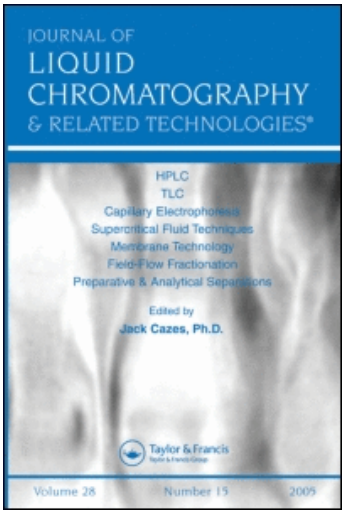

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Catecholamine Quantification in Body Fluids Using Isocratic, Reverse Phase HPLC-CoulArray Multi-electrode Chemical Detector System: Investigation of Sensitivity, Stability, and Reproducibility

Adarsh M. Kumar[a]; Benny Fernandez[a]; Michael H. Antoni[b]; Seth Eisdorfer[a]; Mahendra Kumar[b]
[a] Department of Psychiatry and Behavioral Sciences, University of Miami School of Medicine (M-817), Miami, FL, USA [b] Department of Psychology, University of Miami, Miami, Florida, USA

Online publication date: 12 February 2003

To cite this Article Kumar, Adarsh M. , Fernandez, Benny , Antoni, Michael H. , Eisdorfer, Seth and Kumar, Mahendra(2003) 'Catecholamine Quantification in Body Fluids Using Isocratic, Reverse Phase HPLC-CoulArray Multi-electrode Chemical Detector System: Investigation of Sensitivity, Stability, and Reproducibility', Journal of Liquid Chromatography & Related Technologies, 26: 20, 3433 — 3451
To link to this Article: DOI: 10.1081/JLC-120025600
URL: http://dx.doi.org/10.1081/JLC-120025600

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 26, No. 20, pp. 3433–3451, 2003

Catecholamine Quantification in Body Fluids Using Isocratic, Reverse Phase HPLC-CoulArray Multi-electrode Chemical Detector System: Investigation of Sensitivity, Stability, and Reproducibility

Adarsh M. Kumar,[1,*] Benny Fernandez,[1] Michael H. Antoni,[2] Seth Eisdorfer,[1] and Mahendra Kumar[2]

[1]Department of Psychiatry and Behavioral Sciences, University of Miami School of Medicine, and [2]Department of Psychology, University of Miami, Miami, Florida, USA

ABSTRACT

Catecholamines (CATs) neurotransmitters regulate a number of physiological and behavioral functions. These amines are released from the nerve endings and are present in ultramicro quantities in plasma and other body fluids. For meaningful evaluation of their concentration, it is essential that the technology used for their measurement is highly sensitive, efficient, and stable. Among various methods used for their measurement, recently developed high performance liquid chromatography (HPLC-ECD),

*Correspondence: Adarsh M. Kumar, Department of Psychiatry and Behavioral Sciences, University of Miami School of Medicine (M-817), P.O. Box 016960, Miami, FL 33101, USA; E-mail: akumar@med.miami.edu.

DOI: 10.1081/JLC-120025600
1082-6076 (Print); 1520-572X (Online)

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

combined with a CoulArray multielectrode detector system, is found to be the most suitable for detecting picogram concentrations of CATs in small samples of human plasma and other body fluids, as well as tissues. In this paper, we describe systematic investigations of various factors involved in contributing to the reliability and reproducibility of CATs measurement. These factors include sensitivity of detection, limits of linear relationship between concentration and the response factor, stability of the system and extracts, as well as, other factors that need to be controlled in order to achieve reliable results.

Key Words: Catecholamines; HPLC-ECD; CoulArray.

INTRODUCTION

Catecholamines (CATs) are low molecular weight, biologically active monoamines and include dopamine (DA), norepinephrine (NE), and epinephrine (E). The three catecholamines have been implicated in the regulation of various physiological and behavioral functions of the central (CNS), as well as the peripheral nervous system (PNS). Dopamine is involved in the regulation of motor responses to stimuli, motivation, reward, reinforcement, withdrawal systems, and cognitive functions. Norepinephrine is involved in the regulation of many physiological functions and behaviors, such as mood, anxiety, arousal, sleep–wake cycle, pain, learning, and memory, as well as brain metabolism, and E is implicated in the regulation of cardiovascular activity, anger, anxiety, and a number of metabolic functions. Dopamine and NE mediate flow of impulses between neurons and are, therefore, recognized as important neurotransmitters.

All three CATs contain a catechol nucleus (3,4-dihydroxyphenyl) and an amino acid side chain. A common precursor aminoacid, *L*-tyrosine obtained from dietary sources, is required for the synthesis of the three CATs. Synthesis of DA and NE takes place in the CNS and sympathetic nerve endings of the PNS.[1,2] *L*-tyrosine is converted to *L*-dihydroxyphenyl-alanine (L-DOPA) by the rate limiting enzyme, *L*-tyrosine hydroxylase present in the neuroectodermal tissues. The enzyme, DOPA-decarboxylase converts L-DOPA to form 3,4-dihydroxy phenyl-ethylamine (dopamine). Dopamine is converted to 3,4-dihydroxy-phenyl-ethanolamine or NE by the enzyme, dopamine-β-hydroxylase in the synaptic vesicles of neurons in locus coeruleus in the midbrain, as well as in the sympathetic ganglion in the PNS. Norepinephrine is converted to E in the final step in the adrenal medullae by the enzyme, phenylethanolamine-*N*-methyltransferase (PNMT) in the presence of *S*-adenosyl methionine.[3] Epinephrine is formed exclusively in the adrenal medullae because the enzyme, PNMT is present only in the enterochromaffin granules of the adrenal medullary

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

pheochromocytes, although a small quantity of E is also produced in the brain and other tissues by methylation of NE. The synthetic and structural similarities between three CATs are given in Fig. 1.

Levels of CATs in plasma, CSF, and urine serve as important markers for identifying the changes in the functions of central, peripheral, and autonomic nervous systems (ANS). Circulating CATs levels are sensitive to changes in various environmental, physiological, and genetic factors, such as acute and chronic stress, tissue injury, dysregulation in cardiovascular functions, decreased blood volume, inadequate availability of precursors, uncontrolled secretion of CATs by the tumors of pheochromocyte in the adrenal gland (pheochromocytoma), defects in receptor functions, and/or genetic expression of enzymes involved in their synthesis and metabolism. Any one, or more, of these factors may affect catecholaminergic functions per se, and also the functions of other organs and systems, which are regulated by these monoamines. For example, the sympathetic system is activated in stressful situations, such as in the "fight or flight" response to a threatening environment when an increased release of CATs influences the heart to beat faster, increase cardiac output, and increase consumption of oxygen to generate more energy required to cope with the situation. On the other hand, a decrease in the availability of CATs leads to impairment in the function of heart and other organs. For instance, deficits in central DA have been associated with motor disorders of Parkinson's and Huntington's diseases and drug abuse. Recent studies have also reported that HIV-1 infected individuals exhibit impaired release of NE in response to β-adrenergic laboratory stressors, indicating dysfunction of the ANS.[4] Decreased levels of DA have also been reported in

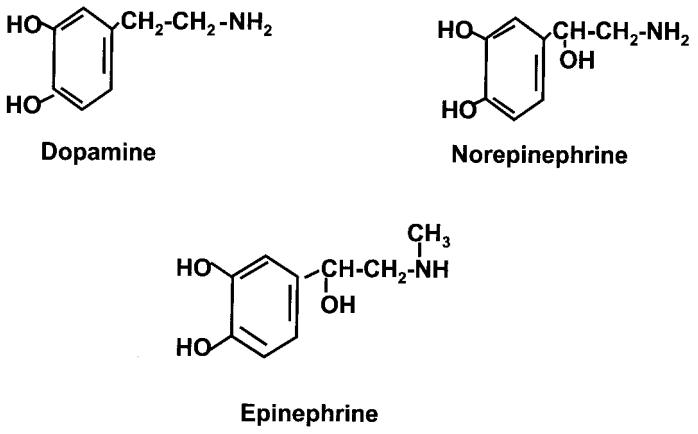

Figure 1. Structural formulae of CATs (dopamine, norepinephrine, and epinephrine).

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

the cerebrospinal fluid (CSF) of HIV-1 infected individuals.[5] However, it is not clearly understood whether decreased DA levels in CSF are associated with motor dysfunctions that occur in this disease.

In view of the significance of CATs in the regulation of many physiological and behavioral functions, there have been constant attempts to develop technologies, which are sensitive, reliable, simplified, and convenient to measure ultramicro quantities of these amines present in body fluids and tissues. A variety of earlier technologies were used to study localization, and distribution of CATs in different brain regions. For example, histochemical techniques combined with light microscopy[6,7] and fluorescence with formaldhyde vapors, was used for mapping the distribution of CATs, as well as serotonin, in the sympathetic system of experimental animals.[8] Subsequent techniques for quantification included autoradiography,[9,10] electron microscopy,[11] radioenzymatic methods,[12–14] fluorimetric,[15,16] immunosorbent assays (ELISA), and various chromatographic techniques, such as gas chromatography with mass spectrometry and fragmentography.[17,18] These techniques made important contributions to our understanding of some of the roles of catecholamines in the functioning of CNS and PNS, however, they lacked sensitivity required for measuring these compounds during life in small samples of plasma or CSF, which can be obtained during experimental investigations in the laboratory. Recent development of high performance liquid chromatography (HPLC) combined with fluorometric (FL), ultraviolet (UV), and electrochemical detection (ECD) offers significant improvement in sensitivity and specificity for measurement of monoamines and their metabolites in small samples of biological fluids and tissues. Among these technologies however, HPLC-ECD has gained much popularity because of the simplicity in preparation of samples required for analysis, high sensitivity of the method to detect and measure picogram quantities, as well as specificity for detecting the compounds in their natural form, without time consuming chemical transformation or derivatization steps needed for other techniques.[13,19] A number of reports have used HPLC-ECD for quantification of CATs in health and disease using human body fluids and tissues.[4,5,20,21]

High performance liquid chromatography with ECD, an amperometric system consists of a single glassy carbon working electrode, a refillable Ag/AgCl, reference electrode (used for setting up a stable potential for operation of the working electrode), and an auxillary electrode. This system, with a single working electrode, although more sensitive and specific than the FL and UV system, is limited in its efficiency for oxidation/reduction reaction and results in low recovery of analytes of interest from plasma and other body fluids. Other frequent problems encountered with this system are drifts in the baseline, and shifting of the retention times of analytes during analysis. These inherent problems with single working electrode system can result in inconsistent reproducibility and

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

recovery of analytes during the same day and between days analysis. Moreover, the glassy carbon working electrode used in this system is embedded in borosilicate glass, and because the eluent passes over the electrode rather than through it, there is a build up of oxidation/reduction products on its thin surface, which needs frequent cleaning and/or changing of the electrode. The surface build up occasionally leads to appearance of erroneous peaks, which interfere in the detection of compounds of interest. These difficulties, although resolvable to an extent, are tedious, time consuming, and expensive.

The better resolution of these difficulties has been achieved by a recently developed HPLC-ECD with CoulArray multiple electrodes detection system equipped with low volume analytical cells containing multiple working electrodes, each having a high surface area. The CoulArray system provides higher precision, sensitivity, and specificity for measuring simultaneously ultramicro quantities of a large number of electroactive compounds including circulating CATs, metabolites, and the related biochemicals. Furthermore, investigations can be conveniently carried out in small samples containing picograms to femtogram quantities of CATs or other compounds for investigating changes occurring simultaneously in the levels of many related compounds of interest in response to various experimental procedures in the laboratory. An autosampler added to the system enhances the efficiency for analysis of a large number of samples in one day. In this paper, we describe in detail the procedures for validation of important parameters required for maintaining precision and quality control for successful analysis of catecholamines. We validated sensitivity, specificity, stability of the system, stability of standards and that of extracts, as well as, reproducibility and recovery of CATs, using the CoulArray multielectrode system.

EXPERIMENTAL

Chemicals

All standards including IS, dihydroxybenzylamine · HBr (DHBA), norepinephrine · HCl (NE), epinephrine · HCl (E), dopamine · HCl (DA), and chemicals for mobile phase including sodium acetate, citric acid, sodium octyl sulphonate, disodiumethylenediamintetra-aceticacid (Na_2-EDTA), dibutylamine and methanol, were of HPLC grade and were purchased from Sigma Chemical Co (St Louis, MO).

HPLC Equipment

The HPLC-ECD system (CoulArray) purchased from Environmental Sciences Associates (ESA Chelmsford, MA) and employed for investigations

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

presented in this report, consists of a coulometric multi-electrochemical detector system (Model 5600A CoulArray Detector). This system is comprised of a control module containing 4 electrochemical cells set in series. Each cell is equipped with 4 channels, thus providing a total of 16 channels with working electrodes made of porous graphite, which can be set at increasing potentials appropriate for oxidation and reduction of a number of analytes present in the same extract of a sample. A thermal chamber for housing the cells, the column, and the injector valve, maintains the temperature. The other parts of the system include a dual-pump programmable solvent delivery module (Model 582), autosampler and injector (Model 540), and CoulArray for windows application software. The chromatographic separation of CATs was carried out using the reverse phase stainless steel (3.9×150 mm), 5 μm, C_{18}, resolve column (Waters, Bedford, MA).

Mobile Phase

Mobile phase used for eluting CATs from a standard mixture, plasma, and CSF extracts, consisted of sodium acetate 50 mM, citric acid 50 mM, sodium octylsulphate (SOS) 0.25 mM, disodium-EDTA 0.075 mM, dibutylamine 0.5 mM, and methanol 10%. The solution was prepared in HPLC-grade water, adjusted to pH 4.0, filtered through a 0.2 μm filter, and was degassed with ultrasonic agitation before use. Deionized, HPLC-grade water was obtained from a Millipore Milli-Q (Bedford, MA), reverse osmosis system having a resistance of 18.3 megohm-cm.

Standard Solutions

Standard stock solutions of NE, E, DA, and the internal standard, DHBA, were each prepared at a concentration of 1 mg/mL in HPLC-grade water and stored at 4°C. Working standard solutions were prepared freshly before use and contained a wide range (10–6000 pg/mL) of concentration of NE, E, and DA in order to investigate the lower, as well as, the higher limit of sensitivity of the CoulArray detector for each CAT. Concentration of the working internal standard, DHBA was kept constant at 1000 pg/mL.

Identification of Individual Peaks and Calibration

Equilibration of the HPLC system was carried out by circulation of mobile phase for a few hours to overnight at a flow rate of 1.0 mL/min. Mobile phase was recycled during overnight equilibration but was discarded during all

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

procedures of standardization and analysis of samples. Working standard solutions, 40 μL containing 1000 pg/mL of each NE, E, DA, and DHBA were then injected individually for identification of their respective peaks by their retention times. Calibration was carried out at the optimum potential to achieve the maximum detector response by injecting 40 μL of a standard mixture containing 1000 pg/mL of each component. The optimum potential for oxidation of CATs using freshly prepared mobile phase at pH 4.0 was found to be +300 mV for conditioning cell (equipped with porous graphite electrode), and +50 mV for electrode 1, and −200 mV for electrode 2. Sensitivity was set at 1 nA. Further validation of retention times and peak heights of each compound was achieved by a second injection of the standard mixture.

Standardization, Determination of Sensitivity, Validation, and Stability

Standardization was carried out by injecting a series of standard mixtures containing each CAT, ranging in concentration from 10 to 6000 pg/mL, in order to determine sensitivity at low and high concentration of each analyte. The Coulchem detector conditions for analysis of CATs were established during the calibration procedure as described above, with the flow rate of mobile phase at 1 mL/min, sensitivity set at 1 nA, and potential set as described above. Analysis was carried out at ambient temperature. The response factors for each concentration was calculated from the ratio of peak height of analyte to that of IS. Validation of the procedure was determined by the consistency of relationship between each concentration and the corresponding response factor. Stability in the retention times of each analyte, as well that of extracts subjected to different conditions, was examined by repeated injections on the same day or different days.

Extraction of Catecholamines from Plasma and CSF

Blood samples were collected by venipuncture from individuals through an indwelling catheter after 20 min of rest, and transferred to tubes containing EDTA and chilled on ice. Plasma was prepared by centrifugation at 1600*g* (3000 rpm), and 0.5 mL aliquotes for assay of CATs were stored at −70°C until analyzed. On the day of analysis, plasma samples were thawed and treated with 100 μL containing 1000 pg/mL of DHBA, 350 μL of 2 M Tris/HCl, pH 8.7, and 20 mg (one scoop) of acid washed alumina (Bioanalytical Systems Inc.). Contents of tubes were mixed for 15 min on a mechanical rocker and centrifuged at 4000 rpm for 1 min. Supernatant was aspirated off without

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

disturbing the alumina. Alumina was washed twice with 1 : 100 diluted 2 M Tris–HCl buffer, pH 8.7 and CATs were eluted from alumina with 200 μL of 0.1 M acetic acid. After centrifugation at 14,000 rpm for 3 min, the supernatant was filtered through 0.2 μm filter and 40 μL was injected into the column for separation of CATs.

Samples of CSF obtained by lumbar puncture using the procedure similar to that described earlier,[5] were kept frozen at −80°C. After thawing, the extracts were prepared from 0.5 mL of CSF by the procedure used for plasma (described above). The extract, 40 μL was injected. Before injecting CSF extracts, calibration was carried out with standards as described above.

Statistics

Results are expressed as standard deviation of the mean when applicable. Statistical significance was examined by use of a Students *t*-test.

Results

A typical elution profile of mobile phase (baseline), CATs (NE, E, DHBA, and DA) separated from a mixture of standards, and from the extracts of a sample of plasma and CSF are shown in Fig. 2(A)–(D).

Standard curves (Table 1) were generated with various concentration (pg/mL) of each CAT in order to validate the relationship between concentration and response factor (the ratio of peak heights of analyte to that of IS). A linear relationship was observed between the range of concentration and the response factors, both at the lower and higher limit of detection of each amine. For NE, the relationship was linear between 20 and 6000 pg/mL, (0.8–240 pg/40 μL injected). For E, the linear relationship was observed between 50 and 6000 pg/mL (2–240 pg/40 μL injected), and for DA the detection and linearity was limited between 200 and 6000 pg/mL (8–240 pg/40 μL injected). Among the three CATs, the sensitivity of detection for DA was found to be the lowest (200 pg/mL or 8.0 pg/40 μL). Two standard curves showing linearity at higher and lower concentrations are given in Fig. 3(A)–(B).

Each analyte (NE, E, and DA) detected is presented as the percent of the total concentration of individual compound in the standard mixture injected. Norepinephrine was detected (Mean ± SD) 91.3% ± 13.3% ($n = 8$), of E was 95.13% ± 7.02% ($n = 7$) and DA was 98.12% ± 2.05% ($n = 5$). In this experiment NE was not detected below 20 pg/mL (0.8 pg), E below 40 pg/mL (1.6 pg), and dopamine below 200 pg/mL (8.0 pg).

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

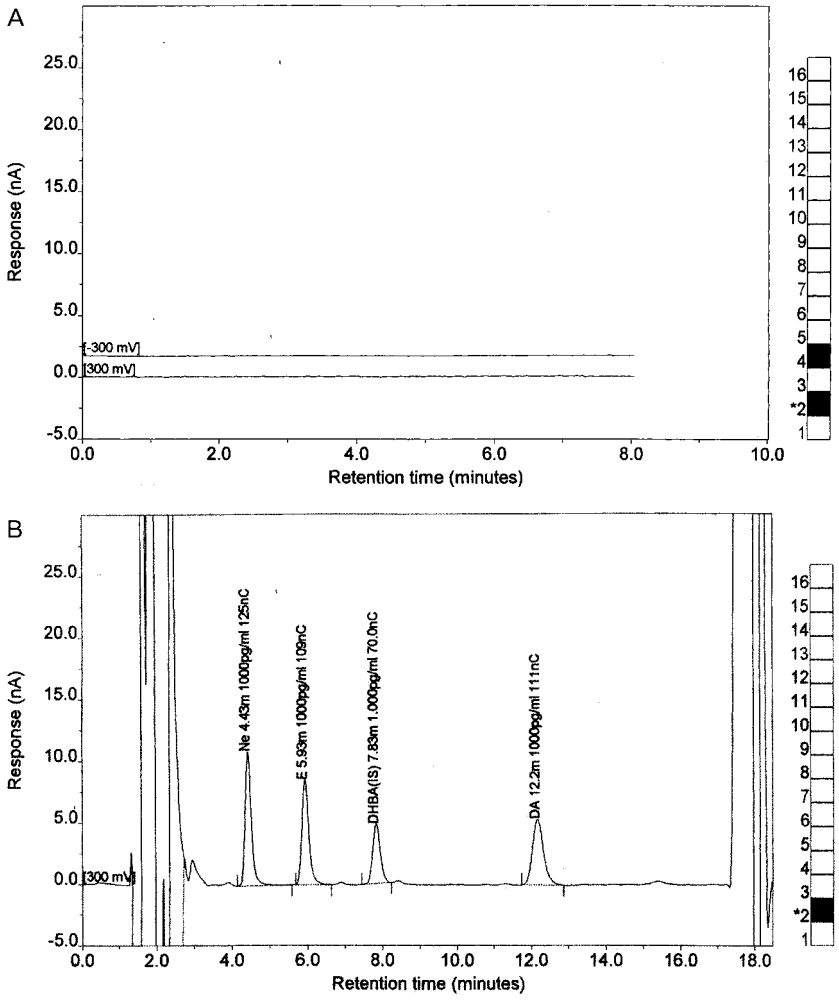

Figure 2. Chromatographic profile of standards of NE, E, DHBA, and DA is shown in order of their retention times. 2A, baseline profile with mobile phase; 2B, separation of analytes from a standard mixture; 2C, profile of a plasma sample, 2D, profile of a CSF sample.

(continued)

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

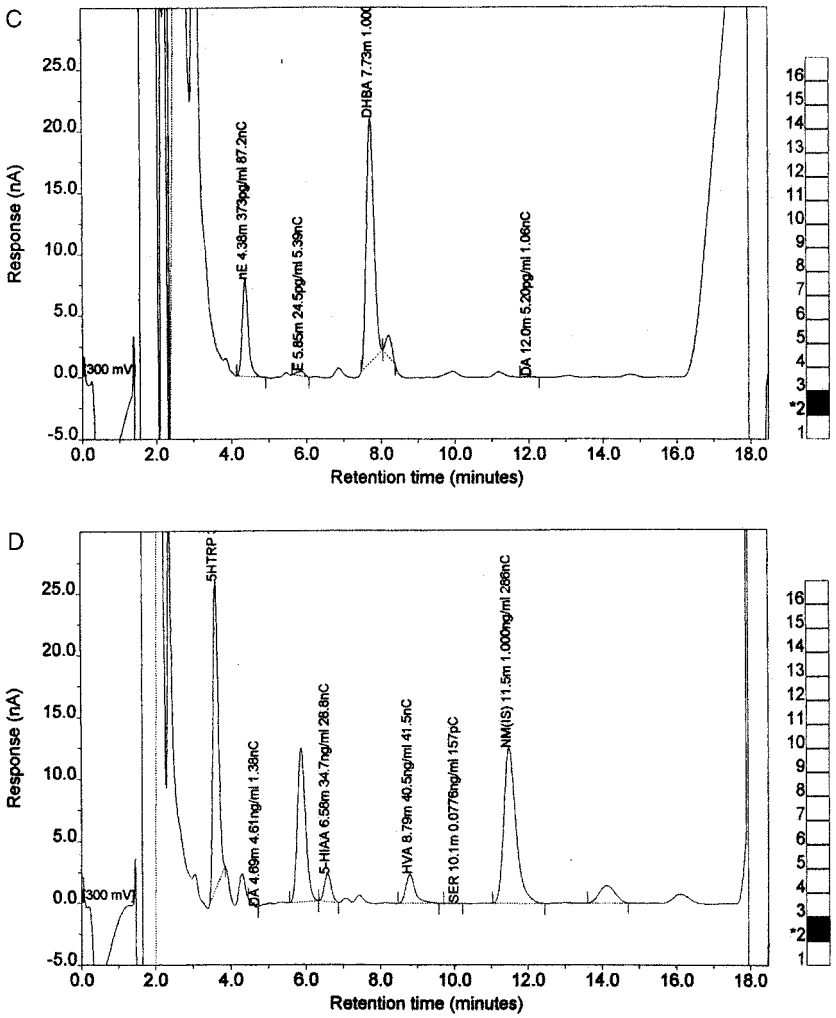

Figure 2. Continued

Retention times for each analyte were found to be stable for each compound whether calibration and analysis of a number of samples ($n = 9$) was carried out on the same day, or on another day ($n = 7$). Slight drifts in retention times of each analyte noticed on different days were found to be insignificant. Data shown in Table 2 demonstrate the stability of RT of all analytes including that of the IS.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Table 1. Standardization data: relationship of concentration and ratio of detector response recorded as peak height (pHt) between the individual analyte (NE, E, and DA) and that of internal standard (DHBA).

	Concentration (NE, E, DA)		% Detected			Ratio (pHt of analyte/IS)		
No	pg/mL	pg/40 μL	NE	E	DA	NE	E	DA
1	20	0.8	66.0	ND	ND	0.0163	—	—
2	50	2.0	71.0	82.0	ND	0.043	0.039	—
3	100	4.0	103.0	87.2	ND	0.12	0.083	—
4	500	20.0	99.0	102.2	94.2	0.61	0.49	0.25
5	1000	40.0	98.4	100.2	99.3	1.21	0.96	0.53
6	2000	80.0	98.0	99.6	100.0	2.26	1.91	1.07
7	4000	160.0	97.6	97.3	98.1	4.81	3.73	2.09
8	6000	240.0	97.4	97.8	99.0	7.19	5.63	3.17

The minimum detection limit (%) of each analyte was different at lower concentrations, but increased with increasing concentration of each analyte and became stable at 500 pg/mL. The minimum level of detection for NE was 20 pg/mL, for E was 50 pg/mL, and that for DA was >100 pg/mL. The data demonstrate a linear relationship between the concentration of each analyte and the response factor (ratio of peak Ht of each analyte/IS).

Stability of Extracts and the Detector System

Stability of CATs in standard solutions was investigated by injecting separate aliquotes of the extract of standards prepared through the same extraction procedure of adsorption with alumina, as that used for extraction of CATs from plasma and CSF. After calibration with the working standards, freshly prepared standard extracts were injected through the autosampler at the beginning, after 25 samples of plasma extracts (approximately 8 hours), and after 50 samples of plasma extracts (overnight). Aliquots of standard extracts were also stored for 3 days and for 1 week at 4°C, and then analyzed. The data presented in Table 3 show that all parameters including the concentration detected, retention times, and peak heights were stable whether the injected extract was freshly prepared, or after it was subjected to different conditions.

Recovery of Catecholamines Standards Spiked into Plasma Samples

Separate aliquotes of pooled plasma samples were spiked with standard solutions containing 400 pg/mL of each NE, E, and DA, and 1000 pg/mL of

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

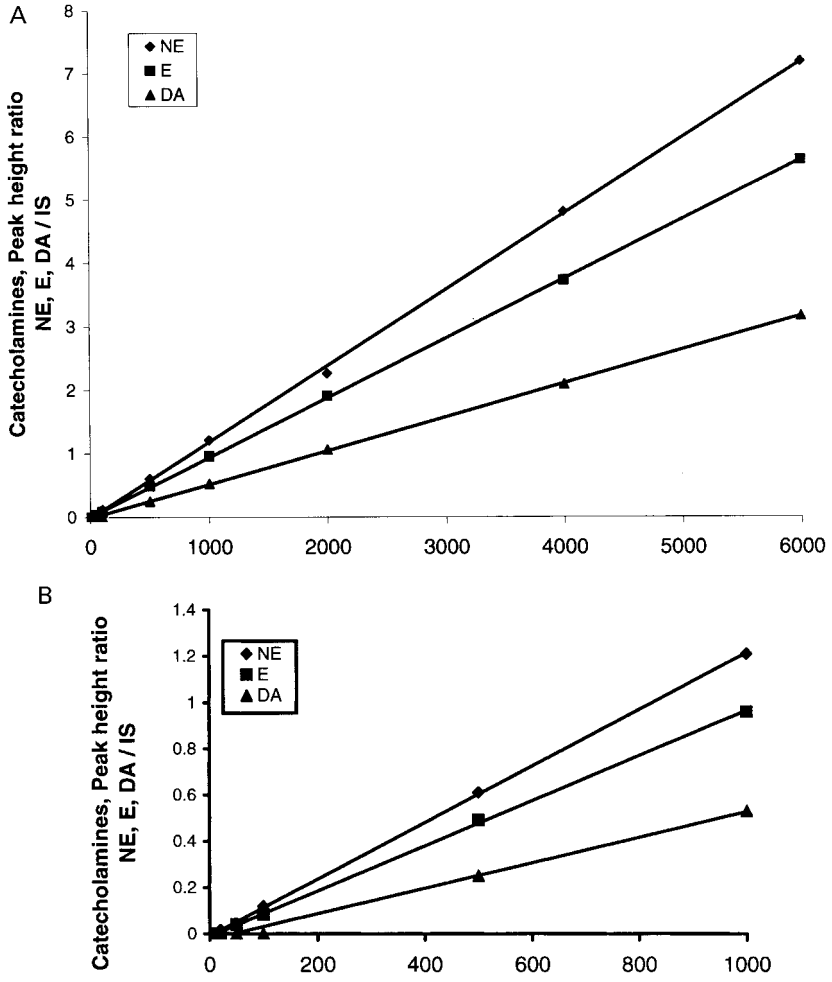

Figure 3. (A) Standard curves of CATs showing a linear relationship between various concentrations of NE, E, and DA (0–6000 pg/mL) and their corresponding response factors (the ratio of peak heights of NE, E, and DA to that of DHBA at each concentration). (B) Standard curve showing the relationship between concentration of CATs at lower range (0–1000 pg/mL) and the response factors (the ratio of peak heights of NE, E, and DA to that DHBA).

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Table 2. Stability of retention times of NE, E, DA, and DHBA with CoulArray detector.

Analyte	Retention time (Day_1) ($n=9$)	% CV	Retention time ($Day_{another}$) ($n=7$)	% CV	*t*-value	*p* value
NE	6.22 ± 0.025	(0.4)	5.80 ± 0.025	(0.5)	35.58	0.000
E	7.97 ± 0.01	(0.125)	7.36 ± 0.03	(0.41)	48.41	0.000
DA	17.66 ± 0.046	(0.26)	16.14 ± 0.08	(0.49)	41.53	0.000
DHBA	11.43 ± 0.03	(0.26)	10.50 ± 0.046	(0.44)	53.45	0.000

Values of retention times (RTs) in minutes of NE, E, and DA (mean $\pm$ SD) are given in order of their appearance in the chromatogram. The RTs of each analyte obtained on day-1 with 9 repeated injections of a freshly prepared standard mixture were not significantly different from the RTs obtained with 7 injections of a fresh preparation on another day ($p = 0.000$).

IS. Catecholamines from aliquots, with and without spiked standards, were extracted with the procedure described above. The extracts were injected and the recovery of the standard was calculated by subtracting the concentration of CATs in the original (unspiked) plasma sample from that in the spiked samples. Recovery of NE, E, and DA standards ranging between 80% and 104% is shown in Table 4.

Reproducibility of the assay for CATs was investigated by injecting extracts prepared from separate aliquotes of the same sample of plasma. The concentration detected in each aliquot of plasma (Mean $\pm$ SD, $n = 4$), as well as, the response factor are presented in Table 5.

Intra-assay coefficient of variance (CV) was determined with seven injections of the standard solution mixture containing 1000 pg/mL of each analyte. Values of the ratio of peak Ht of analyte to that of IS were used to determine %CV. The values, (Mean $\pm$ SD and %CV), for NE, E, and DA were 1.25 ± 0.037 and 2.96%; 0.98 ± 0.01 and 1.02%, and 0.24 ± 0.006 and 2.66%, respectively.

Inter-assay CV was determined by injecting extracts obtained from six separate aliquots of a pooled plasma sample, and the values of the ratio of peak Ht of analyte/IS (Mean $\pm$ SD and %CV), for NE, E, and DA were 2.47 ± 0.21 and 8.5%; 1.03 ± 0.04 and 3.9%, and 0.21 ± 0.02 and 10%, respectively.

DISCUSSION

In this study, we evaluated various parameters that contribute to precision and quality control for detection and quantification of CATs (NE, E, and DA)

Table 3. Stability of CATs standard extracts subjected to different conditions (standard concentration was 1000 pg/mL in each experiment).

	Condition exposed					
	Fresh standards calibration	2nd injection	After 25 injections	After 50 injections	3 days old at 4°C	1 week old at 4°C
Concentration. (pg/mL) detected						
NE	1000	965.9	1002.0	992.3	982.0	1012.6
E	1000	988.0	1015.5	992.4	1015.9	998.2
DA	1000	955.3	1011.5	981.9	993.9	967.9
RT (min.)						
NE	4.43	4.43	4.42	4.41	4.41	4.41
E	5.93	5.93	5.91	5.90	5.90	5.89
DA	12.18	12.13	12.09	12.08	12.07	12.06
Peak heights (nA)						
NE	10.9	10.8	10.8	10.9	10.7	10.8
E	8.48	8.43	8.47	8.47	8.43	8.44
DA	5.32	5.23	5.29	5.28	5.21	5.20
Peak Ht ratio (Analyte/IS)						
NE	2.26	2.27	2.31	2.29	2.26	2.28
E	1.75	1.75	1.81	1.79	1.79	1.78
DA	1.10	1.10	1.13	1.11	1.10	1.10

Standards (NE, E, DA, and DHBA) of known concentration in a mixture were extracted as described in the text. After initial calibration, an aliquot of freshly prepared extract was injected. Injections of the same standard extract were repeated after analysis of plasma extracts of 25 samples and 50 samples. Aliquots of the same standard extracts were also stored at 4°C and then injected after 3 days and after 1 week. Data are given for concentration detected, RTs, peak heights and the response factors for each analyte.

Table 4. Recovery of CATs from plasma samples spiked with 400 pg of each standard.

NE (pg/mL)		E (pg/mL)		DA (pg/mL)	
561.59 (plasma)	Percent recovery ($n=6$)	90.67 (plasma)	Percent recovery ($n=6$)	4.75 (plasma)	Percent recovery ($n=6$)
343	85.7	352.0	88.0	404.0	101.0
336	84.0	348	87.0	416	104.0
331	82.7	344	86.0	395	98.7
320	80.0	337	84.3	381	95.2
368	92.0	375	93.7	437	109.3
373	93.2	356	89.0	413	103.3
345.1 ± 19.23	86.3 ± 4.80	352.0 ± 11.9	88.0 ± 2.94	407 ± 17.5	101.9 ± 4.41

For evaluating the recovery of standards, a plasma sample was divided into 7 aliquots. Six aliquots were spiked with 400 pg/mL of NE, E, and DA and all seven aliquots were extracted as described in the text. Percentage recovery (Mean $\pm$ SD) of standards of NE, E, and DA was calculated by subtracting the concentration of each analyte in the plasma extract from that of spiked samples.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Table 5. Reproducibility of detection of NE, E, and DA. Four aliquots of a plasma sample were extracted separately and injected. Internal standard, 1000 pg/mL was added to plasma before extraction (RF = response factor).

Plasma sample	NE (pg/mL)	RF (NE/IS)	E (pg/mL)	RF (E/IS)	DA (pg/mL)	RF (DA/IS)
1	374.65	0.398	17.24	0.015	4.24	0.0028
2	374.03	0.397	18.03	0.017	2.73	0.0024
3	372.91	0.396	19.4	0.0186	5.20	0.0031
4	369.42	0.393	14.97	0.0143	3.50	0.0026
Mean ± SD	372.7 ± 2.02	0.396 ± 0.0018	17.4 ± 3.53	0.0165 ± 0.002	3.92 ± 0.91	0.0027 ± 0.0002

Reproducibility for the absolute concentration detected and the corresponding response factor for NE, E, and DA was evaluated using extracts from 4 aliquots of a plasma sample. The internal standard concentration was 1000 pg/mL.

in plasma and CSF using a HPLC-CoulArray multielectrode detection system. We used a C_{18} reverse phase 5 micron (3.9 × 150 mm) plasma CAT column, freshly prepared mobile phase at pH 4.0, and the potential used for CATs, as described in the text. The data obtained with this combination of conditions demonstrate a high degree of precision in the overall performance of the system including; (1) a linear relationship between a wide range of concentration (0.8–240 pg) of the three CATs (NE, E, and DA) and the corresponding response factor for each compound; (2) the lower limit sensitivity of detection at 0.8 pg for NE, 2 pg for E, and 8 pg for DA, and the higher limit of sensitivity at 240 pg for all three CATs; (3) remarkable stability of retention times of each analyte during the entire analysis of a large number of sample extracts after initial calibration with known standards; (4) a high percentage recovery (86–102%) of standards of all three analytes obtained from the spiked plasma sample extracts; (5) reproducibility (%CV) of the results within the range desired for maintaining quality control; (6) stability of CATs in the extracts when stored at room temperature for a few hours, and at 4°C for 3 days to 1 week.

The sensitivity of detection with the CoulArray multielectrode-HPLC system used in this study was highest (0.8 pg) for NE, two and half times less for E, and was 10 times less for DA (8 pg). These differences in sensitivity are most likely due to the difference in the polarities and oxidizability of these compounds, NE being more polar than E and DA.

The high sensitivity of detection for the lowest concentration of all CATs in the present system is important for the analysis of small sample volumes (0.5 mL or less). The sample volume used in these assays was 0.5 mL of plasma, as well as CSF, compared to 1.5 mL required for the analysis with the single working electrode HPLC-ECD system. The high efficiency of the present system is attributed to a series of coulometric working electrodes made of porous graphite, which have a 100% capacity for oxidizing or reducing the analytes. In addition, the second and third electrodes in the highly selective analytical cell eliminate the interfering substances, which are reduced at different (higher) potentials than CATs. The retention times of all analytes were found to be very stable (Table 2) compared to that with the shifting retention times in the single working electrode system (multielectrode system vs. single electrode system, % CV, = 0.4 vs. 9.35 for NE; 0.125 vs. 9.23 for E, 0.26 vs. 14.52 for DA, and 0.26 vs. 10.04 for DHBA). Furthermore, the baseline was always stable without any drifts during analysis. Evaluation of factors reported in this paper is an important aspect of validating the methodology used for successful analysis of CATs in small samples of body fluids using a CoulArray multielectrode system.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

ACKNOWLEDGMENT

This study was supported in part by the NIH grants No. RO1 DA12792, RO1 DA13550, R01 NS41205, 1P50 CA 86966, and R01 NS43982.

REFERENCES

1. Fuxe, K. Distribution of monoamine nerve terminals in the central nervous system. Acta Physiol. Scand. **1963**, *64* (Suppl. 247), 37–85.
2. Hokfet, T. Electron microscopic studies on the brain slices from region rich in catecholamine nerve terminals. Acta Physiol. Scand. **1967**, *69*, 119–120.
3. Axelrod, J.; Tomchick, R. Enzymatic *o*-methylation of epinephrine and other catechols. J. Biol. Chem. **1958**, *233*, 702–705.
4. Kumar, M.; Morgan, R.; Szapocznik, J.; Eisdorfer, C. Norepinephrine response in early HIV-1 infection. J. Acq. Immun. Def. Synd. **1991**, *4*, 782–786.
5. Berger, J.R.; Kumar, M.; Kumar, A.M.; Fernandez, J.B.; Levin, B. Cerebrospinal fluid dopamine in HIV infection. AIDS **1994**, *8*, 67–71.
6. Hamberger, B.; Norberg, K.A.; Sjoqvist, S. Cellular localization of monoamines in sympathetic ganglia of the cat. Life Sci. **1963**, *9*, 659–661.
7. Hamberger, B.; Norberg, K.A.; Sjoquist, S. Evidence for adrenergic nerve terminals and synapses in sympathetic ganglia. Int. J. Neuropharmacol. **1964**, *2*, 279–282.
8. Falk, B.; Hillarp, N.A.; Thieme, G.; Torp, A. Fluorescence of catecholamines and related compounds condensed with formaldehyde. J. Histochem. Cytochem. **1962**, *10*, 348–354.
9. Dengler, H.G.; Michaelson, I.A.; Spiegel, H.E.; Titus, E. The uptake of labeled norepinephrine by isolated brain and other tissues of the rat. Int. J. Neuropharmacol. **1962**, *1*, 23–28.
10. Axelrod, J. The enzymatic *N*-methylation of serotonin and other amines. J. Pharmacol. Exp. Ther. **1962**, *138*, 28–33.
11. Aghajanian, G.K.; Bloom, F.E. Electron microscopic autoradiography of rat hypothalamus after intraventricular H-3 norepinephrine. Science **1966**, *153*, 308–310.
12. Passon, P.G.; Penter, J.D. A simplified radiometric assay for norepinephrine and epinephrine. Anal. Biochem. **1973**, *51*, 618–631.
13. DaPrada, M.; Zurcher, G. Simultaneous radioenzymatic determination of plasma and tissue adrenaline, noradrenaline, and dopamine within the femtomole range. Life Sci. **1970**, *19*, 1161–1174.
14. Johnson, G.A.; Kupiecki, R.M.; Baker, C.A. Single isotope derivative (radioenzymatic) methods in the measurement of catecholamines. **1980**, *29*, 1106–1113.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

15. Udenfriend, S. *Fluorescence assay in Biology and Medicine*; New York Academic Press: NY, 1962.
16. Miura, Y.; Campese, V.; Dequattro, V.; Meyer, D. Plasma catecholamines via an improved fluorometric assay: comparison with an enzymatic method. J. Lab. Clin. Med. **1977**, *89*, 421–428.
17. Haroutune, D.; Maas, J.W. An improved procedure of 3-methoxy-4-hydroxyphenylethylene glycol determination by gas liquid chromatography. Anal. Biochem. **1970**, *35*, 13–122.
18. Koslow, S.H.; Racagni, G.; Costa, E. Mass fragmentographic measurement of norepinephrine, dopamine, serotonin and acetylcholine in seven discrete nuclei of the rat tel-diencephalon. Neuropharmacology **1974**, *13*, 1123–1130.
19. Yui, Y.; Kawai, C. Comparison of the sensitivity of various post-column methods for catecholamine analysis by high performance liquid chromatography. J. Chromatogr. **1981**, *206*, 586–592.
20. Ward, M.M.; Mefford, I.N.; Parker, S.D.; Chesney, M.A.; Taylor, B.; Keegan, D.; Barchas, J.D. Epinephrine and norepinephrine responses in continuously collected human plasma to a series of stressors. Psychosom. Med. **1983**, *45*, 471–486.
21. Kumar, A.M.; Kumar, M.; Fernandez, J.B.; Mellman, T.A.; Eisdorfer, C. A simplified HPLC-ECD technique for measurement of urinary free catecholamines. J. Liq. Chromatogr. **1991**, *14*, 3547–3557.

Received June 1, 2003
Accepted June 30, 2003
Manuscript 6167

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016