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Improved Separation of the Denopamine Metabolites Using Capillary Column Gas Chromatography-Mass Spectrometry

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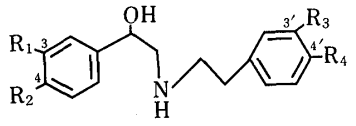
Eight urinary metabolites of the positive inotropic agent denopamine, (−)-(*R*)-1-(*p*-hydroxyphenyl)-2-[(3,4-dimethoxyphenethyl)amino]ethanol, in man were separated and identified by capillary column gas chromatography-mass spectrometry. The metabolites were products of oxidative 3′- or 4′-O-demethylation and/or *meta*-hydroxylation followed by *meta*- or *para*-catechol O-methyltransferase-methylation. Separation of the four isomers of 1-(hydroxymethoxyphenyl)-2-[(hydroxymethoxyphenethyl)amino]ethanol was made possible by the use of a capillary column.

Keywords——denopamine; denopamine metabolite; COMT; monooxygenase; O-demethylation; hydroxylation; methylation; capillary column; GC-MS

Denopamine, (−)-*R*-1-(*p*-hydroxyphenyl)-2-[(3,4-dimethoxyphenethyl)amino]ethanol (I), is a new, orally active, selectively positive inotropic agent.[1] In a previous study on its urinary metabolites excreted after oral administration to man, we identified and quantitated five metabolites (II—VI, Chart 1).[2] The 4′- or 3′-O-demethylation of the dimethoxy function results in II and III, respectively, and ring-hydroxylation at position 3 followed by 3- or 4-O-methylation by catechol O-methyltransferase (COMT) results in IV and V, respectively. The ratio of II and III was 17 : 1, and that of IV and V was 6 : 1.[2] In a subsequent *in vitro* study with rat liver preparations,[3] we demonstrated each enzymatic step of the above reactions, obtaining the isomeric metabolites in similar ratios.



I: $R_1=H$, $R_2=OH$, $R_3=R_4=OMe$
II: $R_1=H$, $R_2=R_4=OH$, $R_3=OMe$
III: $R_1=H$, $R_2=R_3=OH$, $R_4=OMe$
IV: $R_2=OH$, $R_1=R_3=R_4=OMe$
V: $R_1=OH$, $R_2=R_3=R_4=OMe$
VI: $R_2=R_4=OH$, $R_1=R_3=OMe$
VII: $R_2=R_3=OH$, $R_1=R_4=OMe$
VIII: $R_1=R_4=OH$, $R_2=R_3=OMe$
IX: $R_1=R_3=OH$, $R_2=R_4=OMe$

Chart 1

In these studies, the metabolite formed by both O-demethylation and ring-hydroxylation followed by COMT-methylation was characterized as 1-(4-hydroxy-3-methoxyphenyl)-2-[(4-hydroxy-3-methoxyphenethyl)amino]ethanol (VI). However, there should be three other isomers (VII, VIII, IX) of VI in view of the occurrence of the minor O-demethylation and COMT-methylation products III and V. It is possible that these minor isomeric metabolites were actually present but could not be separated from VI by packed column gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC).

Recently, there have been many reports dealing with the high separating power of capillary column gas chromatography. We attempted to analyze a human urine sample collected 4 h after denopamine administration by using capillary column GC-MS, and successfully separated and identified the above three minor isomers, which had hitherto been undetectable.

Experimental

Materials——Denopamine and its metabolites, II—VI, were synthesized and donated by Dr. Iwakuma[4] of the Organic Chemistry Research Laboratory, Tanabe Seiyaku Co., Ltd. Compounds VII, VIII and IX were synthesized by us as described by Iwakuma *et al.*[4] All these compounds were characterized by mass spectrometry. Ethyl acetate and trimethylsilylimidazole (TMSI) were purchased from Tokyo Kasei Kogyo Co. (Tokyo). Ethyl acetate was distilled before use.

Biological Samples——A 10 mg tablet was orally administered to a healthy male volunteer, aged 23 years, about 2 h after breakfast. The urine was collected during the period of 0—4 h.

Enzymatic Hydrolysis——To 1 ml of about 5-fold-diluted urine in a 30 ml glass-stoppered test tube was added 4 ml of 0.1 M acetate buffer (pH 5.2), and the mixture was incubated in the presence of 1000 units of β-glucuronidase (type B-1, Sigma Chemical Co., U.S.A.) and 50 units of arylsulfatase (from *Helix pomatia*, type H-1, Sigma Chemical Co.) at 37 °C for 20 h.

Extraction and Derivatization——The incubation mixture was adjusted to pH 10 with 1 N NH_4OH and extracted with 10 ml of ethyl acetate. The ethyl acetate layer was re-extracted with 4 ml of 0.2 N HCl. The aqueous solution was made alkaline with 1 N NH_4OH, and again extracted with 10 ml of ethyl acetate. After evaporation of the ethyl acetate *in vacuo*, ethyl acetate (100 μl) and TMSI (50 μl) were added to the dried residue. The tube was allowed to stand at room temperature for 30 min. An aliquot of the solution was directly injected into the gas chromatograph-mass spectrometer.

GC-MS——A JEOL DX-300 GC-MS instrument equipped with a DA-5000 data processing system was employed. The column was a cross-linked methylsilicone-coated fused silica capillary column (25 m × 0.2 mm i.d.; coating thickness 0.11 μm, Hewlett-Packard). Injections were made in the split mode. The flow rate of the carrier gas (helium) was 80 ml/min, and the split ratio was set at 95 : 1. The temperature of the column oven was maintained at 260 °C. The mass spectrometer was operated at an ionization energy of 70 eV and an accelerating voltage of 3.0 kV in the electron impact mode.

Results

Figure 1 shows the total ion chromatogram of a TMSI-treated mixture of arbitrary amounts of nine (I—IX) standard samples. All compounds were completely separated from each other in the range of about 7 min to 11 min. It can be seen from Fig. 1 that the *para*-methoxy compounds were more rapidly eluted from the GC column than the corresponding *meta*-methoxy isomers.

The mass spectra of O-TMS derivatives of the four isomers (VI—IX) showed identical fragmentation patterns (Fig. 2). The most prominent ions in these spectra were the fragments at *m/z* 252 and 297 resulting from cleavage between the two carbons of the ethanolamine moiety.

Figure 3 shows selected ion monitoring (SIM) traces obtained by analyzing a hydrolyzed, TMSI-treated urine sample from the volunteer. It can be seen that the trace of *m/z* 252 (representing the O-demethylated phenethylamine moiety) and the trace of *m/z* 297 (representing the hydroxy-methoxybenzylalcohol moiety) both showed four common peaks corresponding to VI—IX, among which the peak of VI was by far the highest.

In order to quantitate each metabolite, a mixture of equal amounts of the respective standard samples (VI—IX) was analyzed by the same method. The relative intensities of the base peak areas of VI, VII, VIII and IX at *m/z* 252 were 1.0, 0.8, 1.2 and 1.1, respectively. By using these ratios for correction, the amounts of VII, VIII and IX excreted in the urine were estimated to be about 3.0%, 2.9% and 5.0%, respectively, of the amount of VI.

The ratios of II to III and IV to V were about 18 : 1 and 7 : 1, respectively, being similar to

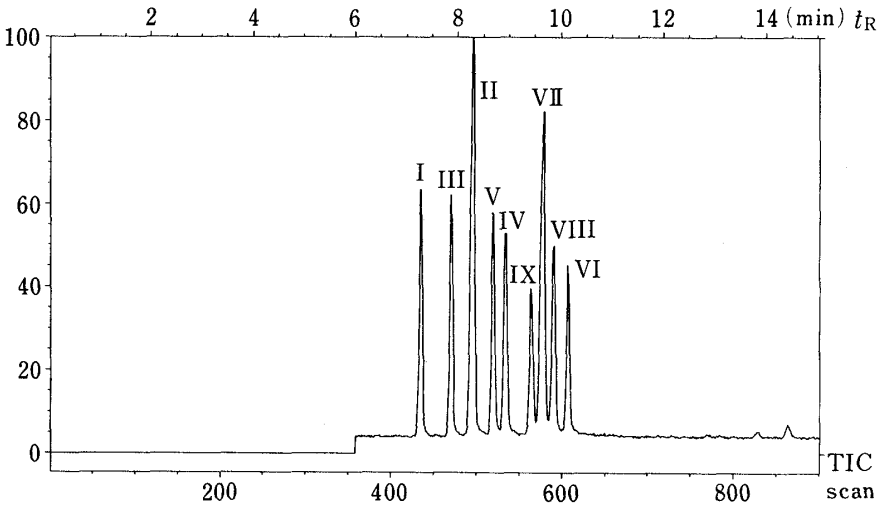


Fig. 1. Mass Chromatogram of O-TMS Derivatives of Denopamine and Its Synthetic Eight Metabolites

TIC, total ion chromatogram.

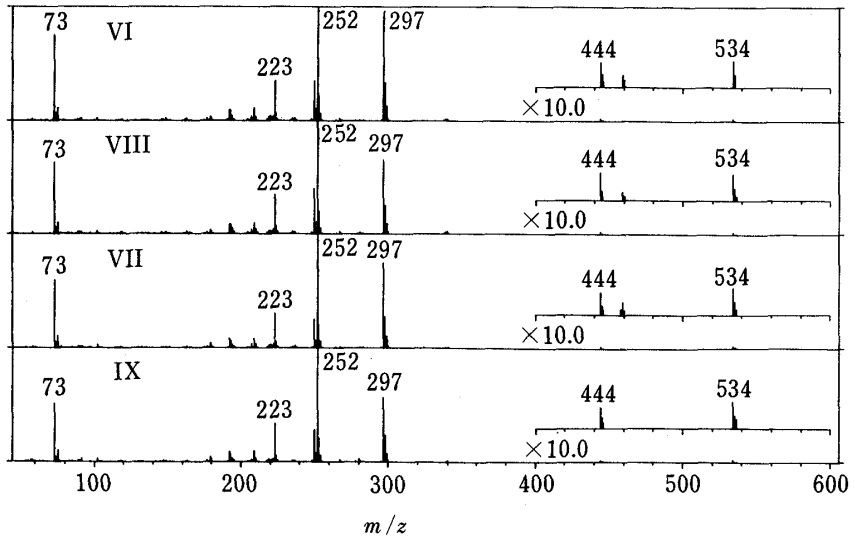


Fig. 2. Mass Spectra of O-TMS Derivatives of Compounds VI—IX

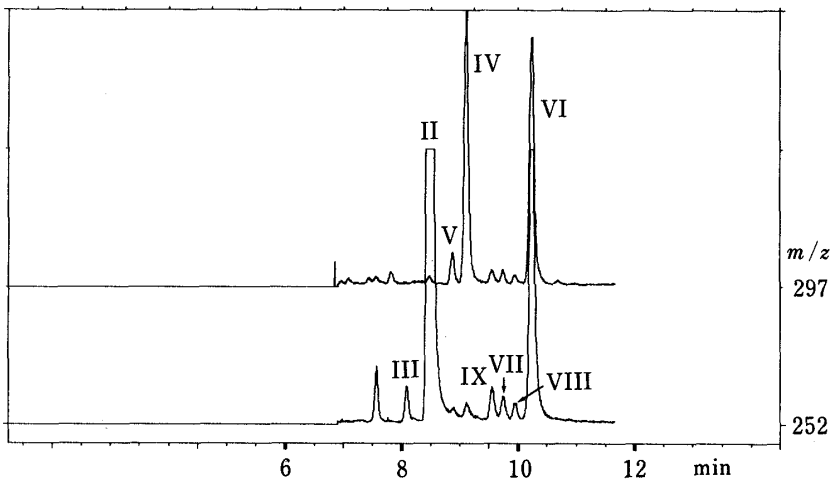


Fig. 3. Selected Ion Monitoring Traces of the Urine Sample Obtained from a Volunteer 4 h after Oral Administration of Denopamine

the ratios previously determined by HPLC.[2)]

Discussion

As denopamine possesses one phenolic hydroxy function on one benzene ring and two adjacent methoxy functions on the other, it is susceptible to ring-hydroxylation, yielding a catechol, and O-demethylation by the monooxygenase system in the liver. The catechol metabolites, *i.e.*, hydroxylated denopamine, are rapidly methylated by COMT. Thus, the two alternative sites in these O-demethylation and COMT-methylation reactions give rise to two pairs of isomers (II, III and IV, V).

There are few studies dealing with minor products of these reactions, because they are usually difficult to separate from the major products.[5,6)] In the previous papers, we have reported separation of these isomers by SIM with GC-MS after HPLC separations.[2,3)] In the present study, the use of capillary column GC-MS made it possible to separate denopamine and all its metabolites including the four isomers of 1-(hydroxy-methoxyphenyl)-2-[(hydroxy-methoxyphenethyl)amino]ethanol, which were not separated from each other by HPLC.

In the previous *in vitro* study, IV was O-demethylated by rat liver monooxygenase to give VI, but ring-hydroxylation of II did not take place.[3)] This is ascribable to the possibility that II, already possessing three hydroxy functions, may be too hydrophilic and may therefore resist further hydroxylation. If we assume that the four bilaterally oxidized metabolites were formed by O-demethylation of the corresponding hydroxy-methoxybenzylalcohols (IV and V), preferential 4′-O-demethylation[3)] should give more of the 4′-O-demethylated (VI, VIII) than of the 3′-O-demethylated (VII, IX) metabolites. In fact, incubation of IV and V with rat liver microsomes produced VI and VII in a ratio of 19 : 1 and VIII and IX in a ratio of 10 : 1 (unpublished observation). Therefore, the excretion of IX in a relatively high amount was unexpected. The distribution ratios of the isomers found in the present study may not necessarily reflect the ratios of their production rates, because the individual metabolites may be further metabolized at different rates.

In conclusion, we have separated and identified eight metabolites of denopamine in human urine simultaneously by means of capillary column GC-MS. This technique has enabled us to determine the three minor isomers of VI, which were undetectable by the combination of HPLC and packed column GC-MS used in the previous study.

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