Determination of Higenamine in the Commercial *Aconiti Tuber* and Its Pharmaceutical Preparations

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Abstract—A cardiac principle of *Aconiti tuber*, higenamine was analyzed with a differential pluse voltammetry employing a glassy carbon electrode. The peak potentials for the oxidation of higenamine were varied depending on the pH of the media. Higenamine at the concentration of $5.4 \times 10^{-7}M$ (0.146 ppm) was easily determined using a pH 7.0 phosphate buffer. The amounts of higenamine in the crude *Aconiti tuber* and tablets containing *A. tuber* were determined by a standard addition method after extracting with methanol, partitioning with a mixture of H_2O and $CHCl_3$, and freeze-drying the aqueous layer.

Keywords—*Aconiti tuber* · higenamine · differential pulse voltammetry · glassy carbon electrode · standard addition method

Aconiti tuber is the root of *Aconitum* sp. (Ranunculaceae) which has been considered as one of the most important medicinal plants having cardiotonic, diuretic and analgesic effects.

Early pharmacological studies of *A. tuber* extracts reported that the cardiotonic activities were shown with the butanol fraction[1]. In 1976 a cardiac principle of *A. tuber*, designated as higenamine was isolated and then identified as dl-1-(4′-hydroxybenzyl)-6, 7-dihydroxy-1, 2, 3, 4-tetrahydroisoquinoline by Kosuge and Yokota[2]. In their work *A. tuber* was extracted with methanol, subsequently followed by partitioning with a mixture of H_2O and $CHCl_3$ to remove poisonous alkaloids into the chloroform layer. The water-soluble portion of the methanol extract was repeatedly subjected to separation by counter current distribution and gel filtration through sephadex LH-20, which led to the colorless plates, higenamine.

Its positive inotropic effect was markedly observed using the isolated heart of rabbit[3]. Potency of higenamine was estimated to be 1,000 times greater than that of the butanol fraction. According to the action mechanism studies of higenamine, the positive inotropic effect was elicited by influencing the movement of calcium ion through sarcolemma[4] and by stimulating cardiac adrenoceptors[5].

Aconiti tuber also contains aconitines, the poisonous alkaloids which are currently utilized in the identification and quantitative analysis on the official documents[6,7]. In order to control the quality of medicinal plants and their preparations, it is more reasonable to use pharmacologically active components as the parameters in the analyses. Considering pharmacological and action mechanism studies of higenamine, it is worthwhile to determine higenamine in the various *A. tubers* and their preparations.

In Korea, various crude *Aconiti tubers* are found in the crude drug market. They include salted and dried type, Yeoum-buja (鹽附子), two different steamed and dried types, Dangpo-buja (唐炮附子) and Kyungpo-buja (京炮附子), and simply dried type, Chooh-du (草烏頭) etc. The various pharmaceutical preparations which contain *A. tuber* as their ingredients are also manufactured in Korea. However, there has been no literature reported on the quantitative analysis of higenamine in either crude *A. tuber* or a pharmaceutical preparation which contains *A. tuber*.

This paper describes the development of an analytical methodology of higenamine with differential pulse voltammetry employing a glassy carbon electrode and its application to the determination of higenamine in *A. tuber* and its pharmaceutical preparations.

Experimental

Apparatus

All electrochemical measurements were made using a EG&G Princeton Applied Research, Model 174A polarograph equipped with a Model 303 electrode system. A glassy carbon electrode was used as a working electrode. A Ag/AgCl electrode (saturated KCl) and a platinum wire were employed as a reference electrode and a counter electrode, respectively. A Houston Instrument, Model RE 0074 Omnigraphic X-Y recorder was used for recording differential pulse voltammetric (DPV) measurements.

Aconiti Tuber and Its Pharmaceutical Preparations

Two different batches of crude *A. tuber*, Dangpo-buja (唐炮附子) were purchased from the crude drug market. Two kinds of tablets (166 mg *A. tuber*/250 mg tablet) manufactured from two different pharmaceutical companies were purchased from a local drug store.

Reagents

All chemicals were reagent grade unless otherwise specified. The various phosphate buffer solutions were prepared by dissolving either the appropriate amounts of H_3PO_4 (Ishizu Pharmaceutical Co., Japan) and KH_2PO_4 (Junsei Chemical Co., Japan) or KH_2PO_4 and $Na_2HPO_4 \cdot 12H_2O$ (Nakarai Chemicals Ltd., Japan) in distilled water at the ionic strengths of 0.200 M.[8] A solution of 0.0100 F H_2SO_4 was made by diluting concentrated H_2SO_4 (Junsei Chemical Co., Japan) in distilled water. Higenamine·HI was synthesized and recrystallized according to the method of Yun-Choi *et al*.[9] A stock solution of $6.3 \times 10^{-4}M$ higenamine (FW 271.3) was prepared by dissolving 0.5 mg of higenamine·HI(FW 399.2) in 2.00 ml of deaerated buffer and was stored under purified N_2 gas. Nitrogen gas commercially purchased (Yugene Gas Co., Seoul, Korea) was passed through a glass scrubbing tower containing acidified vanadous sulfate solution to remove oxygen traces, followed by a second scrubbing tower containing distilled water to remove acid.

Extraction and Preparation of the Test Sample Solution for Analysis

Approximately 100 g of crushed *A. tuber* (or 12~20 g of powdered tablet) were extracted three times with 200 ml (or 100 ml for powdered tablet) of refluxing methanol for 3 hrs. After the removal of the solvent under the vacuum, the residue was partitioned with 100 ml of 1 : 1 mixture of H_2O and $CHCl_3$. Subsequently, the aqueous layer was subjected to freeze-drying.

An appropriate amount of freeze-dried powder (0.0100 g~0.2000 g) was accurately weighed and transferred into a 50 ml of stoppered erlenmeyer flask. Seven ml of deaerated buffer was added to the powder to dissolve it. This solution was well stirred using a magnetic stirring bar, followed by speedy suction-filtration. The filtrate was used as a sample solution for the analysis.

Determination of Higenamine

At the beginning of DPV studies of the day, the glassy carbon working electrode was electrochemically pretreated in the deaerated pH 7.0 phosphate buffer by applying two cycles of $\mp$ 1.1 V for 4 min at each potential. Approximately 7 ml of blank (buffer) or sample solution was accurately transferred into a glass cup which was then placed in the analysis position of the Model 303 electrode system. All of working, reference and counter electrodes should be immersed in the solution of a glass cup which was further deaerated with purified nitrogen gas. For the DPV studies of higenamine the experimental parameters were usually set as follows: Initial potential; −0.100 V, scan rate; +2 mV/sec, and modulation amplitude; 25 mV. For determination of higenamine in the sample solution, the standard addition method was employed to improve accuracy.; 15 μl, 30 μl or 50 μl of 6.3×10⁻⁴ *M* higenamine stock solution was individually added into each sample solution prepared as above. Each of these standard added sample solution was also subjected to DPV. Between sample runs, the working electrode was again electrochemically pretreated by applying

$\mp$ 1.1 V for 2 min to obtain reproducible surface area of the glassy carbon electrode.

Results and Discussion

Differential Pulse Voltammetric Studies of Higenamine

The catechols are electrochemically oxidized to the corresponding *o*-quinones as follows.[10]

$$HO-\text{(ring)}-R \longrightarrow O=\text{(ring)}=R + 2H^+ + 2e^-$$

catechol   *o*-quinone
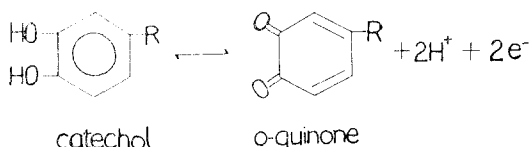
The chemical structure of higenamine contains a catechol moiety, of which electrochemical oxidation involves the hydrogen ions. An initial DPV study of higenamine at the concentration of 1.25×10⁻⁵ *M* is shown in Fig 1, using a pH 7.4 phosphate buffer. This voltammogram shows a very sensitive peak at the potential of 0.120 V which can be used for quantitative analysis by measuring its current. The DPV studies were further investigated using the various pH buffer solutions. The peak potentials (Ep) in the several pH media are summarized in Table I.
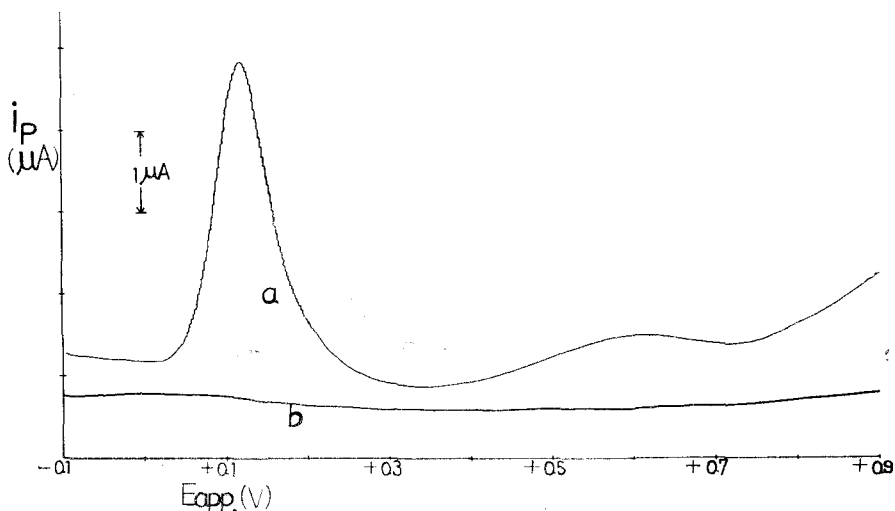

Fig. 1. Differential pulse voltammogram of higenamine.
   a; 1.25×10⁻⁵ *M* higenamine in pH 7.4 phosphate buffer
   b; pH 7.4 phosphate buffer at the ionic strength of 0.200 *M*

Table I. Effect of pH on Ep of higenamine

pH	Ep(V)	pH	Ep(V)
2.0	0.525	5.6	0.325
3.1	0.471	6.1	0.295
4.6	0.371	7.0	0.245

All of media are phosphate buffers with $\mu=0.2\ M$ except a pH 2.2 media prepared using 0.01 F H_2SO_4. Ep (V) was measured *vs.* a Ag/AgCl (sat. KCl) reference electrode.

The peak potentials were linearly shifted to the less anodic potentials as the pH of the media were increased. The peak potential change per pH unit was 64mV/pH, indicating that two protons were involved in the two electron transfer oxidation step. Higenamine was also oxidized in the 1:1 mixture of methanol and H_2O containing 0.01 M KCl with a peak appearing at 0.582$\pm$ 0.0025 V (n=3). But the peak was not symmetrical. Higenamine (demethyl coclaurine) was reported to be un-stable in the basic media.[11] In the present study a pH 7.0 phosphate buffer was preferred as a supporting electrolyte, because higenamine was easily oxidized in this media, and so its peak current was easily measured without any interference. To prevent air oxidation of higenamine a phosphate buffer should be deaerated thoroughly before making a standard solution as well as a sample solution. Even higenamine at the concentration of $5.4\times10^{-7}\ M$ gave a well defined peak in a pH 7.0 phosphate buffer as shown in Fig 2. The detection limit will be in the concentration range of $10^{-8}\ M$. To find out reproducibility of the glassy carbon electrode DPV measurements were performed four times within a day using $2.7\times10^{-6}\ M$ higenamine. The peak current was $5.27\pm0.40\ \mu A$ with % relative standard deviation of 7.6%. Since higenamine has a tendency to be adsorbed on the surface of the glassy carbon eledtrode, it should be polished with fine particle size of
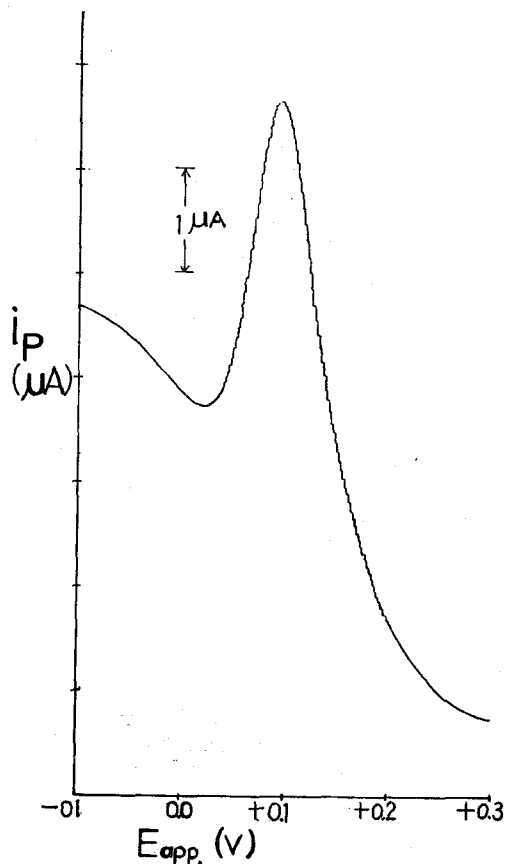

Fig. 2. Differential pulse voltammogram of higen-amine at the concentration of $5.4\times10^{-7}\ M$ in pH 7.0 phosphate buffer.

Al_2O_3 ($3\sim22.5\ \mu$) after its extensive usage.

Determination of Higenamine in *Aconiti tuber* and Tablet Containing *A. tuber*

Fig 3 (a) shows a differential pulse voltam-mogram of the sample solution prepared from well crushed *A. tuber*. In this particular analysis, 99.5 g of *A. tuber* yielded 1.8 g of the water soluble freeze-dried powder and 0.0100g of the powder was dissolved in 7 ml of pH 7.0 phosphate buffer. The pH of the filtrate was remained at 7.0. When the filtrate was scanned from −0.100 V to 0.900 V at a scan rate of 2 mV/second with a modulation amplitude of 25 mV, a well defined peak was appeared at 0.085 V
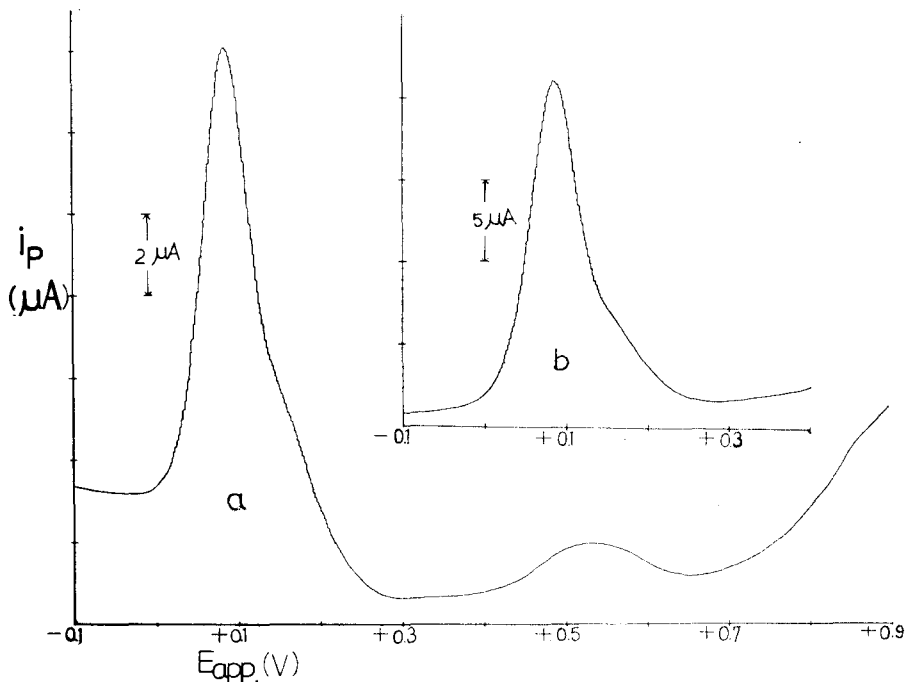
Fig. 3. Differential pulse voltammogram of the sample solution prepared from *Aconiti tuber* (Dangpo-buja).
   a; 99.5 g of *A. tuber* yielded 1.8 g of the water soluble freeze-dried powder and 0.0100 g of the powder was dissolved in 7 ml of pH 7.0 phosphate buffer
   b; 15 μl of 6.3×10⁻⁴ M higenamine was added into the solution of *A. tuber* prepared as a.

vs. a Ag/AgCl reference electrode. In order to identify this peak as the peak of higenamine, 15 μl of 6.3×10^{-4} M higenamine stock solution was added to 7 ml of fresh sample solution prepared as above. The sample solution with standard higenamine added produced the same peak at 0.085 V with increase in the peak current as shown in Fig 3 (b). As the amount of

Fig. 4. Calibration curves of higenamine by the standard addition method.

higenamine added to the sample was increased, the peak current at the same potential of 0.085 V was proportionally increased. Fig 4 shows the calibration curves of higenamine by the standard addition method. The peak current was plotted *vs.* the concentration of standard higenamine added to the sample solution of *A. tuber*. The peak current of zero M of added higenamine was obtained from the sample solution of *A. tuber* itself. The intercept at $i_p=0$ is the concentration of higenamine in the sample solution of *A. tuber*. Triplicate sets of data have different slopes which indicate the sensitivity of the glassy carbon electrode at the time of the measurements being made. The intercept that is the concentration of higenamine in the sample is $3.98 \times 10^{-6} \pm 0.035 \times 10^{-6}$ M (ave.±s.d.) with good precision. From this data, the amount of higenamine in the *A. tuber* was calculated in terms of ppm, that is micro gram of higenamine per gram of *A. tubers* (Dangpo-buja) which were
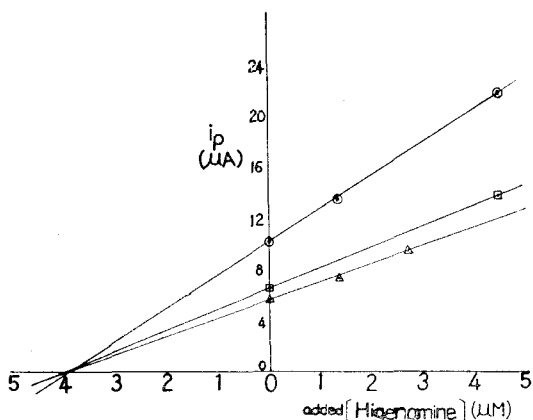
Table II. Amount of higenamine in *Aconiti tuber*, Dangpo-buja(唐炮附子)

Crude drug store	Wt. (g) of *Aconiti tuber*	Wt. (g) of freeze-dried powder	Higenamine (ppm)
(A)	99.5	1.8	13
(B)	96.4	6.8	26

purchased from two different crude drug stores.

The *A. tuber.* purchased from (B) store yielded more amount of freeze-dried powder per weight of *A. tuber* extracted. The higenamine content of *A. tuber* purchased from (B) store was almost twice more than that purchased from (A) store. They seem to be harvested in different seasons. Tablets containing *A. tuber* were also

Table III. Amount of higenamine in tablets containing *A. tuber*.

Tablet	Wt. (g) of powdered tablet	Wt. (g) of freeze-dried powder	Higenamine (ppm)
(A)	12.6	2.8	7.7
(B)	20.8	1.5	21

analyzed for the higenamine content. The analytical results are shown in Table III.

According to the manufacturers, these tablets contain 664 mg of *A. tuber* per gram of tablets. Since *A. tuber* itself contains different amount of higenamine depending upon the harvest season, higenamine contents of these tablets seem to be in a reasonable range.

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