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Measurement of Urinary Catecholamines in Small Samples for Mice

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Abstract

Introduction—Analysis of catecholamines in small samples of urine is difficult and sensitive to stress. Current techniques require pooling of samples or expensive separation by double mass spectrometry. A method for extraction of unconjugated catecholamines in 20µL urine samples has been developed using alumina extraction prior to separation by high performance liquid chromatography (HPLC) and electrochemical detection (ECD).

Methods—Three murine experiments tested the application of the procedure. In the first, collection occurred in the morning and evening prior to handling, and in the morning after three days of handling. In the second, passively obtained urine was compared to stressfully obtained urine in the same mice. Finally, basal collections were compared to urinary catecholamine levels 15 and 30 minutes into novel cage stress. Urine was extracted alongside 2,3-dihydroxybenzoic acid (DHBA) internal standard via alumina and brought to pH 8.5 with tris buffer. The mixture underwent two wash steps for depuration and eluted with perchloric acid for analysis on HPLC with ECD.

Results—This novel extraction method using low amounts of urine yielded 48% recovery in the samples and 60% recovery in the standard extraction on average. With a signal to noise ratio of 3:1, the limit of detection (LOD) of a standard is 1.2pg/mL, which allows for the detection of 3.6pg/mL in urine or 72fg in a 20µL sample. Thus resting catecholamine levels are 216 times higher than the LOD. Unconjugated norepinephrine and epinephrine levels were significantly increased 15 minutes after novel cage stress and epinephrine remained elevated after 30 minutes, but did not show significant differences when comparing collection time, handling exposure, or specific collection technique.

Discussion—The technique is an effective measure for sympathetic activity in micro samples, with a limit of detection in the attomole range for 20µL samples.

Keywords

alumina; catecholamine; epinephrine; extraction; high performance liquid chromatography; methods; mouse; norepinephrine; stress; urine

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1. Introduction

In response to stressors, the central nervous system sets forth a series of physiological responses which activate the sympathetic nervous system (Dallman et al., 1992). These changes are reflected by an increase of unconjugated catecholamines in peripheral organs (Romero, 2004) particularly norepinephrine (NE) and epinephrine (EPI) (Baum, Grunberg, & Singer, 1982). Dietary catecholamines are mostly conjugated before circulation, thus urinary or plasma catecholamines are nearly free of dietary influence and more accurately reflect catecholamines produced within the system (Peaston & Weinkove, 2004). Urinary catecholamine concentrations are ideal for the measurement of the nervous system's stress response because the collection can be passive, induces minimal stress and can be repeated throughout the course of an experiment.

To analyze urinary catecholamines using the commonly available electrochemical detection (ECD) methods, the urine must undergo preliminary extraction via alumina to remove species which cannot be separated using high performance liquid chromatography (HPLC) (Mallols, Lapasi, Camaas, & Ramis-Ramos, 1994; Von Euler & Hellner, 1951). This approach provides recovery rates of 60–70% when volumes of 5mL are available (Claeys, Schepers, Dillen, & De Potter, 1988). However, many research animals do not yield such quantities of urine at one time, which necessitates pooling of urine from the entire group.

This laboratory previously developed a solid phase extraction method over alumina for plasma samples of 20 μ L. The method described herein is a modification of that procedure to accommodate urine (Lucot, Jackson, Bernatova, & Morris, 2009). Other laboratories have used similar techniques requiring sample volumes as small as 100 μ L, but with the costly requirement of double mass spectrometer (MS) separation in addition to the LC and alumina extraction (Li, Rossi, & Fountain, 2000). One study employed a solid phase extraction method using HPLC-MS-MS detection method but pooled the samples to obtain 0.5mL aliquots (Neubecker et al., 1998). Our procedure has also been expanded to measure catecholamine concentrations in white adipose tissue (Rodwan, 2012). Like peripheral organs, the relatively low concentrations of unconjugated catecholamines in fat are shown to increase in response to stress (Migliorini, Garofalo, & Kettelhut, 1997).

The method of sample collection is an important component of the technique. Previous experiments in this laboratory have shown radically increased plasma concentrations of NE and EPI when the collection method induced stress (Lucot et al., 2009). In a study characterizing extraction methods, animals which were anesthetized before collection showed even higher NE and EPI plasma levels than after decapitation (Ueyama et al., 2003; Lucot et al., 2009). Thus, it is essential to reduce the stress induced during sample collection, both to obtain realistic baseline values and to reduce variability between individuals. Urinary collection methods in mice have not been standardized and can range from the use of metabolic cages to more invasive methods. Two common variables in the collection procedure were investigated – variation in collection time and the introduction of handling mice before experimental procedures. Finally, catecholamine levels in response to the mild stressor of a novel cage were investigated to establish the sensitivity of the technique. Mild stress has been shown to increase NE and EPI concentrations in mice.

2. Methods

2.1 Animals

Male C57BL/6 mice (N=24) (Harlan Laboratories, Indianapolis, IN), 6–8 weeks of age, were used in the present experiments. The mice were housed at 22 °C with a 12:12h dark–light cycle with light onset at 6AM. Animals used in the first (n=9) and second (n=9)

experiments were individually housed in plastic cages with wooden shavings while animals in the third experiment (n=6) were group housed in a single cage. Both standard pellet diet (Harlan Teklad, 0.5% sodium by weight) and tap water were available ad libitum. After one week of acclimatization, urine collection began. Collection and handling occurred in the same room as the home cage. All procedures were approved by the Laboratory Animal Care and Use Committee of Wright State University, Dayton, OH.

2.2. Animal Handling

Individually housed animals were removed from the home cage, placed on a towel and held by the tail. One corner of the towel was folded over the mouse and the mouse was rubbed with two fingers on either side of its body. This took place for three minutes each morning for three consecutive days.

2.3. Experimental Collections

Three experiments were conducted. The first experiment was designed to compare samples collected during the morning with samples collected during the afternoon and samples collected with or without prior handling. Urine was collected from mice (n=9) on three different days. The first collection point was at 9 AM, prior to being subjected to the handling method. The second collection point was on following day at 3PM, prior to being subjected to handling. The animals were then handled for three days at 9AM and the final collection taken on the following morning. For urine collection, each mouse was placed in a clean empty cage with water and observed until it urinated; the urine was immediately collected and stored at -80°C until extracted.

The second experiment was designed to compare collection techniques. Urine was collected from mice (n=9) on two different days. The first collection was performed as before; each mouse was placed in a clean empty cage with water and observed until it urinated. Two days later, the more common collection technique was employed in which the mice were scruffed between the shoulder blades and held over a clean weigh boat. In both cases, urine was immediately collected and stored at -80°C until extracted.

The final experiment was designed to validate the utility of the technique as an index of sympathetic nervous system activity by evaluating differences in urinary catecholamines following a mild stressor. Urine was collected from mice (n=6) at three time points in a single day. Each collection employed the scruffing and holding over the weigh boat technique. Animals had a basal urine collection at 11AM and were returned to their home cage. At 1PM, animals were removed from their home cage and placed into a novel, clean cage to induce mild stress. Mice were removed for urine collection at 15 minutes and returned to the novel cage until the final collection at 30 minutes. In each case, urine was immediately collected and stored at -80°C until extracted.

2.4. Preparation of Standard Solutions

Stock solutions of catecholamines were prepared at 0.05mg/mL of the base by dissolving the dry chemical or its salt in 0.1N HCl. Dilution to the working concentration of 0.5 $\mu\text{g/mL}$ was achieved by volumetrically diluting 1mL of the stock to 100mL with 0.1N HCl. Finally the injection standard analyzed on the HPLC for the purpose of calculating the results was created by a final volumetric dilution, adding 5mL of each catecholamine's working solution and filling to 100mL with 0.1N HCl. The injection standard had a final concentration of 2.5 $\mu\text{g/mL}$; when injected into the 20 μL loop of the HPLC, this would result in an injection of 50ng of each catecholamine (Figure 1A). The order of elution from the column for each catecholamine in the injection standard was consistent, and tracked by regular injection.

Urine samples were extracted with an internal standard of 2,3-Dihydroxybenzoic acid (DHBA), which was prepared using 1mL working solution of DHBA and volumetrically diluting to 10mL with 0.1NHCl to achieve a concentration of 50ng/mL.

Alongside every group of samples which were extracted, a standardized solution of these catecholamines was also extracted. This extraction standard was created by combining 25 μ L of each catecholamine's stock solution and diluting to 10mL volumetrically with 0.1N HCl to achieve a concentration of 125ng/mL. Extraction standard concentrations were chosen to produce a similar range of peak heights after extraction as seen in sample extractions. Extraction of this standard solution was used to determine the post-extraction recovery of each catecholamine in relation to the internal standard DHBA.

All standard and stock solutions were stored at 4C until use.

2.5. Extraction Procedure

Each urine sample was extracted via neutral alumina, activity grade I, from Sigma Chemical Co. product A-9003. Extractions took place in 1.5mL microtubes with 50mg of alumina, 200 μ L of 0.05N HCl containing 5mM sodium metabisulfite, 20 μ L urine, 40 μ L of 50ng/mL DHBA internal standard and 200 μ L of 0.2M tris buffer. Tris buffer contains Trizma Base (tris[hydromethyl]aminomathane), reagent grade, from Sigma Chemical Co. product T-1503, and distilled deionized water; this 0.2M Tris buffer was adjusted to pH 9.0 using trace metal grade 1N HCl. Additional tris buffer was carefully added to each microtube to increase the pH to 8.50 $\pm$ 0.05. Due to the small volume of supernatant, a Mettler Toledo micro electrode was used. Samples were then vortexed for one minute and centrifuged at 503xg for one minute. The supernatant was discarded and 1mL of distilled deionized water was added. After again discarding the supernatant, this washing procedure was repeated using 1mL distilled deionized water, vortexing, centrifuging, and discarding the supernatant. The catecholamines were then eluted with 200 μ L 0.1N HClO₄ containing 0.1mM sodium metabisulfite. Each sample was vortexed for 15 seconds, centrifuged for one minute and aliquoted into 0.5mL microtubes. Samples were stored at -80°C until separation and detection on HPLC/ECD. Extractions were performed on five samples at one time as well as a standard extraction. The standard extraction was performed according to the above procedure, replacing the DHBA internal standard and urine with the 125ng/mL extraction standard.

Extractions of fat samples have been performed using the same extraction procedure with additional tissue preparation steps (Rodwan, 2012). Approximately 100mg of tissue was placed into a pre-weighed 1.5mL microtube, weighed, and homogenized in 500 μ L of cold 0.4N HClO₄ acid using a sonicator for 20 seconds, three times. The samples were centrifuged in a refrigerated centrifuge at 16,200xg for ten minutes. Carefully avoiding a layer of fat along the meniscus, 400 μ L of the supernatant was transferred to a 1.5mL microtube to begin the extraction procedure. Due to the higher concentrations of catecholamines, 45 μ L of 50ng/mL DHBA internal standard was used.

2.6. Chromatography

The mobile phase consisted of a stock solution of sodium acetate (8.204g), monohydrous citric acid (11.516g), EDTA disodium salt (0.0584g), and ProClin solution (10mL) in 2L ultrapure water. The pH was adjusted to 4.5 with 1N NaOH. The mobile phase was modified from Xu and Dluzen; during analysis, methanol (35mL) and 1-octanesulfonic acid (0.1288g) were added to the stock, obtaining a final volume of 1L (1998). Samples were introduced into the system using a 20 μ L loop attached to the injection valve. Separation of analytes was accomplished using 300 $\times$ 5mm C18 column (BAS model MF-8954) stationary phase and a

mobile phase flow rate of 0.4mL/min. Measurement of constituents was via amperometric detection using a BASiLC-4C detector optimized at a potential of 700mV maintained across a glassy carbon working electrode and a Radial Flow Auxiliary electrode (BASi MR-1091). The 2.5µg/mL injection standard solution of NE, EPI, DHBA, and dopamine (DA) was injected every four samples to evaluate drift in retention time. Blank extractions, containing neither urine nor any catecholamine standard but undergoing all other procedural steps, were injected to ensure that there were no interfering contaminants. Chromatographic separation was initially recorded using a Houston Instruments Model B5117-2 strip chart set to 0.16 cm/min; DataQ Instruments Model DI-158U, an analogue to digital converter, was employed for all data shown (Figure 1).

2.7. Creatinine Assay

The MicroVue creatinine assay kit from Quidel was used to obtain the concentration of creatinine in collected urine. The procedure provided with the kit was followed. Controls of liquid creatinine in phosphoric acid were provided at the following concentrations to obtain the standard curve: 5mmol/L, 20mmol/L, 40mmol/L. Quality control standards were provided to verify that the values obtained in the laboratory were similar to those obtained by Quidel. For colorimetric detection the color reagent bottle containing picric acid (0.14%) in sodium borate and SDS and stop solution consisting of 1N sodium hydroxide were provided. The plate was analyzed at 490nm optical density using a BIOTEK™ EL808 Microplate Analyzer. Samples were related to the standards to obtain the concentration of creatinine in each (mg/mL).

2.8. Calculation

The catecholamine concentration (ng/mL) was obtained from comparison of sample peak heights from unextracted injection standard peak heights injected throughout the day (Figure 1). For relating the percent recovery of catecholamines within a sample to the percent recovery of DHBA, the ratio of recovery of each catecholamine in an extracted standard was related to that of DHBA. Using the obtained recovery ratio for each catecholamine to DHBA, the samples were multiplied by that ratio to obtain their adjusted catecholamine recovery. This value describes the concentration of each catecholamine per mL of the original sample. Data was further normalized to the creatinine concentration to obtain the ratio (ng/mg) of catecholamine to creatinine.

2.9. Statistical Analysis

Data was analyzed using STATISTICA (Statsoft, v6) data analysis software. Repeated measures t-test was performed using Statistica. An ANOVA could not be performed due to the loss of two samples at the 30 minute time point.

3. Results

The catecholamine recovery was determined using both internal and external standard recovery ratios. This method of determining the recovery was preferred to over spiking, which involves the addition of known standard concentrations of each catecholamine to each sample. In preliminary development, over spiking techniques resulted in the predictable increase in catecholamine levels and did not display signs of interference. This method was deemed unnecessary, as over spiking is usually employed for samples very near the limit of detection (LOD), and neglects the fact each catecholamine is not recovered at the same percent as DHBA. The recovery ratios were found to be fairly consistent to each individual performing the extractions. Recovery within an extraction of standard was 48% on average, while the recovery of the DHBA internal standard within sample extractions was 60% on average. This recovery is less than previous assays, but is well within the LOD which, at a

signal to noise ratio of 3:1, can detect 1.2pg in a standard injection. As little as 3.6pg/mL could be detected in the urine after extraction with 33% recovery; this translates to 72fg or 426 attomoles of NE. This procedure resulted in the greatest recovery in catecholamines; many variables were investigated such as the use of ice during the procedure, different varieties of alumina, elution volume, pH, wash methods, and the pH of the wash. Of each alteration, the time it took to complete the procedure and pH accuracy had the greatest impact on recovery; thus the constraint on pH range and number of samples extracted at the same time.

No significant differences were observed upon comparing the samples that were collected in the AM and PM, nor when comparing those of the unhandled mice to the handled mice. Similarly, there were no differences found when comparing the two collection techniques. Negative data not shown.

The experiment investigating the impact of clean cage stress on urinary catecholamines yielded the following results (Figure 2). A significant increase in NE from the basal after 15 minutes of cage stress was seen; $t(5)=2.74$, $p=.04$. The increase in NE failed to maintain significance at 30 minutes with the loss of two data points; $t(3)=2.07$, $p=.13$. A significant increase was also seen in EPI from the basal after 15 minutes of clean cage stress; $t(5)=3.78$, $p=.01$. This increase in EPI persisted after 30 minutes; $t(3)=4.7$, $p=.02$. No significant changes in DA were observed.

4. Discussion

We describe a method to reliably obtain catecholamines from samples of urine as small as 20 μ L. Measurement of urinary catecholamines is a robust measure of sympathetic nervous system activity in that it is not affected by any but highly stressful collection techniques, is not altered by prior handling and is steady during the light period. It remains sensitive as an indicator of sympathetic nervous system activity.

The extraction method permits obtaining adequate content from 20 μ L of urine for analysis and common analytical techniques are sufficiently sensitive that measurement of smaller volumes could be accomplished by adding liquid to make the volume to the 20 μ L for the extraction process. The lack of effect of collecting the sample in the morning compared with collection in the afternoon could have resulted from of an insufficient time difference during the sleep cycle of the nocturnal mice. However, the negative result does make it clear that there is considerable flexibility in employing this measurement during the normal working day. The mild differences in handling history had no effect as shown by the lack of differences in catecholamine levels between the handled and unhandled mice.

Further, sample collection procedure resulted in no differences between the two collection techniques evaluated. The urine sampled in these two experiments would reflect the catecholamine levels within the animal over the past hours since the animal's last urination. Any stressful impact that the collection may have had, would not be seen in the collections due to this dilution. However, changes in sympathetic activity can be measured within at least fifteen minutes as shown following cage change stress. The basal collection served both as a control and served to empty the bladder. The significant increases in NE and EPI suggest that the mild stressor of a novel cage was sufficient stress for detection using this method. Increased activity in sympathetic nerves is reflected by increases in NE (Udenfriend & Dairman, 1971) and general activation of the sympathetic nervous system initiates changes in EPI (Nesse, Cameron, Curtis, McCann, & Huber-Smith, 1984). The contribution of the NE nerve activity leads to baseline levels comparable to those of adrenally released EPI mirrors the relative levels humans (Eriksson, Gustafsson, & Persson, 1983). The consistent DA over these time points, along with the consistent creatinine levels, suggests

that the increases in NE and EPI are not due to an increase in kidney function or production of urine (McDonald, Goldberg, McNay, & Tuttle, 1964).

This procedure is quick, cost-effective, and can readily be implemented into any experiment which endeavors to measure sympathetic activity. Additional savings, speed, and sensitivity could be obtained through alterations to the HPLC apparatus, for example via employment of a narrow bore column (Mallett & Law, 1991; Janiszewski, Fouda, & Cole, 1995). While the sensitivity in this particular extraction procedure is a non-issue, considering catecholamine levels were 216 times the limit of detection in the lowest sample, further refinement of the procedure in this way may allow for further miniaturization of the required collection volume. This particular technique is sufficiently robust to not reflect the collection technique. The minuscule sample volume required allows for multiple analytical techniques to be employed on individual samples and may decrease the number of animals needed for an accurate measurement.

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Fig. 1. Chromatogram of peak separations for (A) 2.5 µg/mL unextracted standard solution of NE, EPI, DHBA, and DA. The chromatogram is aligned with that of (B) an extracted sample. The retention time (s) for each peak is printed by the name.

Fig. 2.

Mean weight (ng) of NE, EPI, and DA per weight creatinine (mg) in samples of urine (20 μ L) at each time point after clean cage stress. NE and EPI are significantly increased from basal levels at 15 minutes and EPI remains significantly increased at 30 minutes, while DA levels do not change significantly. * $p < .05$