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Identification of Higenamine and its Metabolites in Rat by Gas Chromatography/Mass Spectrometry

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(±)-Higenamine is known as a cardiotonic principle of aconite root (root of *Aconitum* spp., Ranunculaceae). A simple and sensitive detection method for higenamine was developed by using gas chromatography-mass spectrometry (GC/MS). The recovery of higenamine after extraction and concentration with XAD-2 resin column was around 95% from rat biological fluids such as bile, plasma and urine. The limits of detection of higenamine in these biological fluids were approximately 0.1 ng/ml each. It has well been suggested that tetrahydroisoquinolines possessing catechol moiety such as higenamine should be subjected to the catechol-O-methyl transferase (COMT) activity *in vivo*. We detected two major peaks of presumed metabolites of higenamine in the total ion chromatogram obtained from the rat urine sample after the oral adminstration of (±)-higenamine. The scan mass spectrum of one of the metabolites coincided with those obtained from coclaurine (C_6-O-methyl higenamine) and those of the other metabolite are suggestive of isococlaurine (C_7-O-methyl higenamine).

Key words: Higenamine, Coclaurine, Isococlauirne, Gas chromatography/Mass spectrometry

INTRODUCTION

Aconite root (Buja in Korean), root of certain species of *Aconitum* plants which belongs to Ranunculaceae family, has long been considered as one of the most important herbal drugs and used as a heart stimulant, diuretic and anodyne in oriental medicine.

From the plant, Kosuge *et al.* isolated a cardiotonic component, higenamine as hydrochloride salt and identified the structure [**1**, $C_{16}H_{17}NO_3$, dl-1-(4-hydroxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline], as shown in Fig. 1. The higenamine hydrochloride was water soluble, however fairly unstable especially in basic medium (Kosuge *et al.*, 1978). The total synthesis of higenamine was reported by chemists starting either from vanillin (4-hydroxy-3-methoxy benzaldehyde) (Huang *et al.*, 1981) or from β-(3,4-dimethoxyphenyl) ethylamine (Loboeuf and Cave, 1981; Chang *et al.*, 1984).

The mechanism of the inotropic action of *Aconiti* tuber on cardiac muscle has well been investigated. The positive inotropic effect of higenamine was found to be potentiated by extracellular calcium suggesting that higenamine may accelerate calcium influx through the sarcolemma which givdinotropic action (Chang et al., 1981). Park *et al.* (1984) suggested that the positive inotropic effect of higenamine might be elicited by stimulating the cardiac adrenoreceptors. The effects of higenamine on various cardiovascular syh detection limit. The present report describes a simple and sensitive assay method for the analysis of higenamine in biological fluids by using gas chromatography/mass spectrometry.

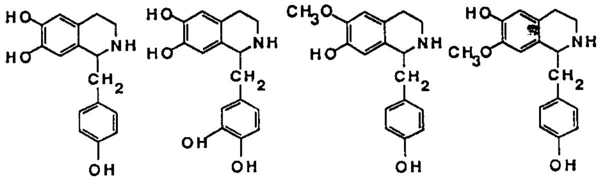
1 **2** **3** **4**

Fig. 1. Structures of Higenamine (**1**), Tetrahydropapaveroline (**2**), Coclaurine (**3**), Isococlaurine (**4**).

The duration of cardiovascular activities of higenamine was observed quite short (5-8 min at the dose of 2-100 μg/kg i.v.) and the short duration of action can be postulated that it, as other physiological catechol amines, might be easily metabolized by catechol O-methyl transferase (COMT) in liver (Kim *et al.*, 1986).

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Moreover, various isoquinolines with -OH groups at C_6 and C_7 positions were observed to be methylated with rat COMT *in vitro*. (S)-Higenamine (**1**, norcoclaurine), on methylation with rat COMT *in vitro*, afforded primarily (S)-coclaurine (**3**), whereas (R)-higenamine afforded mostly the 7-ether isomer, (R)-isococlaurine (**4**) (Rozwadowska *et al.*, 1988 and Sekine *et al.*, 1990). The detection of two presumed metabolites of higenamine is also described in this report from rat urine after the oral administration of (±)-higenamine.

MATERIALS AND METHODS

Reagents

(±)-Higenamine was synthesized according to the previously reported method (Chang *et al.*, 1984) and coclaurine was kindly gifted by Prof. M. Zenk of Pharmazeutische Biologie, University of Munich, Germany. Amberlite XAD-2 resin (particle size; 0.15-0.2 mm) was purchased from Serva Co.(Heidelberg, F.R.G.) and β-glucuronidase and arylsulfatase were from Boehringer Mannheim GmbH Co. Inc. (Mannheim, F.R.G.). (±)-Tetrahydropapaveroline-HBr(THP), N-methyl-N-(trimethyl-silyl)-trifluoroacetamide (MSTFA) and N-methylbis-trifluoroacetamide(MBTFA) were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). All other chemicals used were reagent grade. To protect the decomposition of higenamine and THP, the stock solutions were prepared in 5.0mM HCl containing 3.0 mM dithioerythreitol. All standard solutions were stored at 4℃.

Animals

Adult male Sprague-Dawley rats weighing 300± 50g were purchased and housed in an air conditioned room having a 12 : 12 hour light/dark cycle and were acclimatized to the animal facility and environments for 1 week before use. Tap water and commercial chow(Sam Yang Co., Seoul Korea) were given ad libitum.

Blood was collected from the rat femoral artery into the heparinized polyethylene tube (PE-50) and centrifuged at 500g for 15min to obtain plasma. Rat bile duct was canulated to collect bile and the metabolic cages (Nalgene Co., N.Y., U.S.A.) were used for the collection of urine. For the metabolism study, urine was collected for 24 hrs after the oral administration of 20 mg/kg of higenamine.

Extraction and Derivatization

For the analysis of higenamine and its metabolites in GC/MS, an extraction and derivatization procedure was set up as follows (Scheme 1). A XAD-2 resin (1 g) column activated with acetone, methanol and distilled water was prepared in a pasteur pipette (0.6× 2 cm). Urine or bile sample was incubated with 50 μl of β-glucuronidase/arylsulfatase (5.5 and, 2.6 Unit/ml each) at 50℃ for 1hr just before loading to XAD-2 column. Ascorbic acid (0.5 mM, 25 μl) was added to the biological samples to protect higenamine and THP from oxidation. After the addition of ascorbic acid (0.5 mM, 25 μl) and tetrahydropapaveroline (**2**, THP, 70 μl/ml MeOH, 10 μl) asan internal standard to an aliquot of 500 μl of the urine, 200 μl of the bile or 200 μl of plasma, the mixture was applied to the preconditioned XAD-2 column and the column was washed with distilled water (3 ml). The analytes were collected by elution of 1 ml of ethanol five times and ethanol was evaporated to dryness *in vacuo*. After evaporation of the solvent, the residue was allowed to be thoroughly dried in vacuum desiccator over P_2O_5 /KOH for 30 min and followed by derivatizing with 25 μl of methyl orange and 40 μl of MSTFA at 80℃ for 10 min and then with 5 μl of MBTFA at 80℃ for 20 min. The 2 μl of sample was injected into the following GC/MS system.

Biological fluids (bile 200 μl, plasma 200 μl, urine 500 μl)

- addition of ascorbic acid (0.5 mM, 25 μl) and THP (70 μl/ml MeOH 10 μl)
- enzymatic hydrolysis (urine, bile only) [β-glucuronidase/arylsulfatase (5.5. and 2.6 Unit each/ml) 50℃ 1 hr]
- cool

XAD-2 column

- wash with 3 ml distilled water
- elute with 5 ml EtOH

EtOH eluate

- evaporate in vacuo
- dry in vacuum (P_2O_5/KOH, 30 min)

Derivatization

- Methyl orange 25 μl
- MSTFA 40 μl, 80℃ 10 min
- MBTFA 5 μl, 80℃ 20 min

GC/MSD analysis

Scheme 1. Extraction and derivatization procedure of higenamine from biological fluids.

Analysis with Gas Chromatography/Electron Impact-Mass Spectrometry (GC/EI-MS)

A GC/EI-MS system (5890A/5970B Model, Hewlett Packard Co., CA, U.S.A.) with cross-linked methylsilicone capillary column (SE-30, length: 17 m, inner diameter: 0.2 mm, film thickness: 0.11 µl) was used. The flow rate of carrier gas (helium) was 0.83 ml/min. The temperature of injector and transferline was 280°C and 300°C, respectively. Oven temperature was programmed by increasing 10°C per minute from 180°C to 300°C. Injection mode was split. Higenamine was quantitated by the selected ion monitoring(SIM) mode using THP as an internal standard. The selected ion of THP and that of higenamine were m/z 404 with the retention time of 16.90 min and 15.90 min, respectively. The each scan mass spectrum for higenamine, THP and two metabolites(with the retention times of 14.71 min and 15.73 min) was taken by EI-MS and the presumed metabolites of higenamine were identified as coclaurine and isococlaurine by comparison with EI-mass spectrum.

RESULTS AND DISCUSSION

The analytical method of higenamine (**1**) in biological fluids, such as bile, plasma and urine of rat, using GC/MS was briefly described in Scheme 1. For the quantitative analysis of higenamine in biological samples, THP (**2**) which has similar chemical structure with higenamine was employed as an internal standard during the concentration and purification procedure as well as during the GC/MS analysis. Solid phase extraction with C_{18} was employed for the extraction and

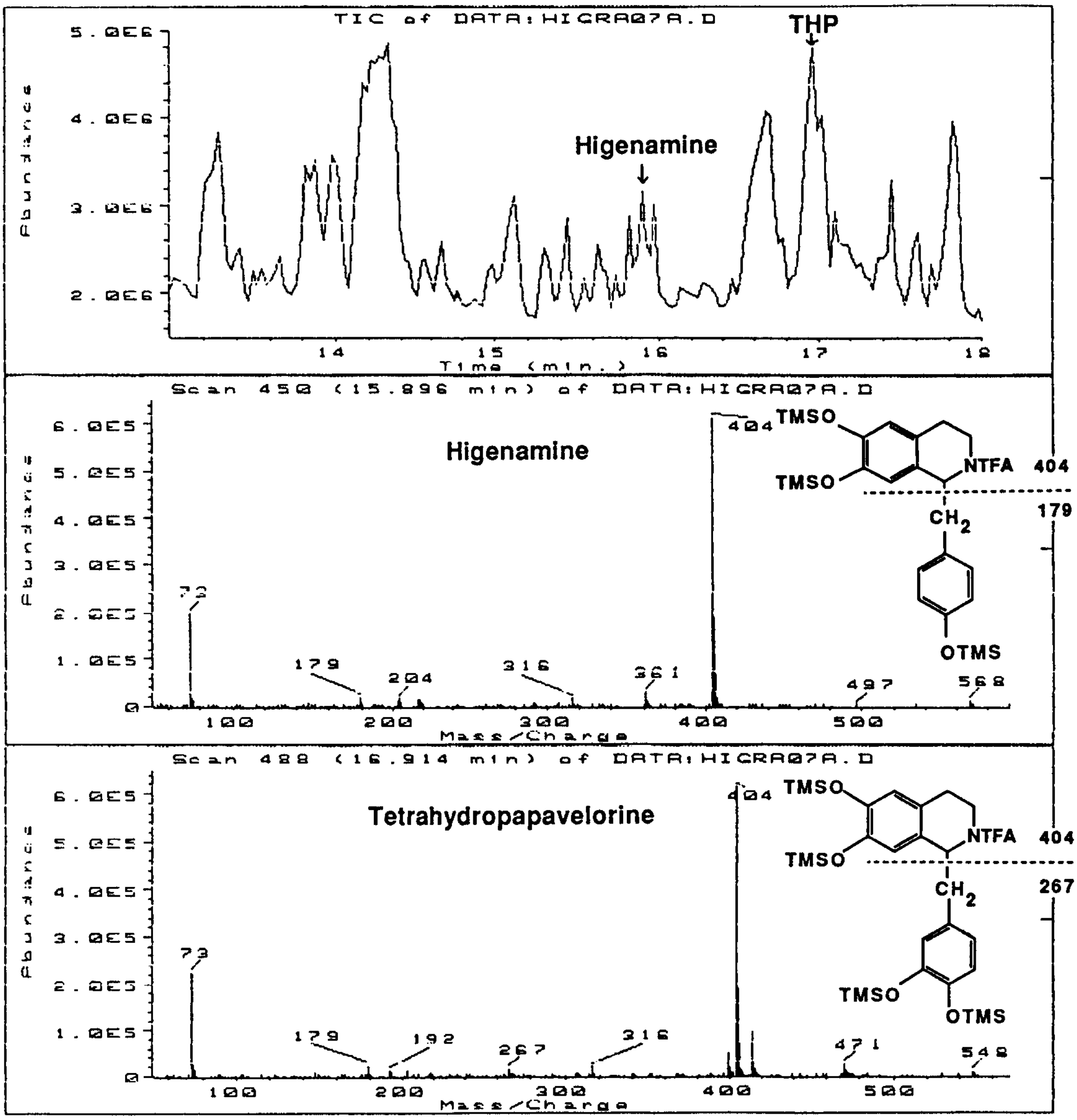

Fig. 2. Total ion chromatogram and EI spectrum of higenamine and tetrahydro-papaveroline extracted from rat urine spiked with higenamine.

concentration of higenamine (Park *et al.*, 1987) and THP (Cashaw *et al.*, 1987). However, the yield of extraction and/or the reproducibility was not satisfactory. When we examined various solid phase adsorbents such as XAD-2, Sep-Pak C_{18}, Bond-Elute C_{18} silica and florisil, recoveries were 95.7%, 48.1%, 34%, 3.2%, 5%, respectively. XAD-2 resin showed the best result for the extraction of higenamine and its metaolites. Higenamine and its possible metabolites from the biloigical samples were concentrated on XAD-2 resin and eluted with ethanol. Because the possible implication of glucuronide and/or sulfate conjugation caused on the hydroxyl groups of higenamine structure in vivo, bile or urine sample was subjected to the enzymatic hydrolysis process with β-glucuronidase and arylsulfatase prior to the extraction. Fig. 2. illustrates the chromatographic separations of higenamine and THP obtained from rat urine spiked with higenamine. The retention times of the higenamine and THP were 15.90 min and 16.90 min, respectively. The base peaks of both higenamine and THP were revealed m/z 404 ion in scan mass spectrum. When the higenamine (1 ng/ml and 10 ng/ml) were spiked to rat bile, plasma and

Table 1. Recovery of higenamine in biological samples

Sample	Recovery (%)*
Bile	97.6 ± 0.25
Plasma	94.8 ± 0.85
Urine	94.9 ± 0.50

*All data were the mean± S.D. of triplicate.

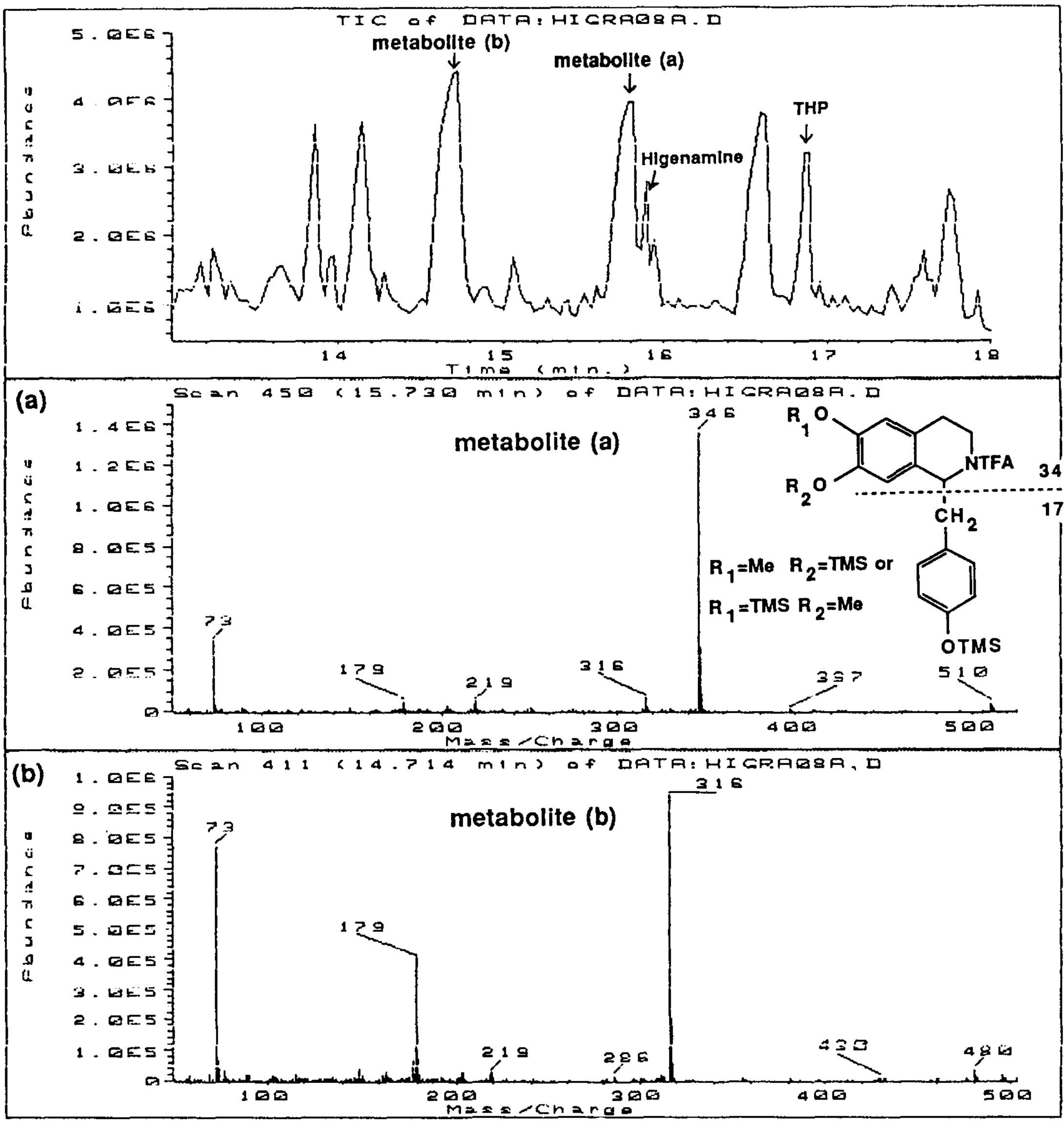

Fig. 3. Total ion chromatogram and EI spectrum of rat urine after the oral administration of higenamine (20 mg/kg).

urine samples, the recoveries from the bile, plasma, and urine samples were 97.6%, 94.8%, and 94.9%, respectively, as shown in Table I. The results implicated that the present extraction and concentration procedure is quite acceptable for the analysis of higenamine in biological fluids. The detection limit of higenamine was also as low as 0.1 ng/ml from all the bile, plasma and urine samples based on the signal to noise ratio of three.

Because higenamine possesses catechol moiety in the structure, it might be metabolized by catechol-O-methyltransferase (COMT) *in vivo* as mentioned previously. After the oral administration of higenamine (20 mg/kg) to rats housed in metabolic cages, the urine samples were collected and the total ion chromatogram and scan mass spectrum were obtained (Fig. 3). Higenamine and THP, an internal standard, were observed at the retention time of 15.90 min and 16.90 min, respectively, as in Fig. 2 and the scan spectrum of higenamine and THP were well matched with those of standard higenamine and THP. The examination of the total ion chromatogram revealed that two peaks with the retention times of 14.71 min (metabolite **b**) and 15.73 min (metabolite **a**) were also detected in addition to the peaks of higenamine and THP as shown in Fig. 3.

The analysis of the fragment ions in the scan mass spectrum of metabolite a (Fig. 3a) suggested that the new peak should be that of one of the mono-methylated higenamine. The methylation should be located in one of the hydroxyl groups (either C_6-OH or C_7-OH) at the tetrahydroisoquinoline nucleus. The total ion chromatogram and the scan mass spectrum were also taken with the mixture of higenamine, THP and coclaurine (C_6-O-methyl higenamine), which is one of the predicted mono-methylated higenamine metabolites. The retention time (15.73 min) and the scan mass spectrum of coclaurine coincided with those of Fig. 3a. So, we assumed that the monomethylated metabolite of higenamine(metabolite **a**) was coclaurine [3, 1-(p-hydroxybenzyl)-6-methoxy-7-hydroxy-1,2,3,4-tetrahydroisoquinoline]. When comparing the scan mass spectrum of metabolite a and metabolite **b** (Fig. 3a and Fig. 3b), it was noted that the base peak with m/z 316 and a peak with m/z 480 in Fig. 3b were 30 mass unit less than the peaks with m/z 346 (the base peak) and mz/ 510(M^+-Me) of Fig. 3a. In addition, both Fig. 3a and 3b showed peaks with m/z 219(316-TFA), 179(4'-hydroxybenzyl unit) and 73(TMS) indicating that metabolite **b** should have 4'-hydroxy benzyl substitution at C_1 with the 1,2,3,4-tetrahydroisoquinoline structure the same as metabolite **a**. And the tetrahydroisoquinoline unit of metabolite **b** was considered to have one methoxyl and one hydroxyl group as that of metabolite **a**, however, the TMS derivative of metabolite **b** might lose two methyl units (m/z 30) easier than that of metabolite **a**. With the above observations, we assumed metabolite **b** as isococlaurine [4,1-(p-hydroxybenzyl)-6-hydroxy-7-methoxy-1,2,3,4-tetrahydroisoquinoline]. Sekine *et al.* (1990) reported that higenamine was monomethylated with rat liver COMT *in vitro*, and in addition with high stereoselective manner; the O-methylation was observed with (−)-(S)- and (+)-(R)-higenamine, affording 80% of 6-O-methylated higenamine and 20% of the 7-O-methylated isomers from the former and the reversed proportion of the 6-O or 7-O-methylated isomers from the latter (Sekine *et al.*, 1990). Our observation confirmed that higenamine is also methylated with COMT *in vivo* and the short duration of action of higenamine (after i.v. injection) can at least partly be explained by the metabolic O-methylation of higenamine *in vivo*.

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