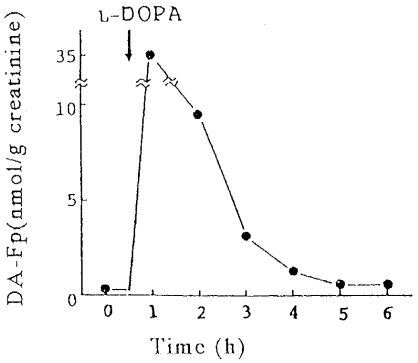

Fig. 7. Time-Courses of Excretion of DA-Fp in Urine of a Rabbit Given L-DOPA

The rabbit was given 25 mg/kg L-DOPA intravenously in the ear. The urine samples were collectected just before administration and each hour thereafter.

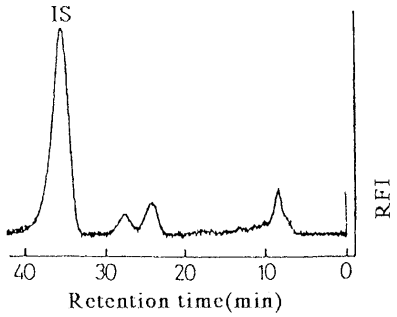

Fig. 8. Chromatogram of a Normal Human Urine Extract

IS is the internal standard.

Table 3. Urinary Excretion of DA-Fp in Men

Subject	Age/year	Weight/kg	nmol DA-Fp/g creatinine
HN	36	60	4.6
AH	23	70	1.0
YM	23	49	3.4
JS	40	54	4.1
AS	27	76	2.0
YN	26	55	1.0
TT	41	53	5.3
TT	32	70	0.8
			Mean 2.8±1.7

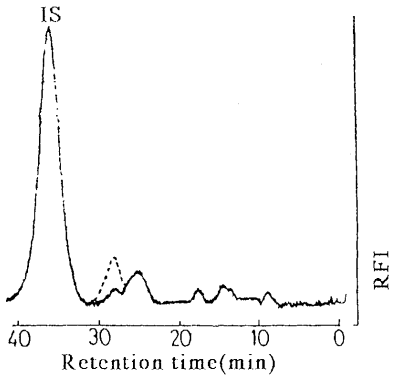

Fig. 9. Chromatogram of a Rat Brain Extract

The DA-Fp peak increased as shown by the dotted line, when 20 pmol standard DA-Fp was injected with the same urine sample. IS is the internal standard.

appeared after several unknown peaks. When standard DA-Fp was added to the urine as described in the recovery test, the peak height increased confirming the retention time. In this way, DA-Fp excretion was measured after the administration of L-DOPA as shown in Fig. 7. Its excretion increased markedly within an hour and gradually decreased over a few hours. No peak, however, was observed in plasma, indicating that the plasma level was less than 5 pmol/ml.

In human urine, DA-Fp excretion was confirmed as shown in the chromatogram in Fig. 8. Eight subjects were starved from 21.00 on the previous day and the second sample of urine excreted the next morning was collected. The mean excretion of DA-Fp was 2.8 nmol/g creatinine as shown in Table 3. We did not know whether the excretion was natural or artificial in the bladder. One of the urines, with or without the addition of 2×10^{-4} M dopamine was incubated at its own pH 6.8 or pH 7.8, adjusted with 4 M NH_4OH at 37 °C. In the presence of dopamine, the artificial conversion into DA-Fp at pH 7.8 linearly increased up to 1.2% in yield in 3 h, while the corresponding figure at pH 6.8 was 0.2%. Without supplementation, no DA-Fp was detected even, after a 5 h incubation. It was unlikely that DA-Fp was artificially produced in the bladder. Further, DA-Fp was found in the normal rat brain as shown in Fig. 9. The mean amount was 8 ± 2 pmol/g wet weight ($n=3$).

In order to confirm the reliability of the values obtained with the above biological samples, we re-chromatographed fractions from respective peaks. The fluorescent derivative of the peak was collected and injected into another column (500 mm × 2 mm, i.d.) of Hitachi gel 3011C at 45 °C. The eluent consisted of 0.05 M citrate buffer (pH 3.8) and methanol (4 : 1, v/v) and its flow rate was set at 0.7 ml/min. The eluent was monitored with the same fluorescence detector. All the DA-Fp peak was completely baseline-separated and showed a single symmetric peak. A working curve was linear from 5 to 200 pmol DA-Fp injected. All the values measured were comparable with those obtained by the first HPLC-fluorescence derivatization.

Discussion

As far as the synthesis was concerned, we obtained all the standard THQs except DA-Fo. When dopamine was condensed with acetaldehyde, types p and o were produced.[12] It was easily confirmed that both types of THQs were produced from norepinephrine with formaldehyde in this paper and from epinephrine in our previous paper,[6] although type p was most abundant. Under the same synthetic conditons, we could not detect type o from dopamine with formaldehyde using HPLC and thin-layer chromatography using varieties of mobile phases. This suggests that type o from dopamine is a very minor component even in biological materials. Thus, we focussed on the analysis of DA-Fp.

The fluorescence reaction of DA-Fp with $NaIO_4$ was a new reaction because its fluorescence maximum was different from that of 3,4-dihydro-6,7-dihydroxyisoquinoline, showing an emission maximum at 480 nm at an excitation maximum of 410 nm, or that of 6,7-dihydroxyisoquinoline showing an emission maximum at 490 nm at an excitation maximum of 330 nm.[13,16] Kaito *et al.*

reported that the THQ from *l*-phenylephrine was converted into a fluorescent derivative with $K_3Fe(CN)_6$ ten times more efficiently than $NaIO_4$.[15] Under our conditions, $K_3Fe(CN)_6$ had no effect on DA-Fp. The fluorescence spectrum of DA-Fp reacted with $NaIO_4$ was different from that of the THQ resulting from *l*-phenylephrine interacting with $K_3Fe(CN)_6$. Then, we tried to purify the derivative by HPLC, but did not succeed because the purification yield was too low. Judging from our experiments, the 1- and 2-position of the fluorescent derivative of DA-Fp are not substituted, because each fluorescent derivative from DA-Fp, En-Fp or DA-Ap had a different retention time when re-chromatographed. Further, the 3- and 4-position of the fluorescent derivative are also not substituted, because the [3-^{14}C]-[4-^{3}H]-DA-Fp we synthesized gave a fluorescent derivative with the same radioactivity ratio of [3-^{14}C]/[4-^{3}H], purified by re-chromatography, as the ratio of the starting material. During purification, the derivative did not adsorb onto the boric gel, which suggests that the catechol moiety of the derivative was not intact. A larger scale purification is necessary to identify the fluorescent derivative.

We found that normal distilled water also contained formaldehyde from the environment, unless it was kept in a glass-stoppered flask as described in our previous paper.[6] This kind of DA-Fp artifact was completely prevented by our sampling and pretreatment. These conditions will be useful for the analysis of catecholamines and related compounds. In additon, the present method is applicable to the determination of dopamine which is converted to DA-Fp.

Thus, the DA-Fp we measured, occurs natural in animals. Its source, however, is either dietary or endogenous. The latter possibilty comes from the observation that the excretion of DA-Fp increased after administration of L-DOPA to the rabbit. The urinary excretion of DA-Ap, so called salsolinol, after L-DOPA administration in man was first reported by Sandler *et al.*[17] DA-Fp, however, was not analyzed in their experiments. In our experiments, DA-Fp was detected after the adminstration of L-DOPA, but DA-Ap was not. It was thought that the excretion of DA-Ap was below the detection limit. Sandler *et al.* injected ethanol into patients to produce acetaldehyde immediately after L-DOPA administration.[17] It was assumed that the acetaldehyde concentration was very low normally. A possible dietary was not ruled out in normal subjects as shown in Table 3, because natural plants contain THQs.[5,17] It is likely that the values of DA-Fp we obtained were the sums of materials from both sources at different ratios. The plasma level of DA-Fp is assumed to be very low, as was the plasma level of dopamine.[18] Further, DA-Fp excretion into human urine was less than one-hundredth that of dopamine. The biosynthetic origin of formaldehye can occur by several routes such as from S-adenosylmethionine,[19] 5-methyltetrahydrofolate,[20] air,[6] smoking,[21] methanol,[19] methylenechloride[22] and possible methylamine derivatives from drugs. The present method will be promising for studying the biosynthesis of DA-Fp.

Further more, the metabolism of DA-Fp is an intriguing problem, because the oxidized products should be toxic like MTPT. It has been reported that THQs, including DA-Fp, are substrates to catechol-*O*-methyltransferase[23] and monoamine oxidase.[24] 1,2,3,4-THQ and 1,2,3,4-tetrahydro-2-methylisoquinoline have been detected in Parkinsonian and normal human brains[25] and are supposed to be the main neurotoxins inducing Parkinsonian syndrome.[26] Finally, dopamine-derived 1,2,3,4-THQs are cytotoxic to a dopaminergic cell model involving clonal rat pheochromocytoma PC12h cell.[27] Several unkown peaks appear in the chromatogram of Figs. 5, 7 and 8. They may offer clues to the detection such products by HPLC. Thus, the present method of determining DA-Fp could help elucidate etiology of Parkinsonism.

References and Notes

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