Determination of Blood Concentration of Higenamine by High Pressure Liquid Chromatography

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Abstract □ A procedure utilizing high pressure liquid chromatography coupled with UV detection is described for the determination of blood concentration of higenamine. Deproteinized serum was pretreated with C_{18} (Sep-pak C_{18} cartridge) and the 70% EtOH eluent was applied onto a reversed-phase column (μ Bondapak C_{18}) with a 15% acetonitrile in 0.05 N NaH_2PO_4-trichloroacetic acid mixed buffer (pH 2.8) as a mobile phase. With the UV detection at 232 nm, the retention times of higenamine and 1,2,3,4-tetrahydropapaveroline, an internal standard, were 5.2 min and 3.9 min respectively. The blood concentration of higenamine was measured at regular intervals after *i.v.* injection of higenamine to rabbit. A drastic decrease in higenamine concentration to 30% of the maximum value obtained immediately after the injection, was observed during the first 1-2 min period and thereafter the rate of decrease was slowed down. The analytical result seemed to coincide with the pharmacological effect of higenamine exerting the maximum chronotropic and hypotensive effect at the completion of the injections which were progressively recovered.

Keywords □ Higenamine, 1,2,3,4-tetrahydropapaveroline, HPLC blood concentration.

Higenamine is a cardiac principle of Aconite root which has long been used as cardiotonic, diuretic and analgesic in Chinese herbal medicine[1,2]. It was also reported to be isolated from *Nelumbo nucifera*[3] and *Annona squamosa*[4]. The structural identification of higenamine by Kosuge and Yokota[5] as *dl*-demethylcoclaurine, 1-(4′-hydroxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, and the subsequent developments of total synthesis of higenamine[4,6,7] made it possible to investigate various biological activities.

Higenamine, *in vitro*, exerted cardiotonic effect, both chronotropic and inotropic, and stimulated adenylcyclase activity and increased the plasma concentration of cyclic AMP[8,9] and was reported to increase cardiac output and heart rate and decrease blood pressure and the systemic vascular resistance when given intravenously to rabbit or dog. These biochemical and pharmacological activities of higenamine were believed to be exerted through the stimulation of adrenergic β_1 and β_2 receptors since the activities were observed to be inhibited by β_1 and β_2 adrenergic blockers such as pindolol, propranolol and atenolol[10-15]. In addition, the duration of activities was quite short possibly because of its rapid transformation to inert metabolites by COMT with which catecholamines are rapidly inactivated[15-17].

This paper describes the analytical method of higenamine by HPLC coupled with a UV detector. The changes in blood concentration after *i.v.* injection of higenamine to rabbit was determined and compared with the pharmacological effects observed with the same dose.

EXPERIMENTAL METHODS

Apparatus

The chromatographic system consisted of a Model 6000 A solvent delivery system, a Model U6K universal injector, a 8×100 mm Radial μ Bondapak C_{18} column which was protected by a guard column of 37-56 μm C_{18} corasil, 4×23 mm, a Model 450 variable wavelength detector and a Model 730 data module from Waters Assoc.(U.S.A.). The Radial column was housed in Flow-max from Young-in Scientific Co.(Korea). All quantitation was performed by the determination of the ratio of the peak heights of higenamine and 1,2,3,4-tetrahydropapaveroline, an internal standard.

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The other instruments utilized are Sorvall RT 6000 refrigerated centrifuge, Gilford system 2600 UV visible spectrophotometer, Datascope 2000 and Datascope 2000 R EKG and pressure monitor and recorder and Bentley physiological pressure transducer.

Chemicals and solvents

Higenamine hydriodide was prepared by the method of Chang *et al.*[7] 1, 2, 3, 4-Tetrahydropapaveroline hydrobromide (THP) was purchased from Aldrich Chem. Co.(U.S.A.) For the preparation of the mobile phase, acetonitrile and the 0.05 N NaH_2PO_4-trichloroacetic acid mixed buffer (pH 2.8) were filtered through a 0.5 μm millipore FH-filter and a 0.45 μm millipore HA-filter respectively before mixing. And the degassing of the mobile phase (15% acetonitrile in the above buffer) was achieved by purging with N_2 gas and then sonicating for 5 min.

Animals

Healthy male rabbits (1.8-2.5 Kg) were purchased and used after 2-3 days of the adjustment period.

Blood sample collection

A three-way tube (Vermed) was connected to a Argyle medicut (Division of Sherwood Medical) which was inserted to the femoral artery of a rabbit locally anesthesized with 1% lidocaine. Appropriate amount of higenamine dissolved in saline (16 mg/1-2 m*l*) was slowly injected into the left ear vein. Blood samples (3 m*l* each) were collected right after the completion of the injection and then at specified time intervals from the three way tube.

Treatment of the blood samples

The coagulated 3 m*l* blood sample was mixed with 1.8 m*l* of 0.05 N phosphate buffer (pH 5.0) containing 0.9% NaCl (PBS-pH 5.0), centrifuged at 1,500g for 20 min at 4℃ and 3 m*l* of the supernatant was mixed well with 0.24 m*l* of 4 M $HClO_4$. Following centrifugation at 1,500 g for 20 min at 4℃ again, 2 m*l* of the de-proteinized supernatant was applied to the EtOH-pretreated Sep-pak C_{18} cartridge (Waters Assoc.). The cartridge was washed with 2 m*l* of PBS-pH 5.0 and then eluted with 1.5 m*l* of 70% EtOH. The 70% EtOH eluent was stored at -5℃ until analysis. No noticeable change was observed during one week period under these conditions.

Chromatographic conditions

To the 300 μl of the above 70% eluent from the Sep-pak C_{18} cartridge, was added 30 μl of 2×10^{-3} M 1, 2, 3, 4-tetrahydropapaveroline (THP) as an internal standard (final conc. of THP: 1.8×10^{-4} M). 30 μl of the aliquot was injected into the HPLC system pre-equilibrated with the mobile phase (15% acetonitrile in 0.05 N NaH_2PO_4-trichloroacetic acid mixed buffer of pH 2.8) and the mobile phase was pumped at the flow-rate of 2.0 m*l*/min with the pressure of 400-500 psi. The absorbance of the eluant was monitored at 232 nm.

Measurement of the heart rate and the arterial blood pressure

After local infiltration of 1% lidocaine, right femoral arterial cannulation was performed with Argyle Medicut as was described for blood sample collection. A EKG monitor was connected to the electrodes (Arbo Softy) which were attached to chest and abdomen and a pressure monitor was connected to the cannulation apparatus via a transducer. An appropriate amount of higenamine dissolved in saline (16 mg/1-2 m*l*) was slowly injected into the left ear marginal vein and the continuous monitoring and recording were maintained for 60 min. The digital recording marked systolic, mean and diastolic blood pressure in mmHg. The heart rate, however, was calculated from the electrocardiogram instead of digital recording which was incorrect due to profound tachycardia.

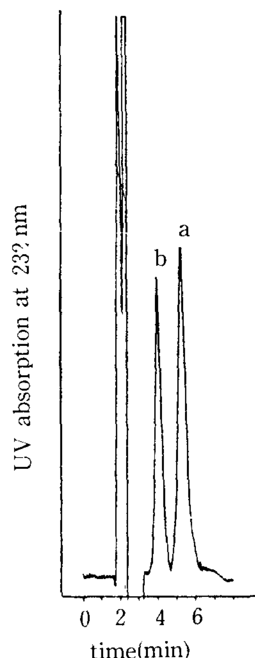

Fig.1. HPLC chromatogram obtained with 30 μl of 200 $\mu g/ml$ solution of higenamine in 70% EtOH containing 1.8×10^{-4}M THP at the specified conditions described in the experimental section.

a: higenamine, b: THP(an internal standard)

RESULTS AND DISCUSSION

Higenamine was reported to increase the cardiac output and the heart rate and decrease the blood pressure and the systemic vascular resistance in rabbit. However the duration of action of higenamine was observed quite short (5-8 min at the dose of 2-100 μg/kg) and this short duration of action was supposed that it, as other physiological catecholamines, was easily metabolized by catechol O-methyl transferase (COMT) in the liver[15). If the short duration of action of higenamine was mainly due to its rapid transformation to it's metabolites in the liver, the blood concentration of higenamine should be reduced at such a rate that can explain rather short activities of higenamine. The possible unstability of higenamine itself at the physiological pH ranges was not excluded also as one of the causes, since higenamine was described very unstable in basic medium having a half life of 45 min at pH 8.0 and less than 10 min at pH 9.0 at room temp[5).

The high pressure liquid chromatographic technique has been widely applied for the analysis of various catecholamines[18-24) which have a common chemical structural moiety and so have similar physico-chemical properties with higenamine. Reversed-phase chromatography systems suitable for the separation of catecholamines have been extensively studied and a reversed-phase column was also adopted to screen plant extracts for cardiotonic amines[25).

Fig.1 shows the chromatogram obtained from a standard solution of higenamine with a reversed-phase column (μ Bondapak C_{18}). 1, 2, 3, 4-Tetrahydropapaveroline (THP) was chosen as an internal standard and the parameters are shown in Table I. The parameters were within the reasonable range for the massive sample screenings. Fig.2 shows the standard curve obtained with the six different concentrations of higenamine; 25, 50, 100, 150, 200 and 250 μg/ml in 70% EtOH. There was a linear relationship between the higenamine concentration and the peak height which was described as

Table I. Parameters of the chromatographic conditions shown in Fig.1 for higenamine and THP.

	retention time (min)	capacity ratio	resolution factor
higenamine	5.2	3.7	1.35
THP	3.9	3.7	0.78

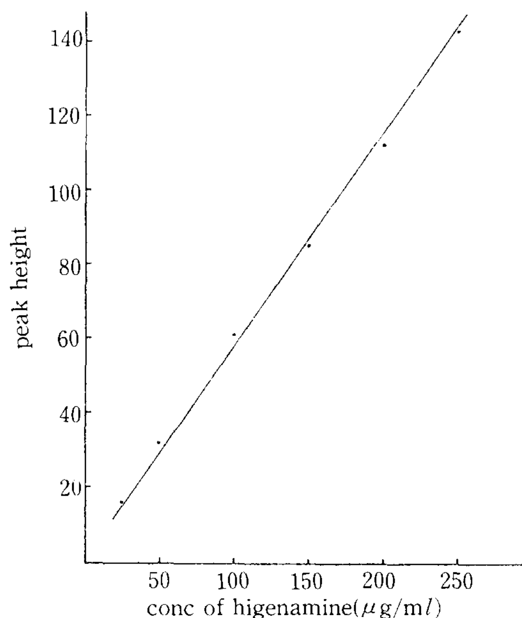

Fig.2. Standard curve for higenamine solution in 70% EtOH.

the % ratio of the peak height of higenamine to that of THP.

Higenamine solutions (5×10^{-5} M for UV detection and 5×10^{-4} M for HPLC analysis) prepared in phosphate buffer (pH 6.0, 7.0, 8.0 and 9.0) were stored at room temperature and the concentration changes were measured with UV and HPLC. No significant changes were observed at all the pH buffer solutions tested during the three hour period which suggested that higenamine itself was reasonably stable at the physiological pH ranges. There were also no significant changes observed at pH 6.0, 7.0 and 8.0 during the 24 hrs period, however at pH 9.0 the UV absorption of higenamine at either 232 and 285 nm decreased to 60% of the initial absorption.

Higenamine concentration in blood was assayed after a series of the purification steps as shown in scheme 1. Most of the interferring materials for the analysis were removed by the adsorption to and then, by the elution from Sep-pak C_{18} following the deproteinization of the serum with perchloric acid. A calibration graph (Fig.3) was obtained with blood samples (3 ml) to which 125, 150, 250, 300 and 500 μg of higenamine was added with the 1.8 ml of PBS-pH 5.0. Each data obtained from the HPLC analysis was applied to Fig.2 and the higenamine concentration in the final analytical solution was determined to get Fig.

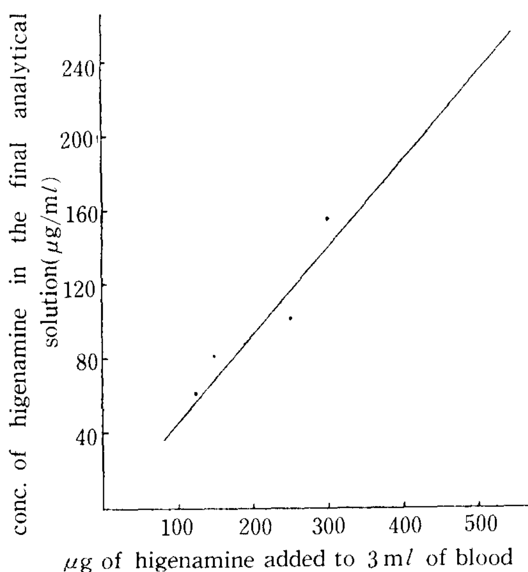

Fig.3. Calibration graph for the determination of higenamine concentration in blood.

3. With the Fig.2 and Fig.3, the higenamine concentration in various blood sample was estimated from the data obtained for higenamine by the HPLC analysis.

Higenamine was *i.v.* injected into a rabbit with a dosage of 10 mg/kg or 40 mg/kg of body weight. The blood samples were collected immediately after the completion of the injection and at specified time intervals. Each blood sample was

Blood, 3 ml
- stand for 30 min in an ice bath
- add 1.8 ml of PBS-pH 5.0, mix well
- centrifuge at 1,500g, 20 min, 4℃

Supernatant, 3 ml
- add 0.24 ml of 4 M $HClO_4$, mix well
- centrifuge at 1,500g, 20 min, 4℃

Supernatant, 2 ml
- adsorbed to Sep-pak C_{18} cartridge
- wash with 2 ml of PBS-pH 5.0
- elute with 1.5 ml of 70% EtOH

Eluent, 0.3 ml
- add 30 μl of 2×10^{-3} M THP

Injection to HPLC, 30 μl

Scheme 1. Purification of higenamine from blood.

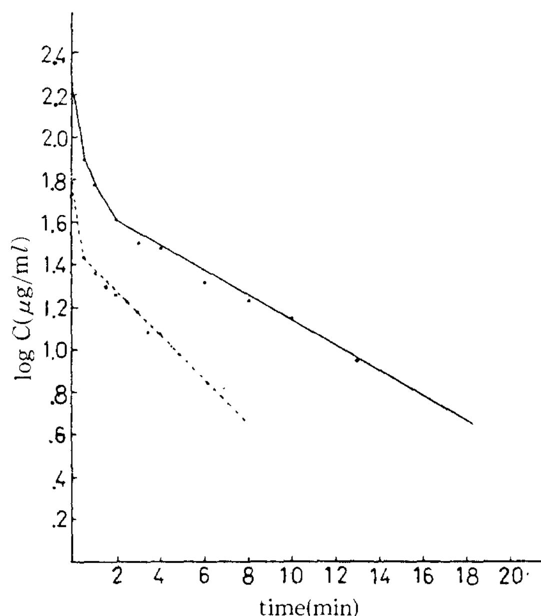

Fig.4. Change of blood higenamine concentration after *i.v.* injection of 10 mg/kg(dotted line) and 40 mg/kg(solid line).

treated as in Scheme 1 and analyzed with HPLC. The peak height for higenamine in the chromatogram was measured as a % ratio to that of THP which was added as an internal standard. And the higenamine concentration in each sample was determined by the application of the % ratio to Fig.2 and then the determined concentration of higenamine in final testing solution to Fig.3. The results are tabulated in Table II. The highest blood concentration detected immediately after the injection was 62 μg/ml (for 10 mg/kg) and 183 μg/ml(for 40 mg/kg) respectively. A drastic decrease in higenamine concentration to 30% of the maximum value was observed during the first 1-2 min period and thereafter the rate of decrease was slowed down. The higenamine concentration at 6 min(for 10 mg/kg) or 13 min(for 40 mg/kg) became as low as 10 μg/ml and 9 μg/ml respectively and the measurement of higenamine concentration beyond this period was not feasible.

Plottng the log concentration of higenamine versus time as shown in Fig.4 shows that after a short equilibration period, the rate of decrease in higenamine concentration became constant. A drastic decrease observed during the first few minutes of equilibration period might be due to the rapid distribution of higenamine to its various deposit sites. And then release of higenamine from the deposit

Table II. Changes of blood concentration of higenamine after *i.v.* injection.

time after injection (min)	10 mg/Kg[a]			40 mg/Kg[a]		
	peak height[b]	blood conc[c] (μg/ml)	% change	peak height[b]	blood conc[c] (μg/ml)	% change
0	50	62	100	149	183	100
0.5	22	27	44	62	77	42
1	18	23	37	48	59	32
1.5	16	20	32			
2	15	18	29	33	41	22
2.5	13	17	27			
3	12	15	24	26	32	17
3.5	10	12	19			
4	10	12	19	24	30	16
6	8	10	18	17	21	11
8				13	17	9
10				11	14	8
13				7	9	5
16				6	8	4
20				6	8	4

a: injected dose, b: % ratio of the peak height of higenamine to that of THP(1.8×10^{-4}M), mean value of data obtained from samples of 3 animals (10 mg/kg) or 4 animals (40 mg/kg), c: value determined by the application of the % ratio to Fig.2 and then to Fig.3.

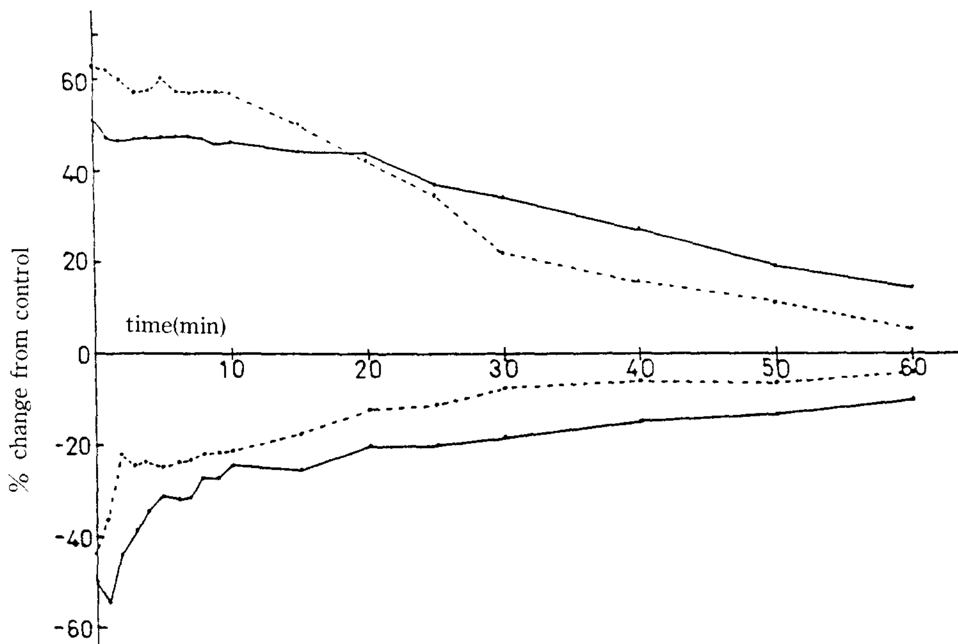

Fig.5. % Change of heart rate (upper portion of the graph) and mean arterial pressure (lower portion of the graph) after 10 mg/kg(dotted line) or 40 mg/kg(solid line) of *i.v.* injection of higenamine.

sites and elimination from blood might be continued at such a rate that the blood higenamine concentration became half of the equilibrated state within approximately 3 min (in case of 10 mg/kg injection) and 4 min (40 mg/kg injection) period. The heart rate and the arterial blood pressure were also monitored after the injection of higenamine (Fig 5) since the injected dosages (10 mg and 40 mg/kg) were 100-8,000 times of the dose level at which minimum pharmacological activities were reported[9,10,12,15]. At the completion of the injections, the heart rates and the mean arterial pressures were measured to be approximately 150% and 50% of the controls respectively which were considered as the maximum responses to observe. And thereafter, the recovery to the normal state progressed slow and was incomplete even 60 min after the injections. These results indicated that although a significant amount of higenamine was not detected by the present method beyond 6 or 13 min after the 10 or 40 mg/kg of higenamine injections respectively, sufficient concentrations of higenamine should be remained in blood to exert the pharmacological activities.

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