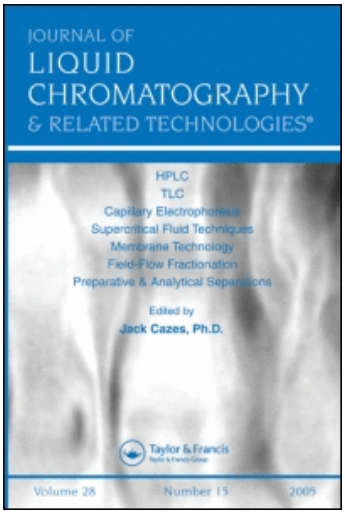

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Adarsh M. Kumar[a]; J. B. Fernandez[a]; Louis Gonzalez[a]; Mahendra Kumar[a]
[a] Department of Psychiatry & Behavioral Sciences, University of Miami School of Medicine, Miami, Florida, USA

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Ultra Micro Quantification of Dopamine and Homovanillic Acid in Human Brain Tissue: Quest for Higher Recovery and Sensitivity with CoulArray HPLC-ECD System

Adarsh M. Kumar, J. B. Fernandez, Louis Gonzalez, and Mahendra Kumar
Department of Psychiatry & Behavioral Sciences,
University of Miami School of Medicine, Miami, Florida, USA

Abstract: Dopamine (DA), a biogenic amine neurotransmitter is involved in regulating a number of functions of the central and peripheral nervous systems, including voluntary and involuntary motor functions and cognitive behaviors. Since the concentration of dopamine in different parts of the brain is extremely small and decreases further in neurodegenerative diseases, such as HIV-1 infection, a highly sensitive and specific methodology is required to quantify DA and its metabolite, homovanillic acid (HVA) in small weights of tissues from different regions of the brain. The method presented in this report used a highly sensitive multielectrode CoulArray HPLC-ECD system for quantification of <4 pg of DA and <10 pg of HVA in homogenates of human brain tissues.

A linear relationship in the calibration curves was observed between a wide range of concentration of standards of DA (50–500 pg/mL) and HVA (0.1–20.0 ng/mL) and the corresponding response factors. Separate aliquots from the same pool of brain tissue homogenate were used to extract DA and HVA by their specific extraction procedure and were quantified separately. Recovery of both DA and HVA from homogenate preparation and aqueous extraction were in the range of 88–98%. Stability of the system is evident from the consistency of retention times of analytes in different experiments. The reliability of the method is shown from the reproducibility of the values of DA and HVA obtained in different aliquots of the brain tissue homogenates.

Keywords: Dopamine, HVA, Brain tissues, CoulArray HPLC-ECD

Address correspondence to Prof. Adarsh M. Kumar, Department of Psychiatry & Behavioral Sciences, University of Miami School of Medicine (M-817), P.O. Box 016960, Miami, FL 33101, USA. E-mail: akumar@med.miami.edu

INTRODUCTION

Dopamine (DA), a biogenic amine and one of the important known neurotransmitters, is present in the central and the peripheral nervous systems (CNS and PNS). Dopamine is involved in a number of important CNS and PNS functions including complex voluntary, as well as involuntary functions of movement, cognition, spatio-visual and working memory, executive functions, and emotional responses.[1] The association of dopamine with the pleasure and reward systems of the brain and experience of feelings of euphoria with drugs of abuse, such as cocaine, methamphetamine, and other stimulants, have been well documented.[2–5] Although the major portion (80%) of dopamine is present in the neurons clustered in a specific area of the midbrain region, the substantia nigra, the neurotransmitter is also present in the other brain regions such as basal ganglia, hippocampus, hypothalamus, ventral tegmental area, and frontal cortex.[6,7]

Regulation of dopamine synthesis and its release is crucial for our mental and physical health. Dopamine deficiency and changes in homovanillic acid (HVA), the major metabolite of dopamine, due to progressive loss of dopaminergic neurons in different regions of human brain, have been reported in aging, chronic alcoholism, and Alzheimer's disease, as well as Parkinson's and Huntington's diseases.[7–10] A decrease in the levels of dopamine have also been found in the cerebrospinal fluid (CSF) of HIV-1 infected individuals with neurological problems,[11] and methamphetamine drug abusers, specifically, those who develop problems of movement disorders and cognitive deficits, similar to Parkinson's disease.[12] With increasing knowledge of the involvement of dopamine in regulation of many important brain functions and association of a number of mental disorders with dysregulated availability of this neurotransmitter, there have been constant attempts over the years to develop simplified, sensitive, and reliable techniques for extraction and quantification of dopamine present in different brain regions of human and animals.

The earlier methods involving fluorometric detection of catecholamines needed larger volumes of body fluids or tissue weights, lacked specificity, and had limited sensitivity ranging between 0.5–20 nanograms.[13,14] Although, the gas chromatography and mass spectrometry combined with mass fragmentography used earlier were highly specific and sensitive, they were time consuming and required expensive instrumentation for derivatization and detection of these bioamines.[15,16] An alternative approach of using HPLC-ECD for analysis of catecholamines first reported by Kissinger et al.,[17] is considered the most suitable for small body fluid volumes and has also been used for measurement of catecholamines in small brain tissue weight homogenates using methods which involved alumina adsorption[13,18] or extraction with organic solvents, butanol/heptane.[19] However, the maximum recovery of catecholamines by these methods was low and remained in the range of 50–60%.[17–19] In order to achieve higher recovery and sensitivity, we have frequently reported modifications in the

methods of extraction, as well as detection, using the single channel and single electrode HPLC-ECD system for measurement of biogenic amines including catecholamines in plasma,[20] urine,[21] whole blood,[22] and CSF.[11]

In a recent study, we used the multi-channel and multi-electrode CoulArray HPLC-ECD system and achieved higher sensitivity of detection, greater stability, and reproducibility for catecholamine quantification in human body fluids[23] than was achieved with the earlier methods.[18,19] However, since dopamine and the other neurotransmitter concentrations are variable in different brain regions of animals and humans, and studies have shown deficits of DA associated with aging and neurodegenerative diseases,[7,11,14] there is a persistent ongoing quest for achieving higher sensitivity and recovery in order to quantify picogram, or even lower concentrations of dopamine in low tissue weights from different regions of the human brain. For instance, recently we applied modifications to the earlier methods[23–25] for quantification of catecholamines in human brain tissues, and found that the levels of dopamine, as well as its major metabolite, HVA were significantly decreased in the brains of cognitively impaired HIV-1 infected individuals compared to that in normal control brains (unpublished data).

It is well recognized that one of the major complications of HIV-1 infection is the progressive neurodegeneration of different areas of the brain;[26,27] it is manifested as impairment of motor and cognitive functions during life. Since the role of dopamine in cognitive and motor functions is well documented,[1] it is pertinent to establish a direct relationship between deficits in central dopamine and impairment of motor and cognitive functions in HIV-1 infection. For investigating such a relationship, the present study was carried out to achieve higher recovery and sensitivity of detection of dopamine at <4.0 pg and that of HVA at <10.0 pg, using highly sensitive CoulArray HPLC-ECD technology, in order to quantify the anticipated concentrations in small quantity of HIV-1 infected human brain tissue.

EXPERIMENTAL

Materials and Methods

Chemicals

All standards including dopamine HCl (DA), dihydroxybenzylamine HBr (DHBA) as an internal standard (IS) for dopamine, homovantllic acid (HVA), and isoproterinol (ISOP) as an IS for HVA, as well as chemicals for mobile phase including sodium acetate, citric acid, sodium octyl sulphonate (SOS), disodium-ethylene-diaminetetra-acetic acid (NA_2-EDTA), dibutylamine and methanol, activated alumina, zinc sulphate, sodium hydroxide, and perchloric acid (PCA) were of HPLC grade and were purchased from Sigma Chemical Co (St Louis, MO, USA).

HPLC Equipment

The CoulArray-HPLC-ECD system (ESA, Chelmsford, MA, USA) was employed for all assays presented in this report. The equipment consists of a coulometric multi-electrode chemical detector system (Model 5600A CoulArray Detector), comprised of a control module containing 4 channels and each channel consisting of 4 electrochemical cells set in series, thus providing a total of 16 working electrodes made of porous graphite. The working electrode can be set at increasing potentials for oxidation and reduction of a number of analytes present in a single sample extract. The cells, the column, and the injector valve are housed in a thermal chamber that maintains the temperature. The other parts of the system include an auto-sampler and injector (Model 540) and a programmable dual-pump solvent delivery module (Model 582), as well as a CoulArray for windows application software. The chromatographic separation of dopamine, HVA, and the internal standards were carried out with the reverse phase stainless steel (3.9×150 mm), 5 μm, C_{18}, Resolve column (Waters, Milford, MA, USA). Two guard columns, 0.45 μ and 0.2 μ, respectively, were placed before the resolve column for trapping any extraneous particulate matter in the sample extracts.

Mobile Phase

Mobile phase for elution of dopamine had the following composition: Sodium acetate 50 mM, citric acid 50 mM, SOS 0.5 mM, disodium-EDTA 0.15 mM, dibutylamine 1.0 mM, and methanol 14%. The solution was prepared in HPLC-grade water and adjusted to pH 4.0.

The mobile phase for HVA consisted of sodium acetate 0.1 M, citric acid 0.1 M, SOS 0.5 mM, Na_2EDTA 0.15 mM, dibutylamine 1.0 mM, methanol 20%. The mixture was adjusted to pH 4.0. Both mobile phase solutions were prepared fresh, filtered through a 0.2 μm filter, and degassed with ultrasonic agitation (Bransonic ultrasound bath) before use in order to eliminate air bubbles, which interfere greatly in the electrochemical assay. Deionized HPLC-grade water used for preparation of all solutions was obtained from an in-house Millipore Milli-Q, reverse osmosis system (Bedford, MA), resistance of 18.3 megohm-cm.

Standard Solutions

Standard stock solutions of dopamine, and HVA, and the internal standard, DHBA for dopamine and ISOP for HVA, were prepared at a concentration of 1 mg/mL in HPLC-grade water and stored at 4°C. Working standard solutions of dopamine and HVA were prepared fresh from stock solutions before use at a concentration of 0.2–500 pg/mL, and 0.1–100 ng/mL respectively. Concentration of freshly prepared working solutions of

internal standards, DHBA and ISOP was kept constant at 100 pg/mL and 4.0 ng/mL, respectively.

Standardization of Dopamine: Test of Linearity

Freshly prepared mobile phase at pH 4.0 was circulated through the HPLC system at a flow rate of 1.0 mL/min for a few hours, or overnight, for equilibration of the system, and was recycled during equilibration phase, but discarded during standardization and analysis of the samples. Calibration and standardization of DA was carried out by injecting a 40 μL aliquot of the working standard mixture containing dopamine (100 pg/mL) and DHBA (100 pg/mL) for initial identification of individual peaks by their retention times. Elution of DA and DHBA was carried out with mobile phase at a flow rate of 1.0 mL/min. All analyses were performed at ambient temperature. In order to achieve the maximum detector response for oxidation/reduction and detection of DA, the optimum potential was set at 220 mV and sensitivity at 2 nA. Validation of retention times and peak heights and peak area under the curve (AUC) was achieved by a second injection of the same concentration of DA and DHBA. Linearity was tested by injecting a range of dopamine standards containing 0.2–500.00 pg/mL and a constant concentration of DHBA (100 pg/mL). The response factor (RF) for each concentration of DA was calculated from the ratio of the peak area of DA to that of DHBA. The HPLC-ECD profile of dopamine and DHBA is given in Figure 1, and a standard curve of dopamine is shown in Figure 2. The validity of the procedure was determined by the consistency of the relationship between each concentration and the corresponding RF value. Retention times of dopamine and DHBA remained stable over time (Table 1).

Recovery of Dopamine Standards from Spiked Aqueous Solution: Extraction by Alumina Adsorption

Recovery and reproducibility was assessed by extraction of 7 aliquots of aqueous solution spiked with the same concentration (100 pg/mL) of dopamine standard and a constant concentration of DHBA, and extracted using activated alumina procedure of Anton and Sayre,[13] and as described earlier for plasma.[23] Briefly, each aliquot of aqueous solution containing DA standard was mixed with alumina and shaken for 15 minutes on a mechanical rocker and centrifuged. The supernatant was discarded and alumina was washed twice with 1:100 dilution of 2 M Tris-HCl, PH 8.7. Dopamine and DHBA were eluted with 200 μL of 0.1M acetic acid and centrifuged. The supernatant was filtered and a 40 μL aliquot was injected into the CoulArray HPLC-ECD system, using the potential and nA settings described above. The percent recovery and reproducibility of aqueous extraction of DA are given in Table 2.

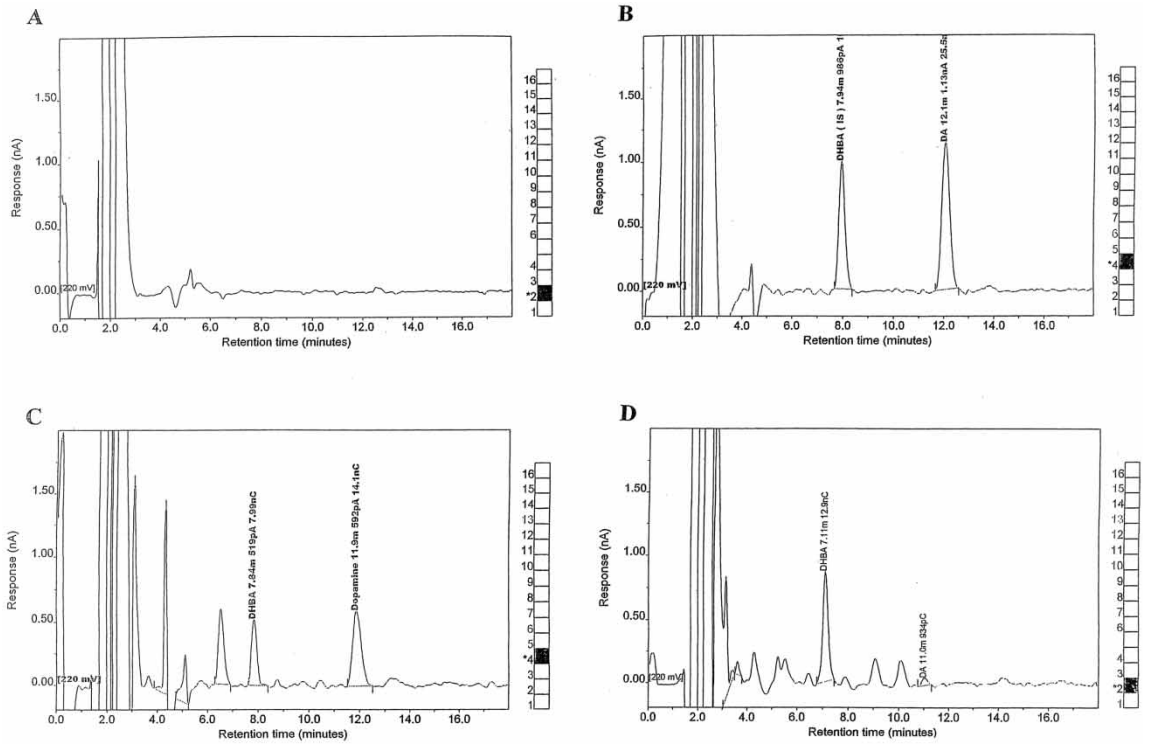

Figure 1. Chromatographic profiles shown are of mobile phase as base line (1A), dopamine standard and DHBA as IS (1B), profile of DA from brain tissue homogenate extracted with alumina (1C) and that treated with $ZnSO_4$/NaOH (1D).

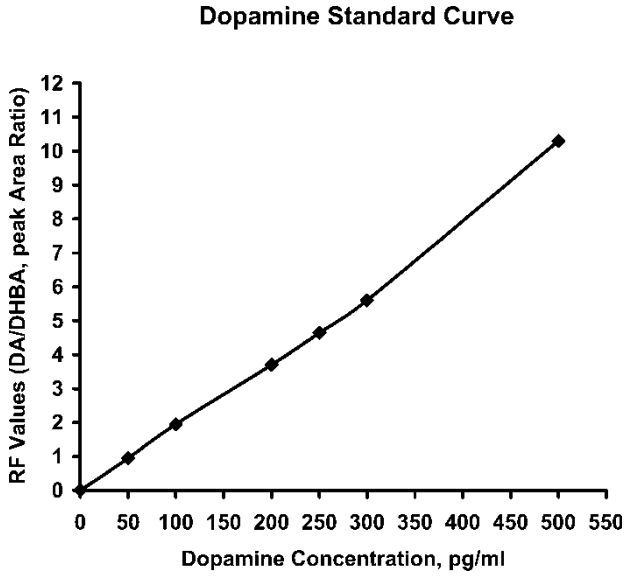

Figure 2. Standard curve of DA showing a linear relationship between a wide range of concentrations (50–500 pg/mL) and the corresponding RF values.

Recovery of dopamine from FC homogenate was examined by spiking seven aliquots of the homogenate with different concentrations of dopamine standards (5–500 pg/mL). Prior to spiking with the standards, dopamine was quantified in aliquots of the same FC homogenate. Percent recovery of dopamine in various aliquots is shown in Table 2.

Dopamine Extraction from Brain Tissues: Alumina Adsorption Method

Brain tissue, frontal cortex (FC) was kept frozen at -80°C until analyzed. Tissues were handled as biohazard materials and the guidelines were followed strictly as proposed by the office of safety and health administration (OSHA). The tissue was weighed into an eppendorf tube and homogenized in chilled phosphate buffer saline (PBS), PH 7.4, with a disposable plastic mini-homogenizer to a concentration of 200 mg/mL. The homogenate was centrifuged at 3000 rpm at 4°C for 15 minutes. The supernatant was transferred to a chilled tube kept in ice. Eight, 1.0 mL aliquots of the supernatant were used for extraction of dopamine by the alumina adsorption procedure as described above, and an aliquot (40 μL) of the filtered eluate was injected for separation of DA and DHBA. Reproducibility of extraction and detection of dopamine in the frontal cortex homogenate is shown in Table 3.

Table 1. Dopamine standard curve & assessment of sensitivity of detection

No.	DA, injected (pg/mL)	DA, detected (pg/mL)	DA detected (%)	DA, RT	IS, RT	R, DA AUC, DA/IS
1	0.2	0.148	74.0	11.23	7.20	0.00032
2	1.0	0.82	82.0	11.24	7.21	0.0184
	1 and 2	Low conc.	No-linear	Relationship		
3	50.0	49.3	98.6	11.24	7.20	0.95
4	100.0	95.6	95.6	11.23	7.19	1.95
5	200.0	194.0	97.0	11.24	7.19	3.70
6	250.0	212.0	84.8	11.23	7.18	4.65
7	300.0	264.0	88.3	11.23	7.19	5.60
8	500.0	478.0	95.6	11.23	7.19	10.33
Mean $\pm$ SD			#3–8 = 93.3 $\pm$ 4.99 CV = 5.35 %	11.24 $\pm$ 0.013 CV = 0.11%	7.19 $\pm$ 0.006 CV = 0.08%	

Table 2. Percent recovery of dopamine spiked into aqueous solution and to brain homogenate using alumina extraction method

Type of treatment	Recovery (%)	DA, RT	IS, RT	RF, DA
DA, spiked 100 pg/mL for aqueous extraction	92.0 ± 2.94 (n = 7) CV = 3.2%	11.63 ± 0.16 (n = 7) CV = 1.38%	7.66 ± 0.19 (n = 7) CV = 2.5%	1.59 ± 0.18 (n = 7)
DA, spiked 5-500 pg/mL for brain homogenate extraction	87.35 ± 7.54 (n = 7) CV = 8.64%	11.95 ± 0.03 (n = 7) CV = 0.27%	7.88 ± 0.02 (n = 7) CV = 0.3%	0.32–8.19
Inter-assay variance	CV = 5.05%	CV = 2.7%	CV = 2.8%	

Dopamine Extraction from Brain Tissue Homogenate Using Alumina or $ZnSO_4$/NaOH

Our investigations on dopamine extraction from tissue homogenate using alumina, revealed that recovery of dopamine from small quantity of human brain tissue was low when less than 1.0 mL of homogenate was treated with alumina. Since the volume of brain tissue homogenate available for DA measurement was approximately 200 μL (available tissue weights were small from specific brain regions, 40 mg/200 μL), we attempted to circumvent the alumina procedure by precipitating proteins in 200 μL of homogenate

Table 3. Dopamine levels in frontal cortex homogenate: reproducibility of extraction with alumina, and of parameters of detection (RTs and RF)

No.	DA, homogenate (pg/mL)	DA, RT	IS, RT	RF, DA
1	82.97	11.88	7.84	1.76
2	79.77	11.93	7.86	1.45
3	94.48	11.93	7.86	2.00
4	86.09	11.93	7.86	1.62
5	97.80	11.93	7.86	2.16
6	90.02	11.94	7.87	1.78
7	86.89	11.93	7.87	1.61
8	97.8	11.93	7.86	2.16
Mean ± SD	89.47 ± 6.31 CV = 7.05 %	11.92 ± 0.016 CV = 0.13 %	7.86 ± 0.008 CV = 0.001 %	1.82 ± 0.25 CV = 13.6 %

with 20 μL of 20% $ZnSO_4$, followed by 2 μL of 5N (20%) NaOH. The mixture was kept in ice for 15 minutes, and centrifuged at 3000 rpm at 4°C. The supernatant obtained was filtered through 0.2 micron syringe filter and 40 μL was injected into the CoulArray HPLC-ECD system. These experiments with different volumes of homogenate were carried out with multiple aliquots drawn from the same pool of pig brain homogenate (0.2 g tissue/mL buffer). The aliquots used were, 7 aliquots of 200 μL each, 3 aliquots of 500 μL each, and 3 aliquots, 1000 μL each. The data on concentration of DA extracted from 200 μL of homogenate treated with alumina or with $ZnSO_4$/NaOH are shown in Table 4, and the data on 500 μL and 1000 μL are described in the results section.

Standardization of HVA

The CoulArray HPLC-ECD system was calibrated for HVA and standardization was carried out by the procedure similar to that of dopamine, but with different concentrations of working standards of HVA and the internal standard, ISOP, each at 4.0 ng/mL. Elution of HVA and IS was carried out with mobile phase at a flow rate of 0.7 mL/min. Since we expected a low concentration of HVA in the small quantity of available tissue from the human brain, the detection system was programmed for a higher level of sensitivity (between 0.00–2.00 nA response and the optimum potential set at 300 mV). The response factor was calculated from the peak area ratio of each concentration of HVA to that of a constant concentration of ISOP (4.0 ng/mL). The chromatographic profile of HVA and ISOP is given in Figure 3. The standard curve showing a linear relationship between the response factors and low concentrations (0.1–2.0 ng/mL), as well as high concentrations of HVA (0.5–20.0 ng/mL), are shown in Table 5 and in Figures 4A and 4B

Extraction of HVA from Brain Tissue: Treatment with PCA

Brain tissue homogenate was prepared as described above for measurement of dopamine, and 6 aliquots from the homogenate pool were used for measurement of HVA. The subsequent steps of centrifugation at 3000 × g and extraction of HVA were carried out at 0–4°C. The internal standard, ISOP (4.0 ng/mL) was added to each 200 μL aliquot of the homogenate supernatant and the mixture was treated with 200 μL of 1.0 M PCA for precipitation of proteins. Extraction of HVA and ISOP was carried out with 750 μL of diethyl ether. The tubes were immediately closed and vortexed for 15 seconds, shaken by hand for 5 minutes, and briefly centrifuged to separate the phases. The ether phase, 500 μL, was carefully transferred to a glass tube (12 × 75 mm) and evaporated to dryness under vacuum for 15 minutes. The residue was reconstituted in 200 μL of sodium acetate, pH 5.0, and filtered through a 0.2 μ syringe filter and a 40 μL aliquot was injected into the CoulArray HPLC-ECD system. Both HVA and IS were detected at the sensitivity range given above.

Table 4. Reproducibility of dopamine quantification in low volumes (200 μl) of brain tissue homogenates: extraction with alumina adsorption procedure or $ZnSO_4$/NaOH treatment

No.	Vol of homogenate (μL)	DA, alumina adsorption (pg/mL)	RF, DA	DA, $ZnSO_4$/NaOH treatment (pg/mL)	RF, DA
1	200	926.86	7.42	1723.87	7.55
2	200	903.24	7.22	1792.62	7.85
3	200	951.32	7.65	1770.56	7.80
4	200	852.36	6.84	1867.95	8.19
5	200	852.36	6.84	1741.22	7.63
6	200	873.96	6.96	1749.69	7.67
7	200	848.9	6.80	1787.19	7.83
Mean ± SD		887.0 ± 37.8 CV = 4.26 %	7.10 ± 0.31 CV = 4.36 %	1776.16 ± 43.9 CV = 2.47 %	7.79 ± 0.19 CV = 2.49 %

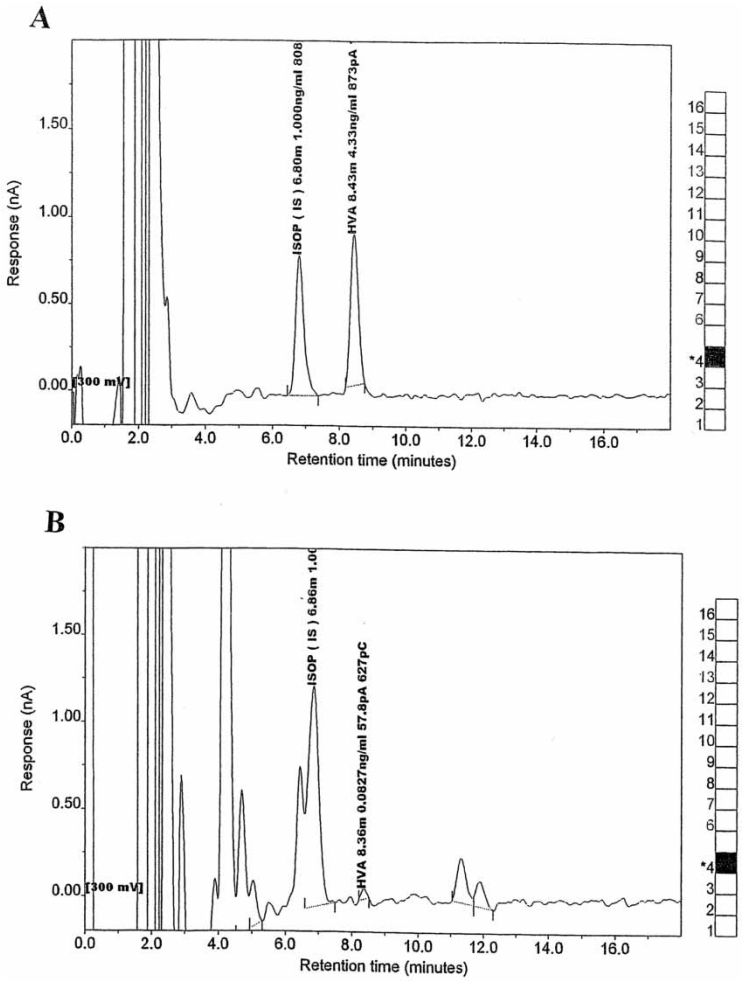

Figure 3. Chromatographic profile of mobile phase for HVA was similar to that of Figure 1A. A) Chromatographic profile of HVA standard and the IS, ISOP; and B) HVA extracted from the human brain tissue.

Recovery of HVA Standards from Spiked Aqueous Solution: Treatment with PCA Precipitation

Recovery and reproducibility of quantification of HVA were evaluated by spiking 200 μL of aqueous solution (buffer) with a range of standards (0.1–100 ng/mL) and a constant concentration of ISOP. The mixture was treated with 200 μL of 1.0 M perchloric acid (PCA), vortexed and kept on ice for 15 min, and centrifuged at 13,000 rpm for 10 min at 4°C, in order to maintain the consistency of the procedure for extraction of HVA from the

Table 5. HVA standard curve: assessment of sensitivity of detection

No.	HVA, injected (ng/mL)	HVA, detected (ng/mL)	HVA detected (%)	HVA, RT	IS, RT	RF = AUC of HVA/IS
1	0.1	0.106	106	8.37	6.78	0.014
2	0.2	0.153	76.5	8.43	6.79	0.0262
3	0.3	0.276	92.0	8.48	6.79	0.046
4	0.4	0.304	76.0	8.43	6.80	0.054
5	0.5	0.418	83.6	8.47	6.80	0.080
6	1.0	0.644	64.4	8.48	6.80	0.175
7	2.0	1.97	98.5	8.44	6.81	0.442
8	4.0	4.33	108.25	8.43	6.80	1.021
9	10.0	14.45	144.5	8.43	6.81	2.752
10	20.0	21.03	105.15	8.43	6.81	5.286
Mean $\pm$ SD			95.49 $\pm$ 21.5	8.43 $\pm$ 0.03 CV = 0.35%	6.8 $\pm$ 0.01 CV = 0.15%	AUC IS = 13.23 $\pm$ 1.67 CV = 12.62%

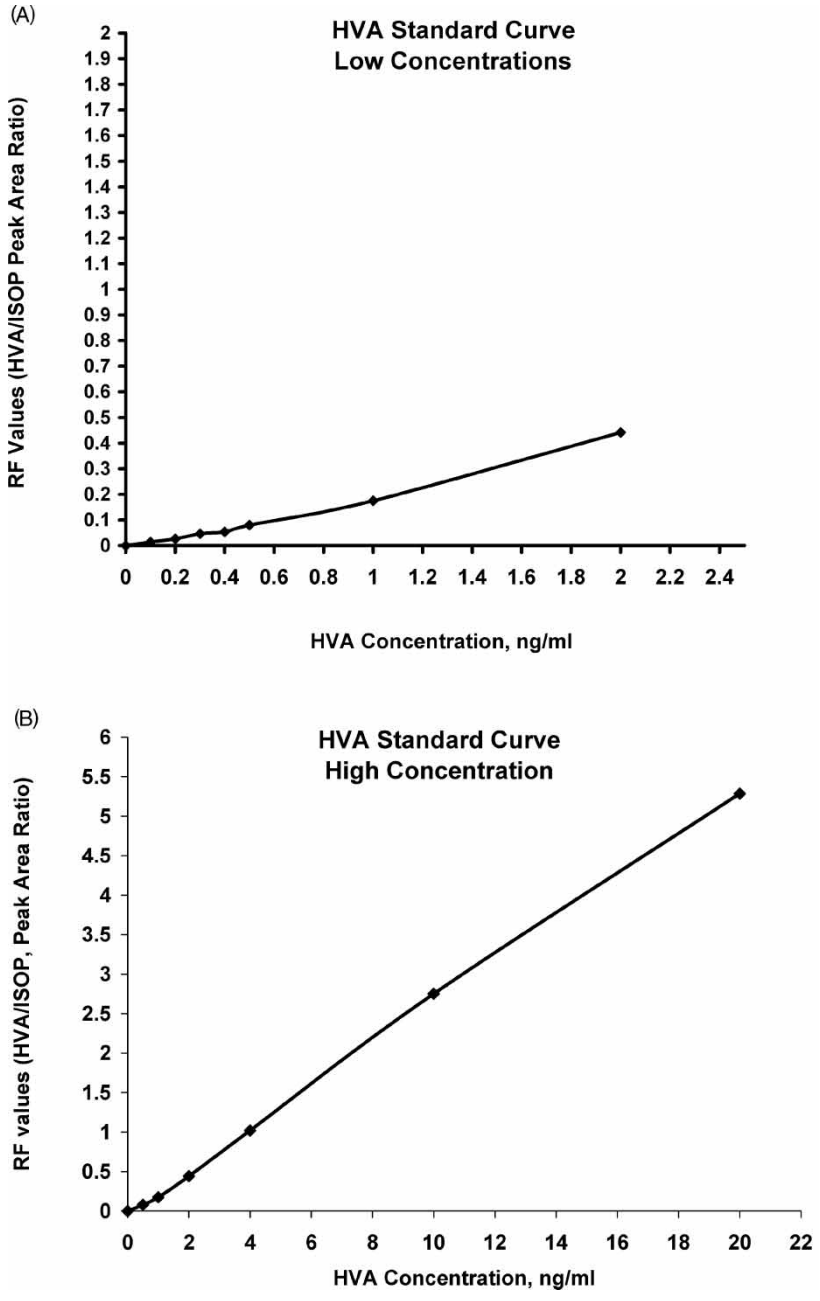

Figure 4. A) Standard curve of HVA showing a linear relationship between low concentrations (0.1–2.0 ng/mL) and the corresponding RF values. B) Standard curve showing a linear relationship between high concentrations of HVA (0.5–20 ng/mL) and the corresponding RF values.

brain tissue. The supernatant containing HVA was treated with 750 μL of diethyl ether and the ether extract processed as described above. An aliquot, 40 μL, was injected for detection of HVA at the above mentioned potential and sensitivity. Recovery of HVA standards from aqueous solution by this method is given in Table 6.

Recovery of HVA from Brain Tissue Homogenate: PCA Treatment

Reproducibility and validity of extraction of HVA was assessed by the recovery of HVA standards (0.1–20.0 ng/mL) spiked into 10 separate

Table 6. Experiments for HVA standard curve, extraction from brain tissue homogenate and percent recoveries from aqueous and tissue homogenates

Type of treatment for extraction and detection/recovery of HVA	HVA, (ng/mL) % detection/recovery	HVA, RT	ISOP, RT
HVA standard curve concentration injected, 0.1–20.0 ng/mL	95.49 ± 21.5 (n = 10) (% Detected)	8.43 ± 0.03 (n = 10), CV = 0.35%	6.8 ± 0.01 (n = 10), CV = 0.15%
HVA, ng/mL in FC homogenate	0.487 ± 0.056 (n = 6) CV = 11.5%	8.45 ± 0.03 CV = 0.35%	6.83 ± 0.005 CV = 0.073%
HVA, % recovery, 0.1–100 ng/mL, spiked for aqueous extraction (PCA treatment)	77.9 ± 16. 3 (n = 11) CV = 20.9%	8.47 ±0.046 CV = 0.5%	6.83 ± 0.06 CV = 0.88%
HVA, % recovery, 0.1–20.0 ng/mL spiked in to FC homogenate (PCA treatment)	78.64 ± 8. 58 (n = 10) CV = 10.9%	8.38 ± 0.015 CV = 0.18%	6.83 ± 0.06 CV = 0.88%
HVA % recovery, 0.1–20.0 ng/mL spiked for aqueous extraction ($ZnSO_4$/NaOH treatment)	99.8 ± 13.13 (n = 9) CV = 13.15%	8.49 ± 0.023 CV = 0.28%	6.85 ±0.013 CV = 0.013%
HVA, % recovery, 0.1–20.0 ng/mL spiked in to FC homogenate ($ZnSO_4$/NaOH treatment)	90.28 ± 9. 24, (n = 10) CV = 10.23%	8.46 ± 0.01 CV = 0.12%	6.86 ±0.01 CV = 0.15%

aliquots of the same homogenate, and HVA was extracted as described above. The percent recovery of HVA by this method is also given in Table 6.

Recovery of HVA from Aqueous Solution: Treatment with $ZnSO_4$/NaOH

Since we observed an improvement in the percent recovery of dopamine with $ZnSO_4$/NaOH as described above for <1.0 mL (200 μL) volume of brain tissue homogenate, we attempted to use the same procedure for extraction of HVA from a small volume of the homogenate. For standardization purposes, however, 200 μL of aqueous solution (buffer) was spiked with different concentrations of HVA ranging between 0.1–20.0 ng/mL and treated with 20 μL of 20% ZnSO4, mixed, and kept in ice for 15 minutes, followed by addition of 2 μL of 20% NaOH. The mixture was vortexed and centrifuged at 13,000 rpm for 10 minutes. HVA in the supernatant was extracted with 750 μL diethyl ether, and the extract was processed as described above. A 40 μL aliquot was injected into the pre-calibrated HPLC system for separation of HVA and IS. Recovery of the aqueous extraction of HVA with $ZnSO_4$/NaOH is also given in Table 6.

Extraction of HVA from Brain Tissue: Treatment with $ZnSO_4$/NaOH

Multiple aliquots ($n = 10$) of 200 μL each from the pool of the brain tissue homogenate supernatant, prepared as describe above (200 mg/mL), were mixed with 4.0 ng/mL of IS, and the mixture was treated with 20 μL of 20% $ZnSO_4$, and kept on ice for 15 minutes, followed by 2 μL of 20% NaOH, mixed and centrifuged. Extraction of HVA and IS with diethyl ether, evaporation of the extract, and reconstitution of the residue with sodium acetate, was carried out as described above. The filtered extract (40 μL) was injected into the pre-calibrated HPLC system. The results of the recovery of a range of standards (0.1–20.0 ng/mL) of HVA are shown in Table 6.

Statistics

Results are expressed as Mean ± SD. Statistical significance was examined by the use of the Student's t-test when applicable.

RESULTS

Standardization of Dopamine

A typical chromatographic profile of the mobile phase as baseline, standards of dopamine and DHBA, and extracts of human frontal cortex are shown in Figure 1A–1D.

A standard curve generated with various concentrations of DA (0.2–500.0 pg/mL) to examine the detection limit at lower and higher concentrations, is given in Table 1. Percent detection of dopamine ranged between 84.8–99% of the injected concentration (50–500 pg/mL). Although, DA could be detected at as low as 0.2 and 1.0 pg/mL (0.008 and 0.04 pg/40 μL injection volume), this limit of detection of DA was not found to be reliable for analysis of the samples. A linear relationship was observed only between concentrations of 50–500 pg/mL of DA and the respective RF values, as shown in Figure 2.

Recovery of multiple aliquots ($n = 7$) of a standard solution of DA (100 pg/mL) after aqueous extraction with alumina was >90% (mean ± SD, 92.0 ± 2.92%), and reproducibility assessed by the coefficient of variance was <4% (CV = 3.2%). Stability of the system was evaluated by the consistency of retention times and AUC of both dopamine and IS. The RF value for 100 pg/mL of DA was 1.59 ± 0.18 (Table 2).

Percent recovery of dopamine from FC homogenate supernatant was measured in 7 separate aliquots spiked with varying concentrations of dopamine (5–500 pg/mL). Recovery was calculated by subtracting the value of DA in homogenate from the total value (DA concentration in homogenate + spiked concentration of DA standard), and the percent recovery (87.35 ± 7.54%, CV = 8.64%) is shown in Table 2. The retention times of DA (11.95 ± 0.03, CV = 0.27%) and IS (7.88 ± 0.02, CV = 0.3%) were also consistent and stable throughout the assay.

Validity of the procedure for the extraction of dopamine from brain tissue, and its detection, was assessed by analyzing 8 separate aliquots of the frontal cortex homogenate supernatant using an alumina adsorption procedure. Reproducibility of the procedure and stability of the system is evident from SD and %CV of DA concentration in 8 aliquots of the homogenate (89.47 ± 6.31, CV = 7.05%), retention times of DA (11.92 ± 0.016, CV = 0.13%) and IS (7.86 ± 0.008, CV = 0.001%), as well as RF values of DA (1.82 ± 0.25, CV = 13.6%) are as shown in Table 3.

The results in Table 4 demonstrate dopamine concentrations measured in multiple aliquots ($n = 7$) of 200 μL of the brain tissue homogenate (drawn from the same pool) treated with either alumina adsorption method for extraction, or with ZnSO4/NaOH for protein precipitation. Although, aliquots of 500 μL and 1000 μL were also treated with alumina, the results presented in the Table are only for 200 μL. Concentration of DA (pg/mL) in 7 aliquots of 200 μL each was almost 50% lower when treated with alumina (887.0 ± 37.8, CV = 4.26%) than when treated with $ZnSO_4$/NaOH (1776.16 ± 43.9, CV = 2.47%). Furthermore, with the alumina adsorption method, DA concentration did not increase proportionately with an increase in the homogenate volume from 200 μL to either 500 μL (937.55 ± 22.85, CV = 2.43%, $n = 3$), or 1000 μL (1744.5 ± 15.29, CV = 0.87%, $n = 3$). Treatment with $ZnSO_4$/NaOH was, therefore, found more favorable for lower volumes of homogenate of smaller tissue weights for measurement of

DA concentration. However, reproducibility for extraction of DA by both the methods was within the acceptable range.

Standardization of HVA

The chromatographic profile of HVA and ISOP is shown in Figures 3A–3B. The elution profile of the mobile phase was similar to that obtained for dopamine (Figure 1A). Retention times of HVA and ISOP are almost two minutes apart.

The standard curve of HVA ranging in concentration between 0.1–20.0 ng/mL is shown in Figures 4A and 4B. The linearity between the lower concentration range of HVA and RF values is presented separately from that of the high concentration, since the RF values of lower concentrations were found to cluster near the baseline if one scale was used to accommodate all values. Percent recovery of HVA standards, however, was more consistent and reliable in the range of 0.1–20.0 ng/mL, compared to that of lower concentrations (0.1–0.5 ng/mL) or of higher concentrations (0.1–100 ng/mL).

The data presented in Table 6 show results of six separate experiments, including an assessment of sensitivity of detection, concentration of HVA extracted from frontal cortex homogenate, percent recovery of HVA extracted from an aqueous solution spiked with standards of different concentrations and treated with PCA, percent recovery of HVA spiked into the aliquots of the same homogenate treated with PCA, and that of aliquots treated with $ZnSO_4$/NaOH before extraction with diethyl ether. The data are presented as Mean $\pm$ SD, and % CV.

DISCUSSION

This study evaluated the efficiency of the methods of extraction and sensitivity of detection for quantification of dopamine and HVA in a small quantity of human brain tissue. With the modifications introduced in the methods of extraction as well as detection, we were able to increase, many fold, the lower limits of quantification of dopamine ($<$4.0 pg) and HVA ($<$10.0 pg) using the highly sensitive multi-channel multi electrode CoulArray HPLC-ECD system. The catecholamine column C_{18} reverse phase 5 μ (3.9 $\times$ 150 mm) was used for separation of both DA and HVA, and was protected by two guard columns (0.45 μ and 0.2 μ) used in series. Dopamine and HVA were extracted from separate aliquots of the same brain tissue homogenates and quantified at specific potential and sensitivity settings required for each analyte. As described in the method section, the mobile phase used for elution of DA and HVA differed in the concentration of some of the components and flow rate for elution.

The chromatographic profile of the elution buffer as the baseline (Figure 1A) was without electrical noise. The profile of dopamine and DHBA in a standard mixture (1B), as well as from the extract of the brain tissue homogenate treated with alumina (1C) or $ZnSO_4$/NaOH (1D), show distinct peaks of both analytes separated by almost 4 minutes. The stability of the HPLC-ECD system is evident from the consistency of retention times (RTs) for DA (11.63 ± 0.16) and DHBA (7.66 ± 0.19), with almost no shifts in the subsequent experiments (Table 2). A linear relationship between a range of concentration of dopamine (50–500 pg/mL) and their respective response factors (RF) demonstrate a high degree of precision in the overall performance of the system and its capability for quantification of as low as 2 pg of DA, a 10 fold increase from 20 pg achieved in the earlier studies.[23,28,29] Although, DA could be detected at as low as 0.2–1.0 pg/mL (0.008–0.04 pg in 40 μL injection volume) at the level of sensitivity and optimum potential of the detector system (0–2.0 nA, 220 mV) used in this study, this limit of 0.2 pg was, however, found to be an outlier in the linear relationship between various concentrations of dopamine standards and the corresponding RF values. In earlier studies using a single electrode HPLC-ECD system, the detection limit for DA was 100 pg and 80 pg, respectively, when the system was set at the highest sensitivity of detection,[18,22] whereas with the CoulArray HPLC-ECD system, we reported the detection of 8.0 pg of DA,[23] which was a 10–12 fold increase from the studies cited above.

The percent recovery of DA (Table 2) in multiple aqueous extractions of 100 pg/mL, and in the spiked frontal cortex with varying concentrations of DA (5–500 pg/mL), show a higher recovery than that reported in the earlier studies whether DA was detected electrochemically[18,19] or fluorometrically.[30]

Dopamine concentration in the HIV-1 infected human frontal cortex used in this study is lower as expected (0.0895 ng/g wet tissue), and is within the lower range of values reported earlier (0.00–100 ng/g tissue) in normal individuals.[6,31,32] Interestingly, when the volume of human brain tissue homogenate available for quantification of dopamine was less than 1.0 mL and the alumina adsorption method was used for extraction, recovery of DA was lower than when the same homogenate volume was treated with $ZnSO_4$/NaOH and the filtered supernatant was injected into the HPLC-ECD system (Table 4). Although, in the elution profile of the $ZnSO_4$/NaOH extract of the homogenate, some unidentified peaks appeared before DHBA as well as DA; these peaks were distinctly separated and did not interfere with the elution of either DHBA or DA (Figure 1D). Retention times of both DHBA and DA remained consistent for both extracts.

For standardization of HVA, the chromatographic profile of standard and the IS (ISOP), and a human frontal cortex tissue extract were obtained at the same sensitivity settings (0.0–2.0 nA) as that for DA. The sensitivity of detection of HVA at <5 pg/mL by the present method is 10–20 fold higher than that reported in earlier studies, where a single electrode system of

HPLC-ECD was used.[22,24,25] The linear relationship was found between a wide range of concentration of HVA (0.1–100 ng/mL) and the respective RF values, as shown separately in Figures 4A and 4B.

Extraction of HVA and ISOP with diethyl ether is a simple and efficient procedure for purification of DA metabolites and the internal standards. The percent recovery of HVA in different experiments, including that of standards (0.1–100 ng/mL) from aqueous extractions and from tissue homogenates in this study (Table 6), is higher compared to that achieved in earlier studies.[24,25,30,33] However, recovery of HVA in this study was found to be higher when homogenate was treated with $ZnSO_4$/NaOH (90.3 $\pm$ 9.24), than when treated with PCA (78.6 $\pm$ 8.58, range 66–97%) prior to extraction of HVA with diethyl ether. Furthermore, neither of the treatments affected the reproducibility of the percent recovery of HVA ($ZnSO_4$/NaOH, CV = 10.24%; PCA, CV = 10.9%).

The higher recovery of HVA in this study may partly be attributed to the high sensitivity of the CoulArray ECD system, and to the difference in the size and the type of column used (3.9 $\times$ 150 mm, 5 μ $C_{18,}$ reverse phase resolve column) for separation of HVA from an ether extract of brain tissue, compared to the column used in earlier studies (3.9 $\times$ 300 mm, μBondapack C_{18}, reverse phase).[24,25] The other contributing factors may include the difference in the composition of mobile phase, pH, % methanol, and the flow rate of mobile phase for elution of HVA.[24,25] The values of HVA obtained from 6 separate aliquots of the frontal cortex homogenate were 0.487 $\pm$ 0.056 ng/mL, and the recovery of spiked HVA standards (0.1–20.0 ng/mL) in ten aliquots of the same homogenate preparation was 78.46 $\pm$ 8.58% (Table 6).

The stability of the HPLC system for detection of HVA and ISOP is evident from the consistency of the retention times of HVA (8.43 $\pm$ 0.03) and that of the ISOP (6.8 $\pm$ 0.01), with minimum shift in their respective retention times throughout the study. The linear relationship between HVA concentrations and RF values, the detection limit, and % efficiency of detection of HVA, demonstrate the reliability of the method.

In summary, this study describes a highly sensitive and a reliable method for quantification of $<$4 pg and $<$10 pg of central dopamine and HVA, respectively, in small regions of human brain tissues of small weights. Using the CoulArray HPLC-ECD system, the detection limit of as low as 0.2 pg of DA and 4.0 pg of HVA could be achieved by changing the settings of the parameters of sensitivity and potential, as well as the mobile phase composition and flow rates. A high degree of precision is demonstrated by the linearity of the relationship between a wide range of concentrations of DA and HVA and their respective RF values. Stability of the system is evident from the consistency of retention times of both analytes throughout the study. Modifications in the procedures for the extraction of DA and HVA were carefully examined for higher recovery from the aqueous solutions and the brain tissues.

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