รายงานสั้น

THE DETERMINATION OF PLASMA NOREPINEPHRINE AND EPINEPHRINE IN DAIRY COWS AND SWAMP BUFFALOES *(BUBALUS BUBALIS)* USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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Abstract

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THE DETERMINATION OF PLASMA NOREPINEPHRINE AND EPINEPHRINE IN DAIRY COWS AND SWAMP BUFFALOES *(BUBALUS BUBALIS)* USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

In the present study, we report on the level of norepinephrine and epinephrine in healthy cattle, using high-performance liquid chromatography with an electrochemical detector (HPLC-EC). Sixteen crossbred Holstein dairy cows (> 87.5% Holstein) and fifteen swamp buffaloes *(Bubalus bubalis)*, which included both sexes, were included. In the dairy cows, the plasma norepinephrine and epinephrine concentrations (mean ± SE) were 3.89 ± 0.51 nM and 0.82 + 0.12 nM, respectively. The concentrations of plasma norepinephrine and epinephrine in buffaloes (mean ± SE) were 3.75 ± 0.51 and 1.82 ± 0.45 nM, respectively.

Keywords : norepinephrine, epinephrine, swamp buffalo, crossbred dairy cow, HPLC-EC

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***ผู้รับผิดชอบบทความ**

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การวัดระดับพลาสม่าอิปิเนฟริน และนอร์อิปิเนฟรินในโคนมลูกผสม และกระบือปลัก *(Bubalus bubalis)* โดยใช้วิธี High Performance Liquid Chromatography with Electrochemical Detection

ในการศึกษาครั้งนี้ ผู้วิจัยทำการวัดระดับของอิปิเนฟริน และนอร์อิปิเนฟรินในพลาสม่า ของโคนมลูกผสมโฮลสไตน์ (> 87.5%) และกระบือ ด้วยวิธี HPLC โดยใช้หลักการของ electrochemical detection พบว่าค่าเฉลี่ยของระดับของนอร์อิปิเนฟริน และอิปิเนฟรินในพลาสม่าของโคนม เพศเมียจำนวน 16 ตัวมีค่าเท่ากับ 3.89 ± 0.51 nM และ 0.82 ± 0.12 nM ตามลำดับ ในขณะที่พบว่าค่าเฉลี่ยของระดับของนอร์อิปิเนฟริน และอิปิเนฟริน ในพลาสม่าของกระบือ จำนวน 15 ตัวมีค่าเท่ากับ 3.75 ± 0.51 nM และ 1.82 ± 0.45 nM ตามลำดับ

คำสำคัญ : อิปิเนฟริน นอร์อิปิเนฟริน กระบือ โคนมลูกผสม HPLC-EC

Introduction

Epinephrine (E), norepinephrine (NE) and dopamine (DA) are main endogenous catecholamines, which can act as neurotransmitters or as hormones. They have been implicated in several types of behaviors and central nervous system (CNS) functions. The plasma levels of catecholamines and their metabolites are required for the evaluation of neuroendocrine disorders and the role of the autonomic nervous system in several physiological and pathological situations. The concentrations of E and NE in cattle have been measured previously during fasting (Frohli and Blum, 1988), heat exposure (Katti et al., 1991) and during chronic pain (Ley et al., 1996). It has been demonstrated that plasma levels of E and NE were elevated in lactating cows subjected to heat stress (Johnson and Vanjonack, 1976; Katti et al., 1991) and in sheep and cows suffering chronic pain (Ley et al., 1992, 1996). An increase in plasma catecholamines can therefore be used as an indicator of stress. However, little information is available regarding normal plasma concentrations of E and NE in dairy cattle and buffaloes raised in tropical areas, while it has been shown that high temperatures and humidity have a great effect on both stress and the level of plasma catecholamines.

The plasma catecholamines can be measured in several different ways e.g. fluorometric, radioenzymatic, mass spectrophotometry and high-performance liquid chromatographic assay. However, high-performance liquid chromatography with an electrochemical detector (HPLC-EC) has been used widely because of its high resolution, sensitivity and because it can measure catecholamines and their metabolites simultaneously using only small samples.

In the present study, we measured the concentrations of NE and E in healthy dairy cows and buffaloes raised in a hot and humid environment, utilizing HPLC-EC. The data will be of beneficial as a database for further studies.

Materials and Methods

Animals and Sample collections

The study was performed on fifteen swamp buffaloes (4 males and 11 females) and sixteen dairy cows from the Veterinary Practice Facility, Faculty of Veterinary Science, Chulalongkorn University in Nakhon Pathom province, under the approval of the University's Institutional Animal Care and Use Committee. Blood

samples (10 ml) were collected from conscious animals, with minimal restraint, from the jugular vein and put into a test tube, containing a 200 µl mixture of ethylene glycol bis (β-aminoethyl ether)-N-N,N',N'-tetracetic acid (EGTA; 90 mg ml^{-1}) and reduced glutathione (60 mg ml^{-1}) as an antioxidant. The collected blood was centrifuged at 1500-x g, at 4 °C for 5 min. The plasma supernatant was transferred into a test tube and kept frozen at -70 °C until analyzed. The analysis for plasma catecholamines was done within 2 months after collection.

Reagents and Solutions

The analytical grade compounds were used throughout the study and all chemicals used in the study were obtained from Sigma (St. Louis, Mo, USA). Ultrapure water was filtered using a 0.22 µm pore size. Stock solutions of epinephrine (E; 0.1 mg ml^{-1}), norepinephrine hydrochloride (NE; 0.1 mg ml^{-1}) and 3,4-dihydroxy-benzyl-amine hydrobromide (DHBA; 0.05 mg ml^{-1}) were prepared by dissolving E, NE and DHBA in filtered, ultrapure water. All stock solutions were aliquoted and stored at -70°C. Standard solutions were obtained by further diluting stock solutions with filtered, ultrapure water.

Extraction procedure for epinephrine and norepinephrine

Extractions were performed according to previously published procedures by Anton and Sayre (1962). One ml of the plasma was placed in a 3-ml column containing frit (Alltech Associates Inc., Deerfield, Il, U.S.A.) along with 20 mg of acid-activated alumina (Sigma), 1-ml of 1.5 M Tris, pH 8.7; (in order to adjust pH) and 100 µl of DHBA (1000 pg), as an internal standard. NE, E and DHBA were allowed to adsorb to an acid-activated alumina by gentle mixing on a rotating mixer, for 20 min. The adsorbed alumina were then washed three times with ice-cold ultrapure water and centrifuged at 3000-x g, at 4 °C for 5 min to remove excesses water. NE, E and DHBA were eluted from the alumina, following the addition of 120 µl 0.1 M PCA (Sigma), suspended by vortex-mixing for 20 min and centrifuged at 3000-x g, at 4 °C for 5 min. The extracts were collected and saved for injection into the HPLC system. All samples from each animal were extracted twice to provide the data of E and NE in duplicate.

Chromatographic instrumentation

An HPLC system with an electrochemical detector, a glassy carbon working electrode and amperometric control (Bioanalytical systems, West Lafayette, IN, U.S.A.) was used to measure the concentration of E, NE and DHBA. A Shimadzu Model LC-10 AD pump (Kyoto, Japan) was connected to a Rheodyne (Cotati, CA, U.S.A.) injector, equipped with a 20 µl fixed loop and a 25-cm spherisorb® column, packed with 5-µm particles. The mobile phase solution was composed of 1.5 mM heptane sulfonate, 100 mM NaH_2PO_4, 1 mM Na_2EDTA and 4% methanol, adjusted to pH 4.2 with saturated citric acid. The mobile phase was filtered through a 0.22-µm filter, degassed by ultrasonic agitation and pumped at a flow-rate of 0.8 ml min^{-1}. The amperometer was set at a positive potential of 0.700 V with respect to the Ag/AgCl reference electrode, with a sensitivity of 0.2 nA. The extract (40 µl) from the plasma samples was injected into the HPLC-EC system to separate NE, E and DHBA. Data were collected and analyzed by Delta 5.0 software (Digital Solutions, Margate, QLD, Australia).

Analytical procedures

Standard solutions at different concentrations were injected into the HPLC system. The retention time was evaluated by injecting both standard catecholamine individually and by the injection of a standard mixture. The recovery of NE, E and DHBA after alumina extraction was calculated from the peak area before and after extraction. Standard solutions of the same concentration were injected repeatedly everyday for several days to verify the repeatability of the assay.

To obtain plasma calibration curves, plasma samples from either cows or buffaloes were pooled separately. Several amounts of NE and E with a fixed amount of DHBA (as an internal standard) were added to 1 ml pooled plasma. The mixtures with different concentrations of standards were then treated similarly to the plasma samples. The absolute level of catecholamine was calculated as the percentual ratio between the peak areas of the catecholamines and the corresponding internal standard, after alumina extraction, to yield a plasma calibration curve, after subtraction from the baseline endogenous E and NE. The level of E and NE in each species were presented as a mean ± S.E.

Results and Discussion

The retention time of catecholamine

The mixture of standard catecholamines which included E, NE and DHBA (internal standard), was injected directly, passing through the EC detector of the HPLC. The peaks of all substances were recorded and are shown in figure 1. The approximate retention times for E, NE and DHBA were 7.40, 11.60 and 15.00 minutes, respectively.

When the plasma pool was extracted through the alumina column, the extract was injected directly into the HPLC. E, NE and DHBA were added to the plasma extract and the peaks of catecholamine were spiked in order to find their exact location after being processed through the alumina. The retention times of all substances in the plasma extract are shown in figures 2a and 2b. The approximate retention times for E, NE and DHBA (internal standard) in the buffalo plasma pool were 7.57, 11.83 and 15.45 minutes, respectively. Slightly different values were found in the dairy cow plasma pool, 7.41, 11.65 and 15.02 minutes for E, NE and DHBA.

The recovery of catecholamines and the standard curves

The percentage recovery of E, NE and DHBA after passing the alumina extraction process were 35%, 40% and 52%. The calibration curves were obtained by adding different amounts of E and NE into the plasma pools from the dairy cows and the buffaloes and endogenous E and NE were subtracted. The r^2 values of 0.982 for NE and 0.995 for E were obtained from the dairy cow, plasma pool, standard curve. While the r^2 values of 0.989 for NE and 0.993 for E were obtained from the buffalo, plasma pool, standard curve. These calibration curves were then used to estimate the catecholamine found in this study.

Plasma norepinephrine and epinephrine levels in dairy cows and buffaloes

From the dairy cow and buffalo plasma calibration curves for NE and E, the average plasma NE and E levels in the crossbred Holstein dairy cows were 3.89 ± 0.51 nM and 0.82 ± 0.12 nM, respectively (Table 1). The average plasma NE and E levels in the swamp buffaloes were 3.75 ± 0.51 nM and 1.82 ± 0.45 nM (Table 1).

Plasma E and NE have been previously reported in both steers and dairy cows under both normal physiological and pathological states. From our results, the average plasma levels of E and NE in crossbred Holstein dairy cows (>87.5 % Holstein) were different from those obtained by other investigators (Frohli and Blum, 1988; Katti et al., 1991; Ley et al., 1996). This may possibly be due to individual laboratory differences, since different methods have varying sensitivity or specificity which leads to different values: to counter this the E:NE ratio was used for comparison. When comparing plasma E and NE in our study, with the work in steers by Frohli and Blum (1988), our data was about 2 and 3 times higher for E and NE. Sex and breed could account for such a differences. The study in Holstein dairy cattle (100% pure) during normal lactation conditions by Katti et al. (1991) revealed a much lower value, being 3 times lower in E and 7 times lower in NE. Comparing the E:NE ratio, the ratio of 0.21 in this study was lower than others (0.34-0.44).

Under certain conditions such as heat stress, both plasma E and NE concentrations and the E:NE ratio were elevated although at different magnitudes (Johnson and Vanjonack, 1976; Katti et al., 1991). From Katti et al.

Fig. 1 Typical chromatogram of a catecholamine standard mixture (10 ng ml^{-1}) containing norepinephrine (NE), epinephrine (E) and DHBA with retention times of 7.40, 11.60 and 15.00 minutes for NE, E and DHBA, respectively.

Table 1 Plasma concentrations of norepinephrine and epinephrine from dairy cows and swamp buffaloes

Species	Concentration (mean ± SE; nM)		E: NE ratio
	NE	E	
Dairy cows	3.89 ± 0.51 (16)	0.82 ± 0.12 (16)	0.21
Swamp buffaloes	3.75 ± 0.51 (15)	1.82 ± 0.45 (15)	0.49

The number of animals is given in brackets.

Fig. 2 Chromatogram of catecholamine extract from dairy cow (a) and buffalo (b) plasma pools, with added E and NE, showing retention times of NE, E and DHBA as 7.41, 11.65 and 15.02 minutes for the dairy cow plasma pool and 7.57, 11.83 and 15.45 minutes, for the buffalo plasma pool.

(1991), it was shown that of these three values, E was increased more dramatically than NE during heat exposure resulting in an increased E:NE ratio. However, after exposure to heat for 40-72 hours, the levels of E, NE and the ratio declined to normal values despite continuing heat exposure. This indicated that animals are able to acclimatize and it would be of interested to see whether heat exposure for a period longer than 72 hours could

affect plasma E and NE levels.

It is known that E and NE are mainly released from different sites; E is mainly produced by the adrenal gland, which plays a major role in hypothalamic-pituitary-adrenal axis, especially during stress (Minton, 1994). It was also demonstrated by Ley et al. (1992) that chronic stress, such as lameness (> 1 week), could elevate both E and NE and the levels remained high even after the lameness had resolved for 21 days; although E tended to decrease while the NE level was unchanged. This implied that E was more susceptible to adaptation.

From this, it is possible to conclude that the high levels of E and NE in this study could be due to prolonged heat exposure, as the animals are raised in the hot environment of Thailand. The explanation for a lower E:NE ratio could partly result from a lowering of plasma E and a high level of NE, in response to heat acclimation. Moreover, as our cows were crossbred Holstein (87.5% pure); the mechanism of heat adaptation, and the plasma catecholamine levels could be different from purebred animals.

We found a higher value of both E and the E:NE ratio in buffaloes compared with the cows. It is possible that buffaloes are more susceptible to heat, as a study in buffaloes with low heat tolerance has been reported previously (Chaiyabutr et al., 1990). Since there was no available data on plasma E and NE in swamp buffalo *(Bubalus bubalis)*, a native animal of Thailand, it was not possible to make any comparisons. Despite the difference in plasma E and NE concentrations, the ratio of E:NE was over a close range, as seen in other species (0.49 vs.0.34-0.44) (Frohli and Blum, 1988; Katti et al., 1991; Ley et al., 1992, 1996). We concluded that this study would be beneficial when used as reference values for buffaloes and cows raised in similar conditions.

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