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Journal of Pharmaceutical and Biomedical Analysis
15 (1997) 1241–1247

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Chiral analysis of 3-methoxy-4-hydroxyphenylglycol in human urine[1]

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Received 21 August 1996; accepted 30 September 1996

Abstract

Since 3-methoxy-4-hydroxyphenylglycol (MHPG) is a neutral metabolite from norepinephrine, it will be a diagnostic marker for mental diseases such as depression. For the development of an immunoassay, the natural enantiomer of MHPG would be required to prepare its antigen and to examine the specificity of the antibody. A natural enantiomer synthesized, however, has not been obtained so far. In this paper, we attempted to enantioseparate synthetic DL-MHPG and to assign D-enantiomer from the optical rotation of MHPG purified from human urine, because endogenous norepinephrine occurs as D-enantiomer which should metabolically generate D-MHPG. Enantioseparation conditions were tested using a Ceramospher Chiral RU-1 column (4.6 × 250 mm) at a flow rate of 0.5 ml/min. The resolution was adequate for the analysis and purification of synthetic DL- and the urinary MHPGs using methanol as a mobile phase and the column temperature at 0°C, where DL-MHPG was detected as two peaks. The earlier peak (peak 1) showed (−) optical rotation, while the latter gave (+) optical rotation. After being treated with β-glucuronidase, the normal human urine was extracted with ethyl acetate and then evaporated to dryness. The residue was suspended in water and the supernatant was analyzed and purified by a reversed phase column with a multi channel detector. A peak corresponding to MHPG was collected and concentrated to dryness. The pooled residues were dissolved in methanol and enantioseparated on the chiral HPLC. The urinary MHPG appeared as a single peak which was corresponded to the earlier peak of DL-MPHG and showing (−) optical rotation. Thus, the urinary MHPG was found to be D-(−)-MHPG. Then the absolute configuration of enantioseparated MHPGs were assigned to each optical rotation, judging from the chemical data and the metabolic pathway of the urinary D-MHPG. These enantiomers will be useful for studying on biochemistry and immunoassay. © 1997 Elsevier Science B.V.

Keywords: D-(−)-3-Methoxy-4-hydroxyphenylglycol; DL-3-Methoxy-4-hydroxyphenylglycol; Enantioseparation; Human urine; Optical rotation

1. Introduction

3-Methoxy-4-hydroxyphenylglycol (MHPG) is a major metabolite from norepinephrine in the

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[1] Presented at the Seventh International Symposium on Pharmaceutical and Biomedical Analysis, August, 1996, Osaka, Japan.

PII S0731-7085(96)01981-4

brain [1,2]. MHPG is conjugated with sulfate in the brain and excreted in blood via cerebrospinal fluid. Norephinephrine as well is secreted from the peripheral nervous system and adrenal gland and metabolized in various organs to MHPG which is conjugated with glucuronide. Both types of the conjugates are excreted together with free MHPG in urine [2–7]. Thus, MHPG levels in the peripheral blood and urine are considered to reflect the activity of the brain. Then, its levels will be a prognostic marker to classify mental illness such as depression [8,9].

There are several methods to determine MHPG in urine such as gas chromatography and high performance liquid chromatography (HPLC) [10]. These methods are inconvenient to assay many samples. Therefore enzyme immunoassay (EIA) was desirable and developed by Yoshioka et al. using a polyclonal antibody [11]. During screening of specific monoclonal antibodies, it was required to discriminate the natural enantiomer of MHPG with the antibody. The endogenous norepinephrine is D-form of the asymmetric carbon and should generate D-form of MHPG as shown in Fig. 1. However, chemically and optically characterization of MHPG entaniomers have not been reported.

In 1980, Blombery et al. [12–14] described deuterium labeled MHPG – [C^2H_3] such as DL-(±)- and D-(−)-MHPG. The optical characterization, however, was not at all proved, although there was tendency of negative optical rotation in catecholamines and their metabolites such as epinephrine, norepinephrine [15] and vanilmandelic acid (VMA). The absolute configuration of D-(−)-norepinephrine was established by x-ray crystallography [16]. The metabolic study of D-(−)-norepinephrine was extensively carried out in various organs. The isotope labeled (±)- or (−)-norepinephrine was stereoselectively metabolized to MHPG as shown in Fig. 1. Especially, the enzyme of MAO and AR were stereoselective [17,18], whereas COMT was not [3]. Thus, the absolute configuration of the urinary MHPG is considered to be a D-form.

In the present paper, we aimed to preparatively enantioseparate DL-MHPG and to assign either separated form to D-enantiomer purified from human urine judging from optical rotation and chiral HPLC.

2. Experimental

2.1. Materials

DL-3-Methoxy-4-hydroxyphenylglycol hemipiperazine salt was obtained from Sigma (St. Louis, MO). Methanol (99.8%) for the measurement of the optical rotation was purchased from nacalai tesque (Kyoto). β-Glucuronidase type IX-A was purchased from Sigma. All the other reagents were commercially available.

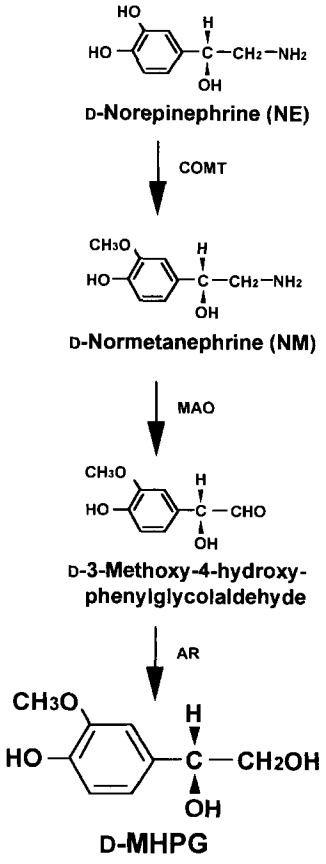

Fig. 1. Metabolism of D-norepinephrine to D-MHPG. COMT, catechol-*O*-methyl transferase; MAO, monoamine oxidase; AR, aldehyde reductase.

2.2. Spectrometories

UV spectra of the purified MHPGs were measured by a UV spectrophotometer UVIDEC 610 (JASCO, Tokyo).

Optical rotations of the purified MHPGs were measured by a digital polarimeter DIP-140 (JASCO) using D-ray of sodium at 20°C. Each sample was dissolved in 99.8% methanol. The concentration was calculated according to the method described in preparation of MHPG solution.

^{1}H-NMR was measured with a NMR spectrometer JNM-GSX 400 (JEOL, Tokyo) in methanol-d_4, using tetramethylsilane as an internal standard. The probe temperature was maintained at 40°C. Chemical shifts are given in ppm and coupling constants (J Values) in hertz (Hz).

Electron ionization mass spectrometry (EI-MS) was carried out by a mass spectrometer JMS-DX-300 (JEOL, Tokyo). The ionization voltage was 30 eV. The temperature of a sample chamber was 260°C.

2.3. Preparation of MHPG solution

For the preparation of 10 mM solution, 2.22 mg of DL-MHPG hemipiperazine salt was dissolved in 977 μl of 0.1 M phosphate buffer (pH 7.0), and then diluted with the same buffer to make a 0.5 mM solution. The molar absorption coefficient was calculated by using the absorbance measured at 280 nm (E = 1.41) and found to be 2820. This value was used to calculate the concentrations of purified MHPG solutions.

2.4. Desalting of DL-MHPG hemipiperazine salt

A column (15 mm, I.D. × 100 mm) of SP-Sephadex C25 was washed with 30 ml each of 0.1 N NaOH and water, and equilibrated with 30 ml of 0.1 M ammonium formate-0.1 M formic acid buffer (pH 2.99) at a flow rate of 1.0 ml min^{-1}. Then 1 ml of 100 mg of MHPG hemipiperazine salt in the above buffer was applied onto the column. The eluate with the buffer was monitored by a UV detector at 254 nm. The flow-through fractions were pooled and lyophilized. The free form of MHPG in these fractions was proved to be not contaminated with piperazine by ^{1}H-NMR. The obtained free form in 88% yield was stored at − 20°C and used for enantioseparation on HPLC and various spectrometries. The chemical shifts of ^{1}H-NMR were as follows:

2.4.1. DL-MHPG hemipiperazine salt

δ: 2.80 (*s*, 4H), 3.59 (*d*, J = 6.1, 2H), 3.86 (*s*, 3H), 4.59 (*t*, J = 6.1, 1H), 6.76 (*d*, J = 8.2, 1H), 6.80 (*dd*, J = 8.2, 1.8, 1H), 6.97 (*d*, J = 1.8, 1H).

2.4.2. Free form of DL-MHPG

δ: 3.60 (*d*, J = 6.0, 2H), 3.86 (S, 3H), 4.61 (*t*, J = 6.0, 1H), 6.76 (*d*, J = 8.0, 1H), 6.80 (*dd*, J = 8.0, 1.8, 1H), 6.97 (*d*, J = 1.8, 1H).

2.5. Enantioseparation of DL-MHPG

Chiral HPLC was performed by a system composed of a pump M-45 (Waters) and a detector 870-UV (JASCO, Tokyo). The free DL-MHPG obtained above was dissolved in methanol to make a 0.3 M solution. The solution (20 μl) was injected into a column (4.6 mm I.D. × 250 mm) of Ceramospher Chiral RU-1 (Shiseido, Tokyo) at 0°C. The mobile phase of methanol was 0.5 ml min^{-1} and monitored at 280 nm. Two peaks appeared and were called peak 1 and 2 in the order of retention time. This enantioseparation was repeated many times for the preparation of purified enantiomers of MHPG and urinary MHPG. The pooled peaks were concentrated to dryness in vacuo. Each oily residue was stored at − 20°C.

2.6. Purification of endogenous MHPG from human urine

Urine of a normal male adult was accumulated for 2 days. Approximately, 1.5 l of the urine was stored on ice and filtered through a filter paper No. 2 (Toyoroshi, Tokyo) to remove infranatants. The filtrate was added 100 mg of β-glucuronidase type IX-A (60 000 unit) and stirred at 37°C overnight. Sodium chloride (500 g) was added and the solution was extracted with 1.5 l each of the ethyl acetate four times. The ethyl

acetate layer was evaporated to dryness in vacuo. The residue was suspended in 1.5 ml of water. The suspension was centrifuged at $7000 \times g$ for 30 min. The supernatant was stored at −20°C. The supernatant (20 μl) was analyzed by the analytical HPLC on a reversed phase to identify the MHPG. For preparative separation, 200 μl of the supernatant was injected into a column (10 mm I.D. × 250 mm) of Capcell pak C_{18} (Shiseido, Tokyo) at 40°C. The elution was carried out under a linear gradient of 0.1% TFA to 0.1% TFA-30% acetonitrile at a flow rate of 3.0 ml min^{-1} for 30 min. A peak corresponding to DL-MHPG was collected and lyophilized. This separation was repeated additionally five times. The lyophilized residue was dissolved in 200 μl of water. A quarter of the solution was applied to a column (4.6 mm I.D. × 250 mm) of Capcell pak C_{18} (Shiseido). The elution was under a linear gradient of 0–10% acetonitrile in water at a flow rate of 1.0 ml min^{-1} for 30 min. The eluate was monitored by a multi channel detector MULTI-340 (JASCO, Tokyo). The peak corresponding to DL-MHPG was collected and lyophilized. This separation was repeated additionally three times. The purified urinary MHPG was dissolved in 1 ml of methanol. The concentration of this solution by measuring UV absorbance at 280 nm was 0.3 mg ml^{-1}. This solution was used for the measuring optical rotation. Then, 200 μl of this solution was evaporated to dryness with N_2, and dissolved in 20 μl of methanol to make a 3 mg ml^{-1} solution. Of the solution, 5 μl was subjected to above enantioseparation (Fig. 4A). Another 5 μl was used for the measuring of EI-MS. After dryness of 400 μl of 0.3 mg ml^{-1} solution, 650 μl of 0.1 M phosphate buffer (pH 7.0) was added to make a 1 mM solution and 25 μl of this solution was loaded into an analytical HPLC.

Results of EI-MS for purified MHPG from human urine were as follows.

High resolution EI-MS *m/z*: Calcd for $C_9H_{12}O_4$:184.07364. Found: 184.07619.

EI-MS *m/z*: 184 $(M)^+$, 166 $(M\text{-}H_2O)^+$, 153 $(M\text{-}CH_2OH)^+$, 137 (base peak), 122, 114, 93.

3. Results

3.1. Desalting of DL-MHPG hemipiperazine salt

Before chiral analysis of DL-MHPG by HPLC, it was necessary to desalt piperazine from the commercial DL-MHPG salt. The flow-through fraction obtained from SP-Sephadex C25 was negative to ninhydrin reaction with piperazine. In a ^{1}H-NMR spectrum of DL-MHPG salt, a signal appearing at 2.80 ppm which was considered as methylene protons of piperazine disappeared in the one of the desalted DL-MHPG. The yield of the obtained free form was 88%.

3.2. Enantioseparation of DL-MHPG

To optimize the condition for enantioseparation of free DL-MHPG, various mobile phases and column temperatures were tested. For the resolution, methanol was the best among methanol, ethanol and various combinations of methanol and ethanol (data not shown). At room temperature the close retention times of DL-isomers eluted with methanol were earlier than those at 0°C. When the temperature was decreased to −20°C, the peaks were diffused broadly and retention times increased. Thus, the optimized mobile phase and the column temperature were determined as methanol and 0°C, respectively. DL-MHPG enantiomers were separated into two peaks as shown in Fig. 2A. Peak 1 seemed to be pure judging from the chromatogram as shown in Fig. 2B. However, peak 2 seemed to be contaminated with peak 1 because of a shoulder before peak 2 as shown in Fig. 2C. Peak 2 in Fig. 2C was repeatedly collected cutting off the shoulder as shown in Fig. 2D. The retention times and UV spectrum of both peaks were the same as those of DL-MHPG in the analytical HPLC under the same condition as described in Fig. 3 (data not shown), while the optical rotations of both peaks were opposite. The compound in peak 1 showed $[\alpha]_D^{20} = -23.0°$ ($c = 0.87$ in methanol), but that in peak 2 gave $[\alpha]_D^{20} = +15.3°$ ($c = 0.99$ in methanol). The equality of both absolute values was dependent on the purity. As shown in Fig. 2, both peaks were eluted as broad peaks. Peak 1 was

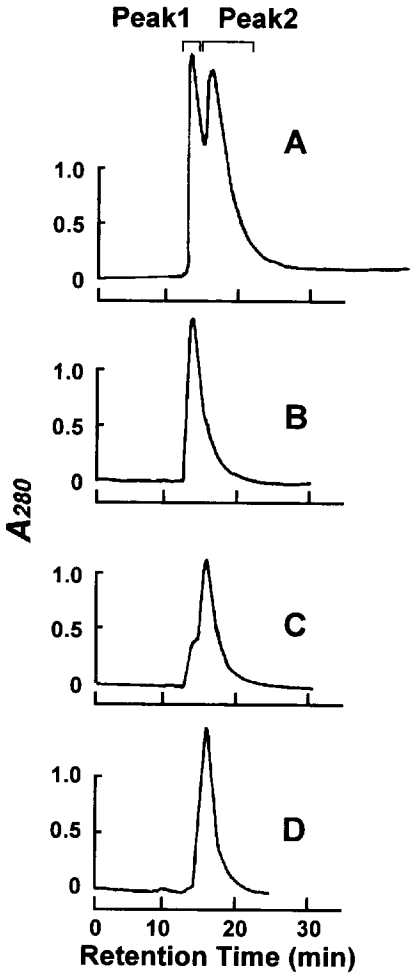

Fig. 2. Chiral chromatograms of DL-MHPG. DL-MHPG was separated (A). Peak 1 was rechromatographed (B). Peak 2 was rechromatographed (C). The fraction of peak 2 in C was rechromatographed (D).

purified by only one chiral HPLC, but peak 2 seemed to contain the peak 1 as shown in Fig. 2C. Then, only peak 2 was applied to the same column repeatedly. Even after 2nd separation, the purification of peak 2 was suspected to be not complete because peak 1 was eluted as a broad peak as shown in Fig. 2B. It was obvious that both compounds in peak 1 and 2 were enantiomers of MHPG and called (−)-MHPG and (+)-MHPG, respectively.

3.3. Purification of endogenous MHPG from human urine

The urinary MHPG hydrolyzed from its conjugate was extracted with ethyl acetate. With or without saturation of sodium chloride, its distribution coefficients were 1.6 and 0.46, respectively, when the DL-MHPG was used. Judging from this result, it was concluded that the saturation was essential for the extraction of urinary MHPG. A portion of this extract was applied to an analytical HPLC to know the recovery of MHPG (data not shown). The extracted MHPG was purified by the preparative reversed phase HPLC (data not shown), and continuously by the analytical HPLC monitoring with a multi-channel detector. The overall yield was 299 μg from 1.2 l of the urine. As shown in Fig. 3, the urinary MHPG was eluted in the same retention time to that of (−)-MHPG and its UV spectrum showed the same pattern to (−)-MHPG as well as (+)- and DL-MHPG. In addition, the molecular ion peaks and the fragment ion peaks obtained from EI-MS were the same as those of (−)-MHPG. These results and optical rotation described in the following lead us to the conclusion that the purification of the urinary MHPG was performed completely and there is (−)-MHPG present in urine.

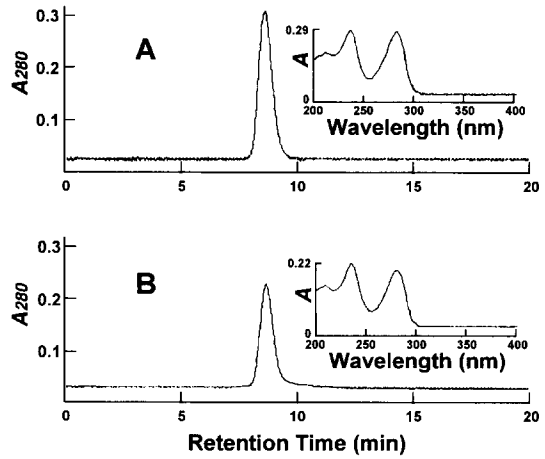

Fig. 3. Reversed phase chromatograms of (−)-MHPG (A), urinary D-MHPG (B) and UV spectra of their peaks. Absorance (A) at 280 nm (A_{280}) was monitored.

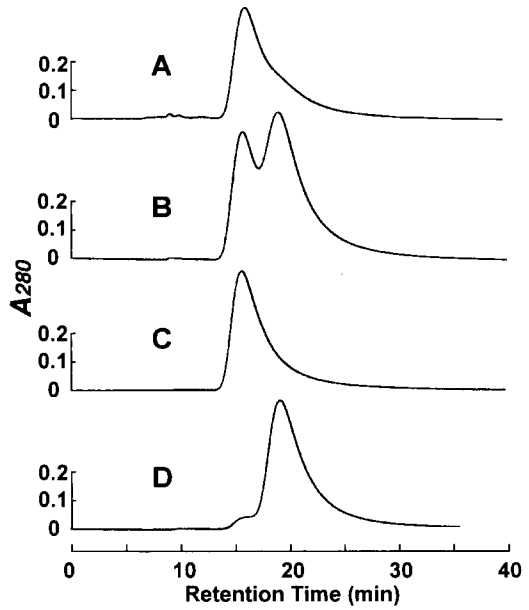

Fig. 4. Chiral chromatograms of various MHPG's. Urinary MHPG (A), DL-MHPG (B), (−)-MHPG (C) and (+)-MHPG (D) were injected, respectively.

3.4. Chiral HPLC and Optical Rotation of Urinary MHPG

The resolution of the chiral HPLC was so sensitive to presence of water that the retention time was not precisely reproducible as shown in Fig. 4. The chiral analysis of the urinary MHPG on HPLC was performed very carefully. As shown in Fig. 4A, only one peak was identified by the monitoring with the UV absorbance at 280 nm, and the retention time of this peak was the same as that of (−)-MHPG (Fig. 4C). In addition, the optical rotation of this peak was negative. ($[\alpha]_D^{20} = -7°$, $c = 0.03$ in methanol). Thus, it was conclusive that there is (−)-MHPG present in urine.

4. Discussion

We have been trying to prepare the specific monoclonal antibody to the endogenous MHPG for a long time, although the specific monoclonal antibody to D-VMA and the one to homovanillic acid was obtained [19]. In our serial studies, we found that the polyclonal antibody recognized the enantiomers of MHPG, but we could not prepare the specific antibody against each enantiomer. In addition, the affinity of the polyclonal antibody against MHPG was less than the level of the urinary MHPG. For these reasons, we thought that the determination of absolute configuration and the preparation of enantioseparated MHPG as antigens were a prerequisite to discriminate the specific monoclonal antibody against the endogenous MHPG. Recently, the chiral column for separation with HPLC has been developed. We attempted the enantioseparation of MHPG by using the chiral column of Ceramospher Chiral RU-1. Fortunately, DL-MHPG were separated into the enantiomers when methanol was used as the mobile phase, but other catecholamines tested such as DL-VMA and DL-norepinephrine were not separated. We used this column very carefully because the resolution of this column was very sensitive against the aqueous phase and the concentration of DL-MHPG. If the sample contained aqueous phase, the resolution was also obscured by an aqueous peak. In addition, the ratio and the retention time of separated D- and L-MHPG were effected by the concentration and injection volume of the sample. We prepared a 3 mg ml^{-1} test solution to compare the retention time on the chiral HPLC (Fig. 2 and Fig. 4). We repeated the enantioseparation many times for the determination of optical rotation of enantiomers and the preparation of enantioseparated MHPG as an antigen for immunization. But, the enantioseparation was not performed completely judging from the difference of the optical rotation of the enantioseparated (−)- and (+)-MHPG. The absolute value of the urinary D-(−)-MHPG was very low in a small amount of the purified urinary MHPG. This low value was not exactly correct, but the direction of the optical rotation was reasonable judging from the results of chiral HPLC in that the urinary MHPG was overlapped with peak 1, (−)-MHPG.

In the present paper, direct evidence of D-(−)-MHPG in urine was attained by both the optical rotation and the chiral HPLC. Meanwhile, a considerable amount of D-(−)-MHPG in circulation

was stereoselectively oxidized to D-VMA and excreted to urine as described by Blombery et al. [12]. In our previous paper for enzyme immunoassay for VMA, we also specifically measured D-VMA in human urine [19]. Thus, it was likely that D-form of MHPG was assigned to (−)-MHPG and stereoselective metabolism of D-(−)-MHPG was substantially completed. The separated MHPGs will be useful for biochemical study of MHPG as well as immunochemical analysis.

Acknowledgements

We are grateful to Miss M. Matsukawa of our laboratory for her valuable technical advice with this work and to Mr S. Yamaguchi of this faculty for mass spectrometry measurement.

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