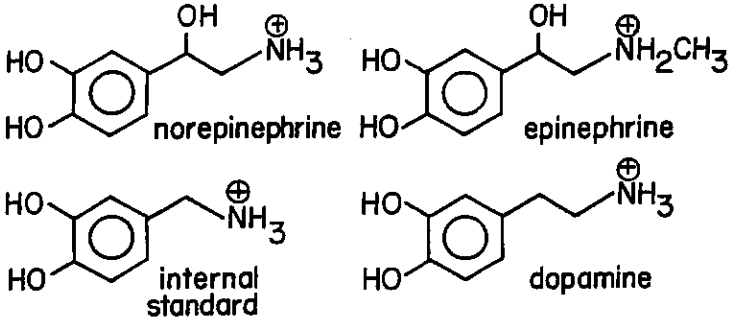
CAPSULES
notes and applications from Bioanalytical Systems, Inc.

Urinary Catecholamines by LCEC

Purpose

One of the primary pathways in mammalian tyrosine metabolism is the production of DOPA and its immediate metabolites dopamine, norepinephrine, and epinephrine.



The two crucial parameters of any catecholamine assay are its selectivity and detection limits. The first requirement stipulates either a specific detector or extensive sample clean-up steps. Most assays have required the latter approach, subjecting the sample to such steps as multiple solvent extractions, liquid-solid adsorption, hydrolysis, enzymatic radiolabeling, derivatization for GLC, etc. Generally, the less selective the detector, the more involved is the isolation scheme prior to instrumental analysis. The second parameter, very low detection limits, is an absolute necessity for catecholamines, since samples may contain concentrations less than 1 ng/mL. Clearly such considerations require a sensitive analysis system. Liquid chromatography/electrochemistry meets these requirements. The first urinary catecholamine LCEC experiments were done by BAS chemists over a decade ago.

Reference

The included mobile phase is optimized for Biophase ODS 5 µm columns (Bioanalytical Systems). Other columns may require additional fine tuning.

Reverse-phase columns containing 3 µm packing materials exhibit a broad range of applicability to the determination of catecholamines and indoleamines, and their metabolites. BAS has recently introduced a new ODS 3 µm column. The utilization of this column requires some adaptation of existing methodologies.

Equipment

Mobile Phase Filtering Kit (BAS MF-6126)
200 µL, 500 µL mechanical pipettes
100 µL glass syringe (Hamilton)
0.5 - 5.0 mL mechanical pipette (continuously adjustable)
vacuum aspirator
5 mL conical reaction vials (BAS MF-7000)
vortex mixer
reciprocal shaker
small centrifuge (BAS MF-5063)
Microfilters (BAS MF-5500)
RC 58 membranes for Microfilters (BAS MF-5658)
pH meter
25 mL beakers
Ion Exchange Kit (MF-6100)

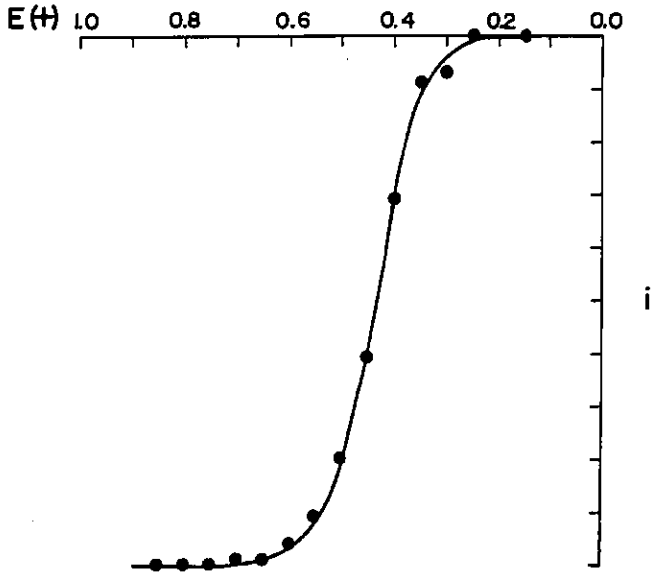


Figure 1. Current-voltage curve for repetitive injections of 20 ng of norepinephrine. All potentials are vs Ag/AgCl.

2701 Kent Ave • W. Lafayette, IN 47906 • (765) 463-4527 • FAX (765) 497-1102 • bas@bioanalytical.com

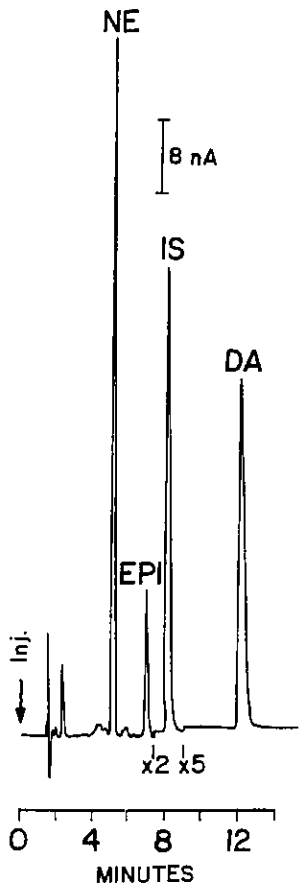


Figure 2. Representative chromatogram of urinary catecholamines. The urine sample contained 75.6 ng/mL norepinephrine, 15.6 ng/mL epinephrine, and 307 ng/mL dopamine.

Reagents

All chemicals should be reagent grade. Solvents should be HPLC grade. Water should be glass distilled and de-ionized.

2 M $(NH_4)_2SO_4$: Dissolve 66 grams ammonium sulfate in 250 mL distilled water. Refrigerate.

0.7 M H_2SO_4: Dilute 4 mL concentrated sulfuric acid in 100 mL distilled water. Use caution!

EDTA/GSH stabilizing solution: Dissolve 1 gm glutathione and 100 mg Na_2EDTA into 100 mL distilled water. Use stirring to dissolve the Na_2EDTA. Refrigerate.

Phosphate buffer, pH 7: Dissolve 4.32 g anhydrous Na_2HPO_4, 1.18 g KH_2PO_4, and 10.0 g disodium EDTA in distilled water and dilute to 1.0 liter. Adjust to pH 7.0 if necessary. Refrigerate.

3M Tris/EDTA buffer, pH 8.6: Dissolve 36.3 g Trizma base (Sigma, p/n T-1503) into 100 mL distilled water. The solution may require warming and stirring for complete dissolution. Add 5 gm Na_2EDTA and stir. When dissolved, transfer solution at room temperature to a pH meter and add 6 M HCl until the pH is 8.6. Refrigerate.

Acid washed aluminum oxide: Use as received (BAS AAO, p/n CF-8010). After opening, keep vial closed and in a dessicator between uses.

0.1 M $HClO_4$: Place 0.85 mL concentrated $HClO_4$ into a volumetric flask containing 50 mL distilled water. Mix well and dilute to 100 mL.

Cation exchange resin: This resin is treated prior to use by washing with successive volumes of 3 M HCl, 3 M sodium hydroxide, 3 M acetic acid, and 0.1 M, pH 6.5, phosphate buffer. The washing steps are carried out with gentle manual agitation. Use about twice the resin volume for each wash. A magnetic stirrer is not recommended. The pH is adjusted to 6.5 during the last wash if necessary. The plastic isolation columns are loaded just prior to analysis. Only the narrow lower portion is filled with resin. Used resin may also be recycled for future use by the above procedure.

Catecholamine standard solutions:* The following standards should be prepared in 0.1 M $HClO_4$. A composite standard solution containing norepinephrine, epinephrine and dopamine will be required, as well as a separate internal standard solution containing 3,4-dihydroxybenzylamine. A stock solution of norepinephrine, epinephrine, and dopamine is prepared by dissolving 36.5 mg norepinephrine HCl, 10.0 mg epinephrine free base, and 111.1 mg dopamine HCl in 100 mL of 0.1 M $HClO_4$. The working standard is prepared by diluting 1.0 mL of the stock solution to 50.0 mL, with 0.1 M $HClO_4$. The resulting concentrations (µg free base/mL) of catecholamines is as follows:

norepinephrine, 6.0 µg/mL; epinephrine, 2.0 µg/mL; and dopamine, 18.0 µg/mL. Refrigerate.

Internal standard solution:* Dissolve 15.8 milligrams dihydroxybenzylamine hydrobromide (DHBA) in 100 mL of 0.1 M $HClO_4$. Prepare the working internal standard solution by diluting 1.0 mL of this stock solution to 10.0 mL using 0.1 M HClO4. The resulting DHBA concentration will be 10 µg/mL (free base). Refrigerate.

** Hydrochloride, bitartrate, etc., salts of these compounds may be used but the concentrations should be corrected for the free base if concentrations are expressed in nanogram/mL or similar units. For example, if norepinephrine bitartrate • H_2O (MW = 337.3) is used instead of the hydrochloride (MW = 205.7), then 36.5 x (337.3/205.7) mg would be required to get an equivalent concentration.*

Standard urine pool: A standard urine pool is used to calibrate detector response. Collect approximately 500 milliliters of urine from healthy individuals, and acidify to pH 3 with 6 M HCl. Add to this pool 5 milliliters of the EDTA/GSH stabilizing solution. Store 20 milliliter aliquots of this pool at -35°C in glass scintillation vials until analysis. The concentration of each of the catecholamines in this pool will be determined by a standard addition procedure using the assay described below.

Conditions

System: BAS 400, BAS 460, or BAS 200 chromatograph

Mobile Phase: 91% 0.1 M MCAA, pH 3.0, 2 mM Na_2EDTA, and 300 mg/L SOS: 9% CH_3CN (v/v). Flow rate 1.5 mL/min.

Column: Biophase ODS 5 µm (250 x 4.6 mm, BAS p/n MF-6017)

Electrode: Glassy carbon

Potential: +0.65 V vs Ag/AgCl

Injection volume: 50 - 100 µL

Sample Collection and Preservation:

A defined volume of urine sample collected over a known period of time is acidified to pH 3 with 6 M HCl. The pH can be determined with sufficient accuracy with pH paper. If the analysis is not done immediatley, transfer aliquots (ca. 20 mL) to glass scintillation vials containing 150 microliters of the EDTA/GSH stabilizing solution. Store the specimens at -35°C prior to analysis for a period not to exceed two weeks.

Sample Preparation

1. For every sample to be assayed, prepare an isolation column by pouring a slurry of the prepared cation exchange resin into the plastic columns. The resin should fill the lower portion of the column as completely as possible. The bed dimensions of the cation exchange resins should be uniform. There should be no voids along the side of the columns, and the heights should be ±2 millimeters of one another.

2. Transfer 5.0 mL of urine to a 25 mL beaker. Add 50 µL of working internal standard solution and 15 mL of 0.1 M, pH 7 phosphate buffer. (If the conjugated forms are also to be quantitated, run a separate sample as follows. Add 200 µL of 12 M HCl to 5.0 mL of acidified urine (pH 3) in a 12 mL glass centrifuge tube. The resulting pH should be about 1. After heating for 30 minutes on a water bath at 85 - 95°C, allow to cool completely. Transfer to a 30 mL beaker, then add 50 µL of working inter-

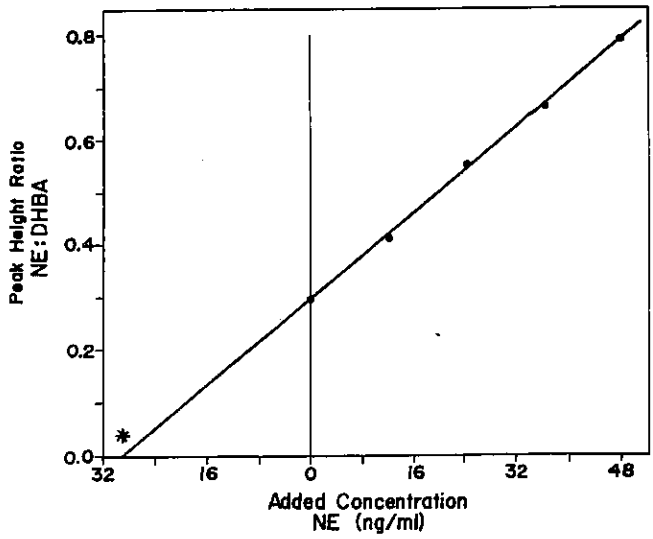


Figure 3. Determination of norepinephrine concentration in a urine pool from a plot of peak height ratios versus added concentration of norepinephrine.

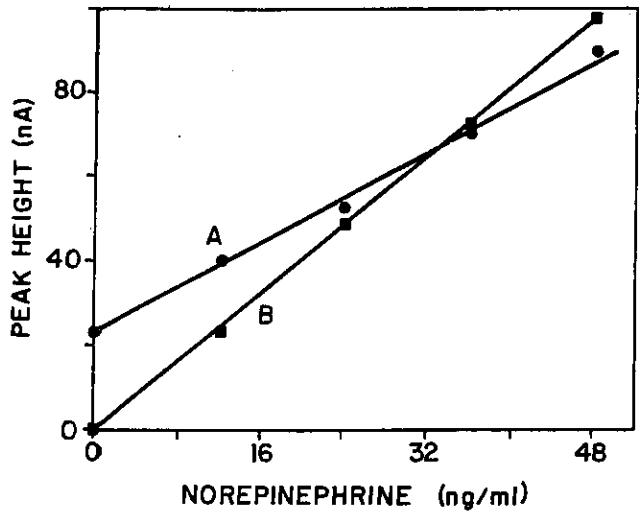


Figure 4. Linearity of response for norepinephrine in spiked urine (A) and in NE standard representing 100% recovery (B).

nal standard solution and 20 mL of phosphate buffer. Adjust pH to 6.5 using 3 M NaOH).

3. Pour the contents of the beaker onto a cation exchange isolation column. Allow the urine to drain completely. Wash the columns with about 10 mL of distilled water. Note: the pH of an overly acidified sample will not respond to the addition of the pH 7 phosphate buffer used in step 1. Recoveries will be very low. When in doubt, check the pH of the sample after adding the phosphate buffer; it should be at least 6.0. If not, titrate it to pH 6.5 with 3 M NaOH.

4. When the columns have completely drained, add 1.3 mL of 0.7 M H_2SO_4. Since less than one column volume is added, elution of the displaced amines does not occur. The subsequent addition of 2 M $(NH_4)_2SO_4$ washes the amines off the column. (When using the assay for the first time, test effluent pH with pHydrion paper. It should not be red. If it is, reduce the volume of acid to 1.0 mL. The lower barrel of the column should be completely filled with resin.)

5. Allow the columns to drain completely, then place a 5.0 mL reaction vial under the column exit. Elute the catecholamines with 4.0 mL of 2 M $(NH_4)_2SO_4$. Collect this effluent.

6. Place 50 mg of alumina in to each reaction vial.

7. Add 500 µL of the Tris/EDTA buffer to the reaction vial. Immediately thereafter, screw on the cap and shake vigorously for 8-10 minutes.

8. Remove the vials from the shaker and allow the alumina to settle.

9. Remove the supernatant by aspirating it to waste.

10. Wash the alumina once with distilled water. The alumina must be thoroughly agitated by the wash solution. This may be easily done by placing the water in a wash bottle and squirting vigorously into the reaction vial. Allow the particles to settle, then aspirate the solution to nearly dryness, without removing any alumina.

11. Add about 0.5 - 1.0 mL of water to each sample, then transfer the slurry to a Microfilter loaded with an RC-58 membrane. A disposable pasteur pipette is ideal for this transfer.

12. Place the Microfilter in a centrifuge and spin it to dryness at 1000 x g for 30 seconds.

13. Put a clean, dry receiver tube on the Microfilter and add 200 µL of 0.1 M $HClO_4$ to the Microfilter sample compartment. Vortex briefly, let stand for a few minutes, and vortex again.

14. Centrifuge the Microfilter at 1000 x g for 1 minute. The acidic extract in the receiver tube contains the catecholamine extract ready for injection into the LCEC Analyzer (F2).

Data Handling

Pooled Sample Calibration:

To accurately quantitate catecholamines in urine, it is necessary to compare the results of unknown samples to standards whose reponse on the LCEC analyzer is known.

Table 1. Standard Additions for Calibration of Urine Pool

Volumes Per Sample*				
Sample Number	Urine Pool	NE Std.	EPI Std.	DA Std.
1	5.0 mL	0.0 µL (0.0)	0.0 µL (0.0)	0.0 µL (0.0)
2	5.0 mL	10.0 µL (12)	10.0 µL (4)	10.0 µL (36)
3	5.0 mL	20.0 µL (24)	20.0 µL (8)	20.0 µL (72)
4	5.0 mL	30.0 µL (36)	30.0 µL (12)	30.0 µL (108)
5	5.0 mL	40.0 µL (48)	40.0 µL (16)	40.0 µL (144)

* These figures in parentheses represent the increase in concentration (in ng/mL) due to the spike, based on the values for the catecholamines standards given in Reagents. Use of standards of different concentration will require new values to be calculated.

Since recoveries vary very little from sample to sample, pooled specimens whose concentrations have been previously determined (usually by standard addition methods) are employed. One pooled sample is run in parallel with every group of five (or less) unknown samples; using the peak height ratios and the known concentrations in the pooled sample, the unknown concentrations may be ascertained.

Quantitation in this manner allows higher sample throughput, since only one standard is required for every five unknowns, rather than one for every unknown if standard addition techniques were used exclusively.

Urine. From a pool as directed in Reagents. Assay catecholamine concentrations in the pooled sample in the following manner.

Spike 5.0 mL aliquots of pooled urine with the aqueous catecholamine working standards listed in Reagents, according to Table 1. Add 50 µL of the working internal standard solution to each aliquot.

Determine the concentrations of each of the compounds in the urine pool by running all five samples through the procedure. Plot the peak height ratios of catecholamine to DHBA versus the catecholamine concentration added to each of the components; extrapolate to zero peak height ratio to obtain the concentration in the urine pool.

Example. A plot of peak height ratio versus added concentration of norepinephrine is shown in Figure 3. The magnitude of the x-intercept (denoted by * in Figure 3) is the norepinephrine concentration in the original urine pool; this value will be subsequently used for quantitation of unknown samples and should be retained. A least-squares fit gave a value of 28.8 ng/mL. Similar curves would be drawn for epinephrine and dopamine based on their respective peak heights and added concentrations.

Table 2. Sample Chromatographic Data for Urinary Catecholamines

Urine sample identity	peak heights (in nA) NE	EPI	DA	IS
5.0 mL unknown (not hydrolyzed)	22.1	3.1	181	100
5.0 mL unknown (hydrolyzed)	66.3	12.9	323	103
5.0 mL urine pool (not hydrolyzed)	20.9	8.6	122	101

Once calibrated, a freshly thawed urine pool sample is run through the procedure with every five unknown samples to be analyzed. A urine pool aliquot should be used for that day's analyses only. Do not refreeze for future use.

Typical Data and Calculations

Free and Total Urinary Catecholamines. A twenty-four hour urine specimen was collected and analyzed in parallel with a urine pool sample according to the procedure. Calibration of the urine pool, as described earlier in this section, gave values of 28.,8 ng/mL NE, 6.7 ng/mL EPI, 138 ng/mL DA. The chromatographic data is summarized below (the entry for IS represents the peak height of the internal standard):

Using the data of Table 2 and expressions of Table 3, the calculations are as follows. The free catecholamine concentrations are:

$$\text{NE: } \frac{22.1/100}{20.9/101} \times 28.8 = 30.7 \text{ ng/mL}$$

$$\text{EPI: } \frac{3.1/100}{8.6/101} \times 6.7 = 2.44 \text{ ng/mL}$$

$$\text{DA: } \frac{181/100}{122/101} \times 138 = 207 \text{ ng/mL}$$

Catecholamine levels are often expressed relative to the creatinine concentration or total sample excretion time as a "normalization" factor for changes in the urinary volume or the collection period of the specimen. In the previous sample, the creatinine level was 0.92 ng/mL; the volume was 1760 mL collected over 24.0 hr. The values for total NE would therefore be:

$$\text{NE (ng/mg creatinine)} = \frac{89.6}{0.92} = 97.4 \text{ ng/mg creatine}$$

$$\text{NE (ug/day)} = \frac{89.6 \times 1760 \times 24}{24 \times 10^3} = 157 \text{ mg/day}$$

Table 3. Catecholamine Equations

$$\text{catecholamine conc. (ng/mL)} = \frac{\text{pk. ht. ratio (unknown)}}{\text{pk. ht. ratio (urine pool)}} \times \text{urine pool conc. (ng/mL)}$$

$$\text{catecholamine conc. (ng/mg creatinine)} = \frac{\text{catecholamine concentration (ng/mL)}}{\text{creatinine concentration (mg/mL)}}$$

$$\text{catecholamines excreted (µg/day)} = \frac{\text{conc (ng/mL)} \times \text{sample volume (mL)} \times 24}{\text{collection period (hrs.)} \times 10^3}$$

Assay Performance

Urine samples were collected at random from a group of apparently healthy individuals. The samples were prepared and assayed according to the described procedure. Table 4 summarizes the results for a group of 9 samples.

The time required for preparation of 12 samples for injection is approximately 1.5 hours. After preparation, chromatography time for each sample is 12-15 minutes.

Recovery was measured by comparing spiked aliquots of the urine pool to direct injections of standards containing known quantities of catecholamines. Figure 4 shows the standard addition plots used to determine norepinephrine recovery.

Recovery is calculated as the ratio of the slope of line A (actual recovery) to the slope of line B (100% recovery). The calculated recoveries were; norepinephrine, 54%; epinephrine, 49%; and dopamine, 50%. It should be noted that this assay for catecholamines incorporates two safeguards against the possibility of erroneous results. First, analysis of a calibrated urine pool in conjuction with each group of samples compensates for any day-to-day variations in chromatographic conditions, detector response etc.

Secondly, an internal standard. DHBA, is included in each sample. Using peak height ratios in the calculations removes the need to consider absolute recovery as a factor in routine analysis.

The precision of the assay was determined by analyzing five replicates of a urine sample. Table 5 outlines the coefficients of variation obtained for norepinephrine, epinephrine, and dopamine. Calculations were made using the internal standard, as described.

Liquid Chromatography Electrochemistry is a highly useful tool for biomedical research. As with any instrumental technique, the precision and accuracy of the measurement depends on the instrumentation, the skill and knowledge of the operator and the integrity of the sample preparation procedure. Use of these techniques for medical diagnosis and accountability for the same rests entirely with the user of this equipment.

Table 4. Randomly Selected Urinary Catecholamine Concentrations

	NE	EPI	DA
range (ng/mL):	8-125	1.2-15.6	5-334
mean (ng/mL):	53.2	9.1	192
median (ng/mL):	46.0	10.6	177
standard deviation (ng/mL):	42.4	5.3	100

Table 5. Assay Precision for Urinary Catecholamines

	NE	EPI	DA
Coefficients of Variation	1.2%	2.7%	3.2%
Conc. Assayed (ng/mL)	59.3	11.0	372

Related BAS Publications

1. Current Separations, Vol. 2, No. 2 (1980)
2. LCEC Capsule 203 . Tissue Catecholamines
3. LCEC Capsule 204. HVA and DOPAC in Brain Tissue
4. LCEC Capsule 227. Plasma Catecholamines
5. LCEC Capsule 231. Urinary Metanephrines
6. LCEC Capsule 229. Urinary Vanilmandelic Acid
7. LCEC Capsule 154. Urinary MHPG

References

1. R.M. Riggin, P.T. Kissinger, Anal. Chem., 49(1977) 2109- 2111.

2. P. T. Kissinger, Anal. Chem., 49(1977) 883.