[Chem. Pharm. Bull. 22(11)2573—2577(1974)]

UDC 547-314.08

Presence of (±)-δ-(3,4-Dihydroxyphenyl)-γ-valerolactone in Human Urine

Takashi Nishikawa, Hiroaki Kubo, Masayuki Saito,[1a] Shohei Yoshiue, and Zenzo Tamura[1b]

School of Medicine, Kitasato University[1a] and Faculty of Pharmaceutical Sciences, University of Tokyo[1b]

(Received April 13, 1974)

The presence of a new compound in human urine had been suggested from the gas chromatographic assay for urinary catecholamines.[2] The compound was purified and identified as (±)-δ-(3,4-dihydroxyphenyl)-γ-valerolactone mainly by the ultraviolet, infrared, mass and nuclear magnetic resonance (NMR) spectra, and chemical functional group tests. The compound was excreted in urine of most men and women, and predominantly in conjugated form.

On the gas chromatogram of human urinary catecholamines,[2] a peak often appeared at the retention time different from those of known catecholamines.[3] This report describes the purification and the determination of the chemical structure of the compound corresponding to this peak.

Experimental

Gas chromatographic assay for urinary catecholamines was performed according to the method of Kawai, *et al.*[2] with a slight modification. The procedure consisted of the following steps: hydrolysis of urine for deconjugation with acid or with the enzyme, β-glucuronidase/arylsulphatase[4]; adsorption of catechol compounds on alumina[5] at pH 8.4 in the presence of ethylenediaminetetraacetate and elution from alumina with 1 M acetic acid in methanol; trifluoroacetylation with trifluoroacetic anhydride; injection into the gas chromatograph. Shimadzu GC-3BE gas chromatograph equipped with an electron capture detector was used.

The ultraviolet and the infrared spectra were measured on a Hitachi 124 spectrophotometer and on a 215 Hitachi grating infrared spectrophotometer, respectively. The mass and the gas chromatographic-mass spectra were determined on a Shimadzu LKB GC-MS 9000, and the NMR spectrum was measured on a JMN-MH-100 (Japan Electron Optics Laboratory Co.). Optical rotation was measured on a DIS-SL polarimeter (Japan Spectroscopic Co.).

Results

Detection of Unknown Peak (X) on the Gas Chromatogram of Human Urinary Catecholamines

The unknown peak (X) was detected on the gas chromatogram of human urinary catecholamines (Fig. 1). Since the peak (X) appeared after acid or enzymatic hydrolysis, the compound corresponding to the peak (X) was presumed to be present in urine predominantly in the form of glucuronide or sulphate conjugate. The specimens of most normal men and women, however not all the specimens, gave the peak (X). The peak height ratio of the compound to dopamine ranged from 0.0 to 2.5.

1) Location: *a*) *Asamizodai, Sagamihara-shi, Kanagawa*; *b*) *Hongo, 7–3–1, Bunkyo-ku, Tokyo.*
2) S. Kawai and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **16**, 699 (1968).
3) S. Yoshiue, K. Imai, and Z. Tamura, *Nippon Naibumpi Gakkai Zasshi*, **45**, 1587 (1969); S. Yoshiue, K. Imai, H. Kubo, and Z. Tamura, *ibid.*, **47**, 881 (1972).
4) from *Helix pomatia*, Boeringer Mannheim GmbH.
5) Aluminium Oxide Woelm neutral, Woelm Co.

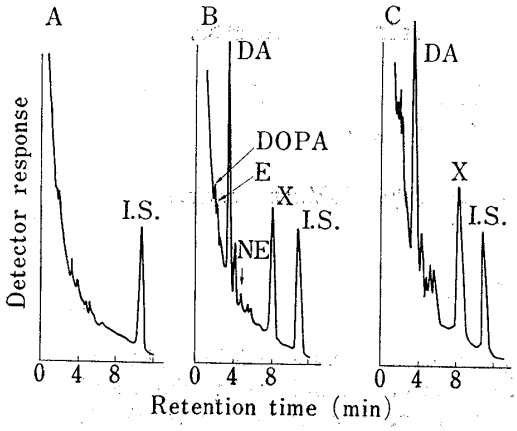

Fig. 1. Gas Chromatogram of Human Urinary Catecholamines

A, B, and C were obtained from the analyses of the same urine specimen.
A: unhydrolyzed urine
B: acid hydrolyzed urine (at pH 1.0, at 100°, for 20 min)
C: enzymatically hydrolyzed urine (at pH 6.5, at 37°, for 12 hr. Five microliters of the aque ous enzyme solution[4] was added to 5 ml of the specimen without addition of any buffer solution.)
The conditions were: 3 mm i.d.×200 cm long, 1% XF-1105 on Chromosorb W (60—80 mesh) packed glass column; nitrogen flow rate 40 ml/min; column temperature 180°; injection port temperature 200°.
DOPA: 3-(3,4-dihydroxyphenyl)-L-alanine
DA: dopamine
NE: (±)-norepinephrine
E: (−)-epinephrine
I.S.: internal standard (dieldrin)

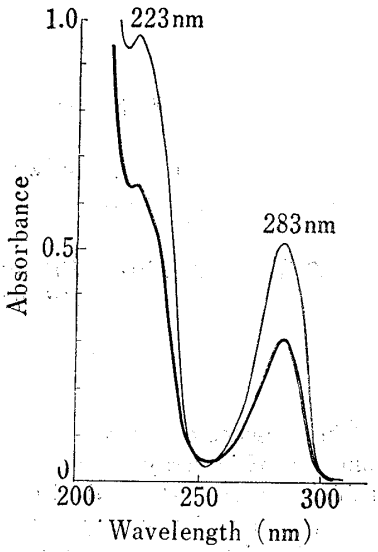

Fig. 2. Ultraviolet Spectra of the Compound and Dopamine Hydrochloride (DA·HCl) in 0.05 M HCl in Ethanol

DOPA, E, and NE gave the similar spectra to that of DA·HCl.
——: the compound
——: DA·HCl

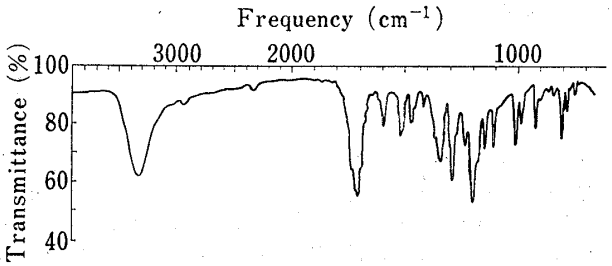

Fig. 3. Infrared Spectrum of the Compound in KBr Disc

Purification of the Compound Corresponding to the Peak (X)

Throughout the purification procedures described below, the compound was detected by the gas chromatographic method. Five liters of stored urine was adjusted to pH 1.0 with 6N HCl, then heated at 100° for 20 minutes. The solution was extracted twice with an equal volume of ethyl acetate. The compound was transferred into the organic phase, leaving catecholamines in the aqueous phase. The organic solvent was evaporated, the residue was dissolved in a mixture of 500 ml of water and 50 ml of 0.2M disodium ethylenediaminetetraacetate, and the pH of the solution was brought to 6.0 with 4M NH_4OH. Then 100 g of alumina[5] was added, and 4M NH_4OH was further added until the pH of the solution reached 8.4. The mixture was stirred for 10 minutes, then the solution was discarded through a glass filter funnel. The alumina on the funnel was washed with 200 ml of water, and the adsorbate was eluted with 200 ml of 1M acetic acid. The eluate was evaporated, the residue was dissolved in 4 ml of a mixture of benzene and methanol (1: 1), applied onto a Silica gel[6] column (25 mm i.d.×400 mm), and eluted with a mixture of benzene, methanol and acetic acid (92: 8: 0.5). The eluate was collected in 5 g fractions, the fractions containing the compound were combined and evaporated. The residue was dissolved in an appropriate volume of methanol,

6) Silica gel 60 extra pure, E. Merck Co.

and applied onto a thin-layer of Silica gel HF_{254}[7] (20 cm×20 cm, 0.4 mm thick) in a line, and developed with ethyl acetate. The compound was detected as a dark band (*Rf* 0.80) under 254 nm light and was eluted with ethyl acetate from the scraped silica gel of the band. The eluate was evaporated and approximately 1.5 mg of white crystals were obtained. The crystals melted at 128—130°, and were freely soluble in methanol and ethanol.

Structure Determination of the Compound

The ultraviolet spectrum of the purified compound was quite similar to those of catecholamines (Fig. 2). Like catecholamines,[8] the compound reacted with ethylenediamine and the product exhibited a yellowish green fluorescence in ultraviolet light. These spectral and chemical properties, and the adsorptivity on alumina indicated that the compound possessed a catechol moiety in its molecule.

Strong absorption bands at 1700 cm^{-1} and 1200 cm^{-1} in the infrared spectrum (Fig. 3) indicated the presence of an ester bond of carboxylic acid. The presence of an ester bond was confirmed by the test with hydroxylamine and ferric chloride,[9] in which, in order to avoid the interference of the color reaction of the catechol moiety with ferric chloride, a sufficient quantity of hydrogen peroxide solution was added after the treatment with hydroxylamine. Therefore the compound should have at least four oxygen atoms which belonged to one ester bond and vicinal two phenolic hydroxyls.

As indicated in Fig. 4, the mass spectrum showed a molecular ion at *m/e* 208 and predominant peaks at *m/e* 123 $[C_6H_3(OH)_2CH_2]^+$ and *m/e* 85. This was supported by the result of the gas chromatographic-mass spectrum of the trifluroacetylated compound which produced fragments of *m/e* 400 (molecular ion), 315 $[C_6H_3(CF_3COO)_2CH_2]^+$ and 85 (Fig. 5).

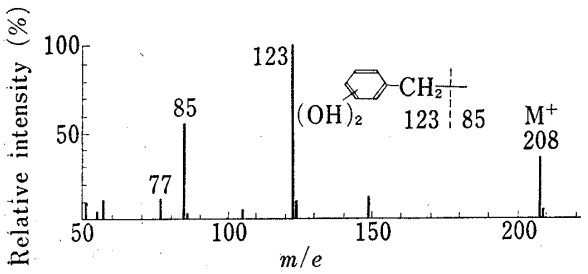

Fig. 4. Mass Spectrum of the Compound

The conditions were: electron energy 70 eV, ion source temperature 250°.

The relative intensities were: 85 (54.3%), 123 (100 %), 208 (34.4%).

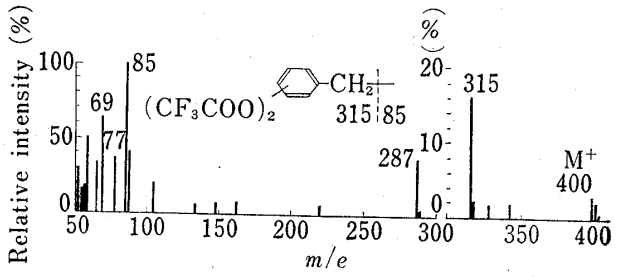

Fig. 5. Gas Chromatographic-Mass Spectrum of the Trifluoroacetylated Compound

The trace amount of the compound was dissolved in ethyl acetate, and trifluoroacetylated with trifluoroacetic anhydride, and an aliquot of the solution was injected.

The conditions were: 3 mm i.d.×200 cm long, 1% OV-1 on Chromosorb W (60—80 mesh) packed glass column; helium flow rate 30 ml/min; column temperature 170°; electron energy 70 eV; ion source temperature 290°.

The relative intensities were: 69 (63.0%), 85 (100%), 287 (37.0%), 315 (15.6%), 400 (3.1%).

The molecular formula was assumed to be $C_{11}H_{12}O_4$ from the data described above and the result of the elemental analysis [Calcd. for $C_{11}H_{12}O_4$ (%): C, 63.44; H, 5.82. Found (%): C, 62.01; H, 5.62; N, 0.00.]. The NMR spectrum in the region of δ 6.7 to 7.1 ppm (Fig. 6) was similar to those of catecholamines, indicating the presence of 3,4-dihydroxyphenyl group, which was indicated also by the ultraviolet spectrum (Fig. 2). Furthermore the NMR spectrum

7) Silica gel HF_{254}, E. Merck Co.
8) H. Weil-Malherbe and A.D. Bone, *Biochem. J.*, **51**, 311 (1952).
9) F. Feigl, "Spot Tests in Organic Analysis," 7th English edition, Elsevier Publishing Co., 1966, p. 214.

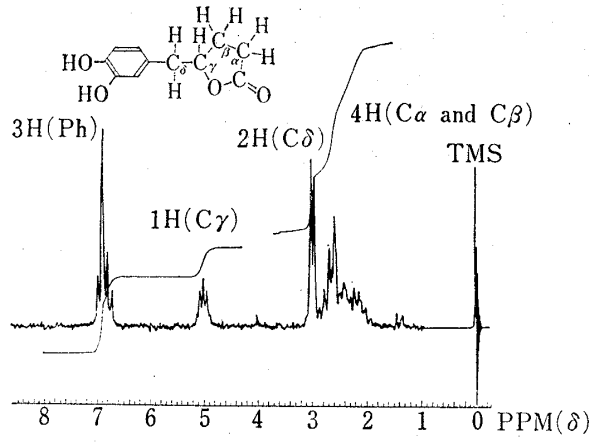

Fig. 6. NMR Spectrum of the Compound in CF_3COOD

Ph=phenyl

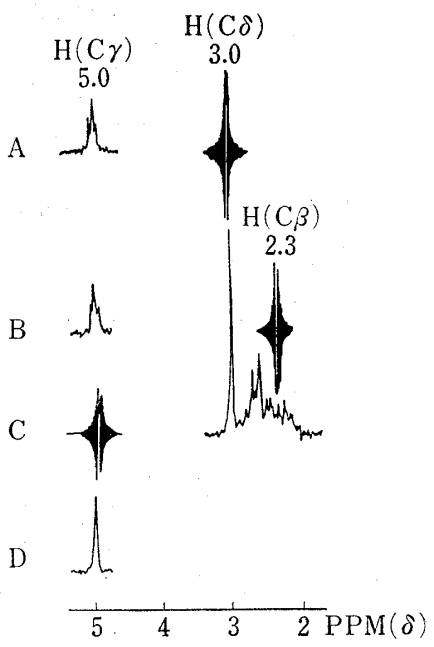

Fig. 7. Spin Decoupling NMR Spectra of the Compound in CF_3COOD

A: H(Cδ) was irradiated to destroy the spin-spin coupling interaction between H(Cδ) and H(Cγ), and the pattern of H(Cγ) was simplified.
B: H(Cβ) was irradiated, and H(Cγ) appeared as a triplet.
C: H(Cγ) was irradiated, and H(Cδ) appeared as a singlet.
D: H(Cβ) and H(Cδ) were irradiated simultaneously, and H(Cγ) appeared as a singlet.

(Fig. 6) indicated that only one possible structure was δ-(3,4-dihydroxyphenyl)-γ-valerolactone. The structure was confirmed by the results of the spin decoupling NMR spectra (Fig. 7).

The compound was found to be optically inactive[10] though it had one asymmetric carbon atom in its molecule. By comparison of the specific optical rotation and the melting point reported by Oshima, *et al.*,[11] the compound was determined as a racemate, (±)-δ-(3,4-dihydroxyphenyl)-γ-valerolactone.

Discussion

This is the first report that describes the presence of (±)-δ-(3,4-dihydroxyphenyl)-γ-valerolactone in human urine.

Oshima, *et al.* reported that optically active δ-(3,4-dihydroxyphenyl)-γ-valerolactone was excreted in urine of rabbit after oral administration of (+)-catechin or (−)-epicatechin.[11] We also purified the compound from the urine of (+)-catechin-administered rat by the same procedure as that from human urine, and identified it with the optically active compound by the measurements of ultraviolet and infrared spectra, and melting point. This fact showed that racemization did not occur during the purification and the compound was a racemate before the treatment of the hydrolysis.

The purified compound was not an artifact produced under the conditions of acid hydrolysis by any other chemical changes than deconjugation, because acid hydrolysis could be replaced by enzymatic hydrolysis, giving the same result (Fig. 1). However the conjugated position in the molecule was not determined.

By the trihydroxyindole method,[12] which was used commonly for the measurement of catecholamines, the compound did not yield a fluorescent product. Therefore the presence of the compound would not interfere with the measurement by this method. However there remains a possibility that the compound is further metabolized into 3,4-dihydroxyphenyl-

10) The optical rotation was measured at 0.5% concentration in ethanol with an optical path of 1 cm.
11) Y. Oshima, H. Watanabe, and S. Kuwazuka, *Bull. Agr. Chem. Soc. Japan.*, **24**, 497 (1960).
12) A. Lund, *Acta Pharmacol. Toxicol.*, **6**, 137 (1950).

acetic acid or some other metabolites of catecholamines and influences the measurement of a metabolite of catecholamines.

The metabolic origin and the biological effect of the compound are not clarified. Since the excreted amount in normal human urine showed marked daily and individual variations, and since the untreated rat fed on the regular solid diet scarcely excreted the compound, it may not be an endogeneous metabolite.