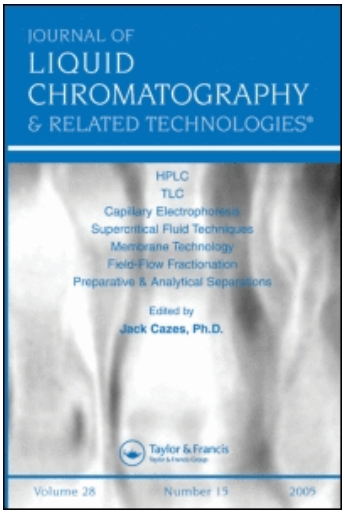

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A Simplified HPLC-ECD Technique for Measurement of Urinary Free Catecholamines

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To cite this Article Kumar, Adarsh M. , Kumar, Mahendra , Fernandez, Jesus B. , Mellman, Thomas A. and Eisdorfer, Carl(1991) 'A Simplified HPLC-ECD Technique for Measurement of Urinary Free Catecholamines', Journal of Liquid Chromatography & Related Technologies, 14: 19, 3547 — 3557
To link to this Article: DOI: 10.1080/01483919108049410
URL: http://dx.doi.org/10.1080/01483919108049410

JOURNAL OF LIQUID CHROMATOGRAPHY, 14(19), 3547-3557 (1991)

A SIMPLIFIED HPLC-ECD TECHNIQUE FOR MEASUREMENT OF URINARY FREE CATECHOLAMINES

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ABSTRACT

A simplified HPLC assay is described for quantification of free urinary catecholamines. The procedure involves exraction of catecholamines, (norepinephrine, epinephrine and dopamine) from urine, using columns filled with Biorex-70. The catecholamines from the extract were separated on a high performance liquid chromatographic system using reverse phase C18, 5 u column and determined by electrochemical detection. Integration and calculations are achieved by a data module using area ratio method with dihydroxybenzylamine as internal standard. Recovery of more than 90% was achieved for each catecholamine. A linear relationship between a wide range of concentrations and ratio of the area of amines to that of internal standard was observed. The method is simple and rapid and therefore can be used to analyze a large number of samples in one day and should prove useful in studies involving the role of catecholamines in different psychiatric disorders.

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INTRODUCTION

The catecholamines are important markers for the central and peripheral nervous systems activity. Measurements of thelevels of these biogenic amines in plasma and urine are important for the diagnosis of abnormalities in the central and peripheral noradrenergic and adrenergic functions. For example, stress induced biological responses such as increased heart rate, blood pressure and glucose levels in blood or urine are known to be mediated by catecholamines. Recent studies show that catecholamine are also involved in various psychiatric disorders (1). However, measurements of catecholamines is difficult and needs a great deal of caution since plasma and urinary catecholamines are present in extremely small amounts ranging in picogram to micrograms quantities. Plasma catecholamines represent one time measure of autonomic reactivity whereas twentyfour hours urinary catecholamines are known to represent the whole body activity through the day. Although urinary catecholamines concentration is in microgram quantity, its quantification is difficult since a number of other biogenic substances present in urine interfere in the analysis. Earlier methods of urinary catecholamine measurements included fluorometric, gas chromatography with mass spectroscopy, radioimmuno and radioenzymatic assays. Fluorometric assay involved derivitization of catecholamines to trihydroxyindoles. This derivitization was found to be difficult to control and large variations in the values were reported by different laboratories (2,3). Analysis of catecholamines by gas chromatography and mass spectrometry (GCMS) (4) is expensive, time consuming and is not suitable for routine catecholamine analysis. Radioimmunoassay of catecholamines has not been practical because of the nonspecificity of the antibodies. Radioenzymatic methods (5), where a radioactive methyl group is enzymatically transfered to the catecholamines, are specific and accurate but are lengthy, since they require purification and pre-concentration steps before the enzymatic reaction can be carried out. In recent years high performance liquid

chromatography (HPLC) methodology has become available and these methods are reported to be sensitive, specific and rapid for the quantification of catecholamines.

In earlier studies, quantification of catecholamines on HPLC was achieved by using UV or fluorometric detectors which were specific but were not very sensitive for these amines. However, HPLC equipped with electrochemical detector (HPLC-ECD) is rapid, sensitive, specific and can be used for the analysis of plasma as well as urinary catecholamines without any chemical transformation to their derivatives (6,7). Although some studies have reported the use of HPLC-ECD for quantification of urinary catecholamines (7,8), there are a number of factors that can contribute to potential errors. These factors include variations in the purification procedures for avoiding interference from other biogenic substances, use of different HPLC columns and calibration of the instrument using aqueous standards rather than the "urine" standard. It is believed that some of these factors may be responsible for variations observed in the values of urinary catecholamines and thus, not making it possible to determine the role of urinary catecholamines in psychiatric disorders. In this communication, we report a simplified procedure for the extraction of catecholamines from urine and quantification of norepinephrine (NE), epinephrine (E) and dopamine (DA), using HPLC-ECD and a C18, 5μ reverse phase catecholamine column. Calibration of the instrument has been achieved by using catecholamine-free urine spiked with known amount of amines and dihydroxybenzylamine as internal standard. Furthermore, integration of peaks by data module ensures the accuracy of the measured areas adding precision to urinary catecholamine values.

MATERIALS AND METHODS

Chemicals

Norepinephrine.HCl (NE), epinephrine (E),and dopamine.HCl (DA) were obtained from Sigma (St. Louis, MO), dihydroxy- benzylamine. HBr (DHBA) was

purchased from Waters (Milford, MA). Biorex-70 ion exchange columns were purchased from Biorad Laboratories. Sodium acetate, citric acid, dibutylamine, ethylenediaminetetra-acetate (Na_2EDTA) were obtained from Eastman Kodak Co. (Rochester, NY). Sodium octyl sulfate was purchased from Aldrich Chemical Co. (Milwaukee, WI). Methanol, HPLC grade (Mallinkrodt), was obtained from Scientific Products (Baxter Health Care Corp).

Standard Solutions

Stock solutions of 1 mg/ml of norepinephrine, epinephrine, dopamine and dihydroxybenzylamine were prepared in 10 mM Tris-EDTA, pH 6.5, and were stored at 4°C. The stock solutions were diluted with deionized water to the desired concentrations before use.

Mobile Phase

Mobile phase for HPLC was prepared fresh in HPLC grade deionized water and contained the following: 0.1M sodium acetate, 0.1 M citric acid, 0.5 mM sodium octyl sulfate, 0.15 mM disodium ethylenediamine tetraacetate (Na_2EDTA), 1.0 mM dibutylamine and 5% methanol. The solution was adjusted to pH 3.9, and was filtered under vacuum using a 0.45um filter. It was degassed at room temperature before use. A minimal mobile phase flow was maintained at 0.2 ml/min at all times to prevent clogging of the column and electrode and to maintain a reproducible separation.

Urine Collection

Urine samples were collected in containers containing 6M hydrochloric acid (1% v/v) as preservative. The samples were adjusted to pH < 2.0 and were stored frozen at -70°C until used. Usually no extra addition of HCl was found to be necessary for adjusting the pH. Urinary catecholamines are reported to be stable at -70° C and low pH for several months (9).

Catecholamine free urine (CAFU)

Urine was made catecholamine-free by adjusting its pH to 10.6 and allowing it to sit for 5 days at room temperature exposed to air and light. Its pH was then adjusted to 6.5 and centrifuged at 15,000 rpm for 10 minutes. The supernatant was passed through disposable microcolumn filled with Biorex-70. Aliquots of the eluate were stored at 0°C. The aliquots when analyzed on HPLC, showed a complete elimination of norepinephrine, epinephrine and dopamine peaks.

Urine Standard

Three milliliter catecholamine-free urine was spiked with 1 μg NE, 1 μg E and 1 μg DA and 3 ug dihydroxy- benzylamine (DHBA, internal standard). The mixture was vortexed and placed on disposable microcolumn filled with Biorex-70 and was allowed to drain completely. The column was washed three times with 10 mM Tris-EDTA buffer, pH 6.5, and bioamines were eluted with 6.0 ml elution buffer (0.6M boric acid). Eluate was vortexed and stored at 4°C. Prior to injection, eluate was diluted appropriately and 20 μl was injected for calibration of the instrument or for the quantification of amines.

Extraction of Catecholamines

Urine samples were thawed, mixed, and centrifuged at 15000 rpm for 5 minutes. The clear supernatant was brought to pH 6.5 with 0.5 M sodium hydroxide. Internal standard, 3 ug, was added to 3.0 ml of urine supernate, mixed and placed on Biorex-70 column. The column was washed off urine three times with 10mM Tris-EDTA pH 6.5. The catecholamines adsorbed on the column were eluted with 6 ml of a 0.6 M boric acid solution. Eluate was diluted appropriately in mobile phase, usually 25 to 50X, so that discernable peaks for catecohlamines are obtained by injecting a 20 ul aliquot on HPLC.

HPLC System and Procedure

High performance liquid chromatography (HPLC) system (Waters Associate Division of Millipore, Milford, MA) was used and consisted of an U6K injector, Model

590, solvent delivery system and an electrochemical detector Model 460, equipped with a glassy carbon working and auxiliary electrodes and a silver-silver chloride reference electrode, and Waters data module 740. The chromatography column (3.5mm x 15cm) certified for plasma catecholamines (Water's) packed with C_{18} hydrocarbon chain bonded to spherical 5u silica particles was used. Separation of the peaks was achieved at sensitivity set at 5nAFS and at a potential of +0.55 V with a flow rate of mobile phase kept at 0.5 ml/minute.

Before injecting the extracts of urine samples, the instrument was equilibrated to obtain an elution profile and to stabilize retention times (RT) of analytes by injecting 20 ul of aqueous standard mixture containing NE, E, DA and DHBA. Total elution times for all the analytes was 15 minutes. Calibration of the instrument was carried out by injecting 20 μl of diluted extract of catecholamine-free urine spiked with a 1 μg NE, 1 μg E, 1 ug DA and 3 μg of DHBA. A 20 μl diluted extract from each sample was used for HPLC analysis.

For validation of the assay, a series of standards ranging in concentrations from 0.25 μg to 4 μg of NE, E, DA and 1 μg of DHBA were added for one ml of catecholamine-free urine. Extracts were prepared as described above, and 20 μl of 50 fold diluted extract containing 50 - 800 pg of each of NE, E, and DA and 200 pg of DHBA was injected to determine the ratio of the area of peaks of NE, E, and DA to that of DHBA.

Calculation

Integration of the elution peaks and calculations of concentration of individual catecholamine (μg/ml) in urine were based upon the ratio of the area of NE, E and DA with respect to that of DHBA and was achieved by preprogramming the data module.

RESULTS & DISCUSSION

Typical chromatograms of aqueous standards of NE, E and DA are shown in Fig.1A. A similar chromatogram obtained from catecholamine-free urine spiked with

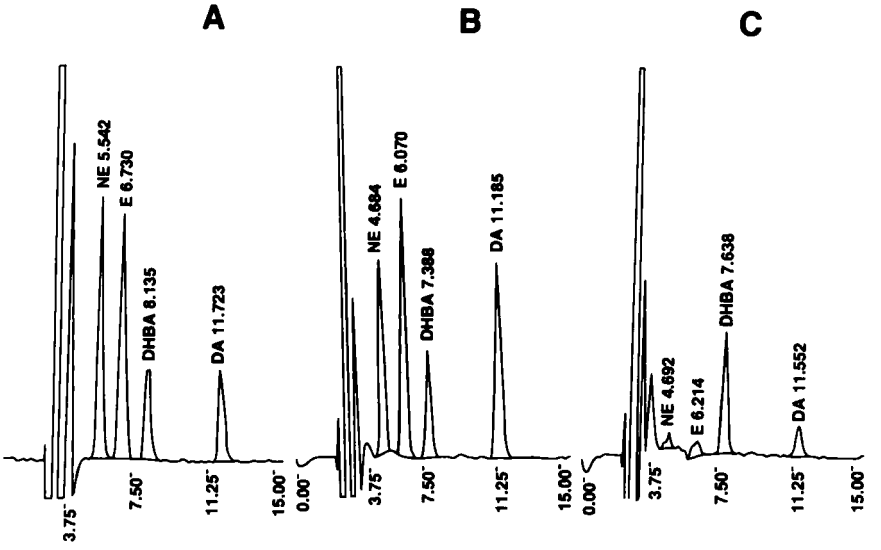

FIGURE 1: Chromatographic profiles of extracts of catecholamines (NE, E, DA) and internal standard (DHBA). Standard catecholamine aqueous mixture (A), catecholamine-free urine spiked with catecholamines (B), and a typical extract obtained from urine (C).

standard catecholamine solutions of known concentrations is presented in Fig. 1B. The retention time (RT) of NE was found to be 4.684 minutes, that of E was 6.070 minutes and that of DA was 11.185 minutes. Retention time of the internal standard, DHBA, was 7.388 minutes. The peaks shown are well separated from each other and were found to have no overlap. A typical chromatographic profile of the catecholamines extract from a sample of urine is shown in Fig 1C.

The calibration curves for catecholamines over the range of 0-4.0 ug each of NE, E, and DA / ml of CAFU or urine were found to be linear (Fig. 2). The HPLC-ECD methodology was found to be most sensitive for NE since the area of the NE peak was always greater than that of either E or DA for the same concentration.

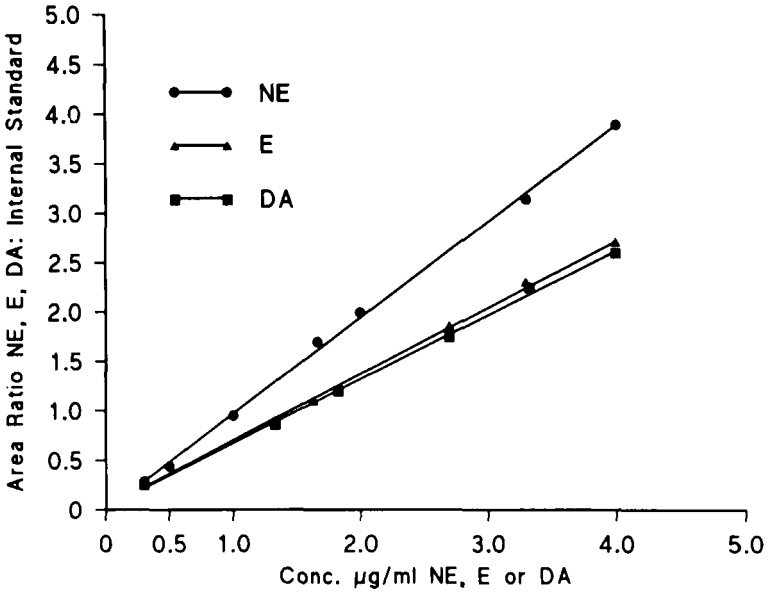

FIGURE 2: A standard curve showing a linear relationship between concentrations of NE, E and DA and the ratio of area of NE, E, DA to that of DHBA.

Recovery of NE, E and DA was evaluated by duplicate injections of extract obtained from CAFU spiked with various concentrations of standards of catecholamines. The recovery for NE was observed to be 84-113% (97.3 ± 11.2, mean±SD), for E, it was 84-108% (100.5 ± 11.4, mean±SD) and 91.0-102% (99.4 ± 9.5, mean±SD) for DA. methodology was found to be most sensitive for NE since the area of the NE peak was always greater than that of either E or DA for the same concentration.

Recovery of NE, E and DA was evaluated by duplicate injections of extract obtained from CAFU spiked with various concentrations of standards of catecholamines. The recovery for NE was observed to be 84-113% (97.3 ± 11.2, mean±SD), for E, it was 84-108% (100.5 ± 11.4, mean±SD) and 91.0-102% (99.4 ± 9.5, mean±SD) for DA.

Reproducibility of the assay method for NE, E and DA was evaluated by injecting repeatedly extracts prepared from aliquots of the same sample of urine. Interassay coefficient of variation (CV) for each amine was achieved by analyzing extracts of 5 aliquots of a single sample of urine and gave a CV of 15.1% for NE, 8.6% for E and 7.9% for DA. Intraassay CV was obtained by assaying six aliqouts of a single extract of a sample of urine and values of 7.8% , 9.5% and 3.2% were obtained for NE, E and DA respectively.

Random samples of urine collected, during working days, from both males and female laboratory workers as volunteers (age 25-35 years), were analyzed for catecholamines. The results show that catecholamine levels (ug/ml) in urine of female volunteers were NE = 0.096$\pm$ .03 (N = 12), E = 0.21 $\pm$ 0.06 (N = 12), DA = 0.36 $\pm$ 0.1 (N = 12). The levels of catecholamines (ug/ml) in urine of male volunteers were NE = 0.77 $\pm$ 0.26 (N = 7), E = 0.37 $\pm$ 0.04 (N = 7) and DA = 0.51 $\pm$ 0.14 (N=7).

In the present report catecholamines were extracted from the samples of urine using Biorex-70 columns and the results obtained were very satisfactory. Some of the earlier studies used large amounts of alumina (0.5g/10 ml of urine) to isolate catecholamines from urine, but this procedure was not found to be reliable (10,8) probably due to losses occurring in elution of adsorbed catecholamines from alumina. Moreover, use of alumina for extracting urinary catecholamines has been shown to cause difficulty with reverse-phase systems because of void volume interferences (11,12). On the other hand, isolation of urinary catecholamines by one step clean up procedure on Biorex-70 minicolumns provided almost pure extracts containing only NE, E and DA. Fig 1C shows that apart from the three catecholamine peaks, no other peaks were detected which further supports the suitability of the extraction procedure.

Elution of catecholamines on HPLC reverse phase column using a mobile phase (pH 3.9) containing 5% methanol and ion-pairing reagent sodium-octylsulfate (0.5 mM)

was found to be adequate and provided a good resolution of all the peaks at a flow rate of 0.5 ml/minute. The electrode potential of +0.55V also was observed to be suitable for detector sensitivity of 5nAFS and gave low background signal. The base line was always found to be stable, smooth and noise-free and no major shifts were observed in the retention time during analysis of catecholamines. Overloading of HPLC columns was avoided by suitablly diluting any extracts containing more than 800 pg/20 ul of catecholamines. To ensure the absence of catecholamines in CAFU, a chromatogram was always run with an aliquot of extract obtained from CAFU prior to calibration with CAFU containing standards. The use of internal standard (DHBA) reduces the variability due to transient changes in the detector sensitivity as well as HPLC column performance since both the internal standard and analytes will exhibit parallel responses at the same time. Since catecholamines levels in plasma and urine are found to differ with varying stressful situations, the significant differences observed in the levels of catecholeamines in the random samples of urine obtained from female and male volunteers could be a reflection of the differences in the ability of females and males to cope stress under similar situations.

The procedure described in this report is not entirely a new technique but has been simplified so that HPLC combined with electrochemical detector can be used efficiently for urinary catecholamine analysis. Since a large number of samples can be extracted simultaneously and analysis of each sample requires relatively short elution time, which is less than 20 minutes, is therefore convenient for the analysis of large number of urine samples in one day. Other salient features of this technique include a good chromatographic resolution of individual analytes of interest, and the reliability, sensitivity and reproducibility of the method.

ACKNOWLEDGEMENT

This work was supported, in part, by NIH grant #HL36588 and VA merit review # 9003.

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