Application of High-Performance Liquid Chromatography to Norepinephrine and Epinephrine Determination in Horse Plasma

Yoshikazu Fujii,1) Toshifumi Hirahara2) and Toyoaki Hayama2)

An experiment was carried out to establish a determination method of catecholamines (CA), which are indicators of sympathomimetic activity, in horse plasma. A high-performance liquid chromatography with the trihydroxyindole reaction commonly used in the human case was introduced, but failed to measure CA in horse plasma precisely. Then a deproteinization under low temperature upon extraction of CA and an improvement of mobile phase were conducted for modification. Following the above modification, an excellent precision was obtained by this method. Furthermore, the mean and standard deviation of normal values of CA in 10 healthy Thoroughbred horses were 184.9±56.7 pg/ml with norepinephrine and 119.4±44.9 pg/ml with epinephrine. They were close to those obtained by other methods. Thus, this modification was evaluated to be very effective in determining CA in horse plasma.

Key words: catecholamines, norepinephrine, epinephrine, horse

Of the circulating catecholamines (CA), norepinephrine (NE) is secreted from sympathetic nerve endings as neurotransmitters, whereas epinephrine (Epi) is released from the adrenal medulla as hormone.1) Therefore the concentrations of CA in human plasma are commonly used as indicators of sympathomimetic activity which respond to physical and mental stress.2–4)

In the present experiment an attempt was made to apply the concentrations of CA in horse plasma as indicators in the same manner as those in human plasma. As a first step the method of CA determination was investigated. A high-performance liquid chromatography (HPLC) with the trihydroxyindole reaction widely used in the human case5,6) to determine CA was adapted. In an effort to establish a determination method of CA in horse plasma the Shimadzu system for CA analysis7) (Shimadzu Seisakusho Co., Ltd., Kyoto) was introduced.

This method failed, however, to measure CA in horse plasma showing high values of the coefficient of variation (CV) in reproducibility, scattering of recovery rate in addition to the frequent appearance of irregularly high values of CA in plasma. Then further research and some improvement over the method were made to finally succeed in determining CA in horse plasma with precision. The modified
method and its result are to be reported in this paper.

This study was conducted on 20 Thoroughbred horses 2 to 5 years of age stabled in the Equine Research Institute and Horse Racing School, Japan Racing Association. Ten of these horses were chosen to determine a normal value of CA, since they were tamed and were not wild at the collection of blood samples. Blood samples were collected when the horses were at rest in the morning before their physical exercises. Blood was placed in a vacuum tube containing EDTA-2Na. Blood samples collected were immediately ice-chilled. Plasma samples were prepared from them by centrifugation at 3 000 rpm for 10 min at 4°C. They were frozen at −80°C and stored. The determination of CA in them was done within one month.

In addition to the flow diagram of the Shimadzu system for CA analysis, the component of a modified mobile phase and reaction agents are illustrated in Fig. 1. Upon determination an extraction of CA by alumina treatment was required and the method used for it was summarized in Fig. 2. First, 1 ml of plasma sample was deproteinized with 1 N perchloric acid, then centrifuged at 12 000 rpm for 3 min at exactly 4°C by using an L8-M ultracentrifuge (Beckman Instruments, Inc., Palo Alto, U.S.A.) for modification. The resulting clear supernate was combined with alumina, which specifically absorbs CA on the alkaline side, and extracted again with 0.4 N acetic acid. Then 50 µl aliquot of solution extracted by the procedures stated above was injected into HPLC shown in Fig. 1. The flow rate of mobile phase was 0.8 ml/min. Then NE and Epi were eluted in this order. They were detected at an excitation wavelength of 410 nm and an emission wavelength of 520 nm and integrated as peak areas. Furthermore,
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they were calculated quantitatively from the values of CA standard with which the test proceeded simultaneously.

The frequent appearance of irregularly high values of CA in plasma was seen no more by the deproteinization at low temperature, as mentioned before. The cause of irregularly high values was not clarified. It might be one possible cause that there were some substances which were influenced by a rise in temperature at the ultracentrifugation in horse plasma. Such substances might be interfering substances which had been reported to be histamine, acetylcholine and 5-hydroxytryptophan in human beings. Then, the mobile phase was improved by decreasing pH, concentrations of base and methanol, to separate a peak of NE from a peak of contamination. The above mentioned improvement caused a delay in the retention time of NE and a separation of 2 peaks, as demonstrated in Fig. 3. A stable ground line was obtained despite of high sensitivity. It was concluded that a determination of CA in horse plasma was possible by making a few modifications in the conventional method of determination of CA in horse plasma.

The improved method of determination was studied on its precision. The standard calibration curve for the determination of both NE and Epi possessed lineality up to 1 ng, as shown in Fig. 4, and continued to show similar lineality up to 10 ng. The reproducibility of concentrations of standard and CA in plasma (n=6) was satisfactory when CV values were 3.1% and 9.3% in NE and 2.8% and 7.6% in Epi, respectively. The recovery rate of CA in horse plasma was hardly scattered, being 70.8±4.7% with NE and 59.3±5.5% with Epi. The recovery rate of CA in human plasma was low, or 50–80%. The dissolution of a part of catechol was considered as a reason
for the low rate, because pH moved from acid side to alkaline side upon alumina absorption.\textsuperscript{10}

Furthermore, a normal value of CA was determined in 10 well-trained and healthy Thoroughbred horses (2-year-old). The mean and standard deviation of plasma NE level were 184.9±56.7 (94–301) pg/ml and those of plasma Epi level 119.4±44.9 (58–215) pg/ml. Hodson et al.\textsuperscript{11} and Beadle et al.\textsuperscript{12} reported these levels determined by a sensitive double isotope enzymatic method. Of these levels, plasma Epi level alone was slightly higher than that obtained in this experiment. When Hardee et al.\textsuperscript{13} determined both levels by HPLC with an electrochemical detector, they obtained almost the same results as the authors.

This modified method was evaluated to be very effective for determining CA in horse plasma precisely. The normal value determined by it was close to that estimated by some conventional methods.

**Literature Cited**


高速液体クロマトグラフィーによるノルエピネフリンおよびエピネフリン測定法の馬血漿への応用（短報）：藤井良和1）・平岡敏史2）・田山豊秋2）（1）日本中央競馬会競走馬総合研究所、2）東京農工大学農学部獣医学科家畜薬理学教室）——交感神経活動の指標とされる血漿中カテコールアミンの測定法を馬において確立するために，ヒトで広用されているトリハイドロキシインドール法による高速液体クロマトグラフィーを導入し，種々検討した。前処理における低温下での除蛋白操作および移動相の組成について改善し，良好な精度が得られた。また，健常なサラブレッド種10頭の平均値および標準偏差は，ノルエピネフリンは184.9±56.7 pg/ml，エピネフリンは119.4±44.9 pg/ml であり，他法の成績と比較してほぼ同様の値であった。以上の結果から，この改良した方法は馬血漿中カテコールアミン分析法として有用であることが示唆された。