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HPLC electrochemical detection and quantification of monoamines and their metabolites in rat brain tissue samples

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The monitoring of monoamines and their metabolites in CNS samples can be very valuable in pharmaceutical and biomedical research. A specific high performance liquid chromatography, coupled to a coulometric electrochemical detection method, for the assay of monoamines (dopamine, norepinephrine, epinephrine and serotonin) and their metabolites in rat brain tissue samples was developed. The chromatographic separation was achieved on a C8 reversed phase column with a mobile phase consisting of 0.1 M sodium formate buffer, 5 mM sodium 1-heptanesulfonate, 0.17 mM ethylenediaminetetraacetic acid disodium salt and 5% v/v acetonitrile (pH ±4.0). The detection was achieved through electrochemical detection, with a coulometric cell potential setting of +650 mV. The flow-rate was at 1 ml/min and the total run time was 50 min. The method was validated according to validation guidelines. The method was found to be linear (R^2 > 0.99) over the analytical range (5 to 200 ng/ml) for all monoamines and their metabolites. All the other validation parameters were acceptable and within range. The method was applied to three rat brain areas (pre-frontal cortex, hippocampus and striatum), where the monoamines (except for epinephrine) and their metabolites were easily detected.

1. Introduction

In the central nervous system (CNS), monoamines are an important group of biogenic amines, which include dopamine, noradrenaline, epinephrine, serotonin and their metabolites (Raven and Johnson 2002; Koolman et al. 2005; Noback et al. 2005; Burtis et al. 2012). Their main function is neurotransmission by means of neuronal or hormonal signals in a variety of physiological processes (Burtis et al. 2012). The imbalances and dysfunction of monoamines are associated with a variety of CNS disorders (Booij et al. 2003; Kurian et al. 2011; Ng et al. 2015). These monoamines are mainly synthesised from two amino acids, tyrosine and tryptophan (Koolman et al. 2005; Burtis et al. 2012). The metabolic pathways of the monoamines are shown in a simplified diagram in Fig. 1.

The objective of this study was to develop a high performance liquid chromatography (HPLC) method to detect and quantify the monoamines in CNS samples, more specific rat brain tissue samples. The method was therefore developed and validated for the detection and quantification of dopamine and its metabolites (3,4-dihydroxyphenylacetic acid, 3-methoxytyramine and homovanillic acid), norepinephrine (noradrenaline) and its metabolite (3-methoxy-4-hydroxyphenylglycol), epinephrine (adrenaline) and serotonin and its metabolite (5-hydroxyindoleacetic acid).

2. Investigations and results

2.1. Method validation parameters

The method was validated according to the guidelines of the FDA concerning linearity, sensitivity (quantification and detection limits), precision (reproducibility), accuracy (repeatability), recovery and stability (US FDA 2013). Selectivity was performed by injecting samples of solution A without any standards or brain tissue. Surrogate matrices (for example artificial cerebrospinal fluid) were used instead of the authentic sample matrix as it is free of monoamines (van de Merbel 2008).

2.1.1. Linearity/calibration curve

Linearity was tested by preparing eight standard concentrations as described in section 4.2.2. Six replicates of each standard were injected to establish linear regression for each analyte. The linear regression value (coefficient of determination, r^2) for the calibration curve must not be less than 0.95 for endogenous biomolecules (Shabir 2005). For calibration, the internal standard method was used with 5-HMT as the chosen internal standard.

2.1.2. Quantification and detection limits (sensitivity)

The lower limit of quantification (LLOQ) and the lower limit of detection (LLOD) can be defined as the minimum concentration where the signal-to-noise ratio is at least 10:1 and 3:1 greater than the average background noise of an unspiked blank (only containing the internal standard), at the retention time of each analyte, respectively (Shrivastava and Gupta 2011).

2.1.3. Precision and accuracy

Four concentrations (5, 10, 75 and 200 ng/ml) were chosen and six determinants for each were done for precision and accuracy. Precision results were expressed in %RSD (relative standard deviation from the mean) and the acceptability criterion for each concentration level was not to exceed 15% (US FDA 2013). The accuracy results for each concentration level were determined by comparing the closeness of the mean test concentration result to that of the true concentration value. The accuracy results were expressed as % recovery. The acceptability criterion for accuracy for each concentration level was to fall between 90 to 110% (US FDA 2013; Shabir 2005).

2.1.4. Recovery

The percentage recovery of the extraction procedure was determined by preparing four spiked concentrations (5, 10, 75 and 200

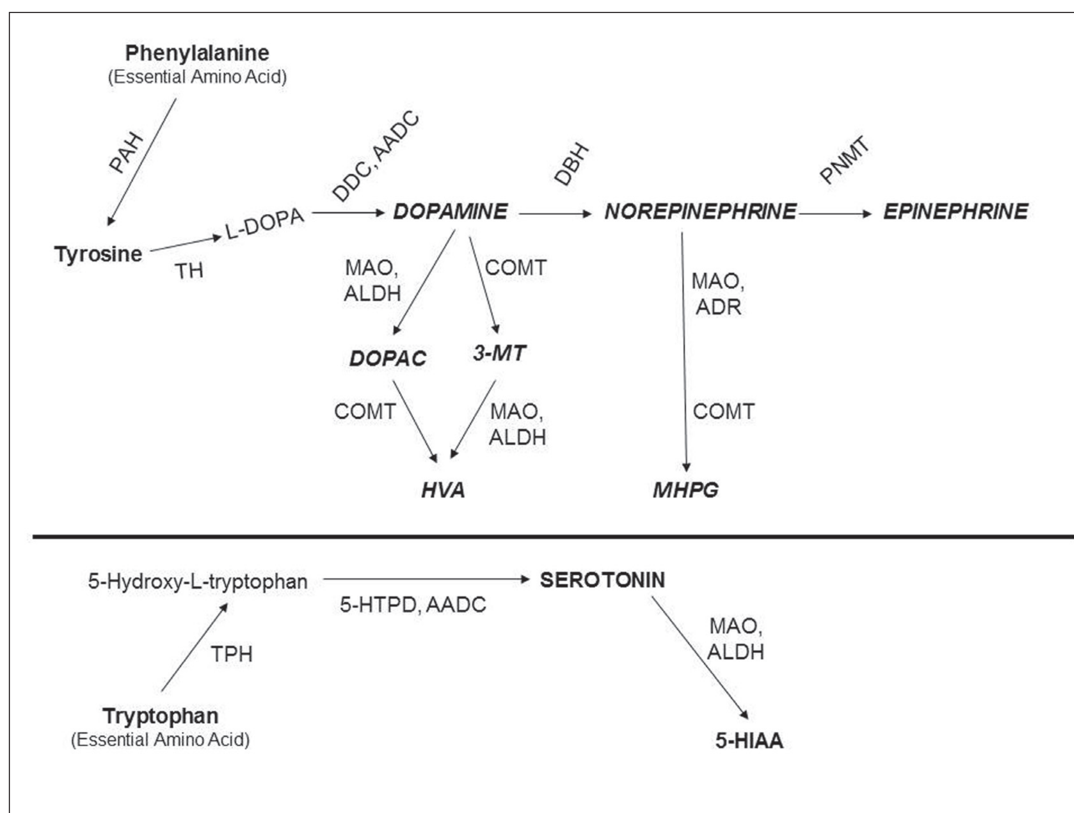

Fig. 1: Monoamine metabolic pathways. PAH: Phenylalanine hidroxylase; TH: Tyrosine hidroxylase; L-DOPA: L-3,4-Dihydroxyphenylalanine; DDC: DOPA decarboxylase (Aromatic L-amino acid decarboxylase/AADC); MAO: Monoamine oxidase; ALDH: Aldehyde dehydrogenase; DOPAC: 3,4-dihydroxyphenylacetic acid; COMT: Catechol-O-methyltransferase; 3-MT: 3-Methoxytyramine; HVA: Homovanillic acid; DBH: Dopamine ß-hydroxylase; ADR: Aldehyde reductase; MHPG: 3-Methoxy-4-hydroxyphenylglycol; PNMT: Phenylethanolamine N-methyltransferase; TPH: Tryptophan hydroxylase; 5-HTPD: 5-Hydroxytryptophan decarboxylase (Aromatic L-amino acid decarboxylase/AADC; 5-HIAA: 5-Hydroxyindoleacetic acid (Modified from literature (Allen et al. 2009; Burtis et al. 2012).

ng/ml) in solution A. The peak areas were compared to those of prepared standards. This was done in triplicate for each concentration and the mean % recovery was calculated. The % recovery for each concentration must be above 90% and consistently reproducible to adhere to the acceptance criterion.

2.1.5. Stability

Stability was determined by injecting the eight standard solutions and two CNS samples directly after sample preparation. The initial injection set was assayed immediately and served as reference values. The samples were reinjected after 24 h and 48 h, while keeping the samples at room temperature in the HPLC auto-sampler tray. Percentage stability for each analyte will be given as the mean % stability of the samples analysed and ±SD at the given hour.

Table 1: Linear regression line equation and coefficient of determination

Monoamines	y = mx + c	Coefficient of Determination (R^2)
Norepinephrine	y = 0.0042x + 0.0208	R^2 = 0.9988
MHPG	y = 0.0069x + 0.0345	R^2 = 0.9989
Epinephrine	y = 0.0029x + 0.0167	R^2 = 0.9989
Dopamine	y = 0.0052x + 0.0288	R^2 = 0.9991
Dopac	y = 0.0040x + 0.0234	R^2 = 0.9989
3-MT	y = 0.0045x + 0.0250	R^2 = 0.9986
HVA	y = 0.0047x + 0.0346	R^2 = 0.9988
Serotonin	y = 0.0073x + 0.0309	R^2 = 0.9993
5-HIAA	y = 0.0064x + 0.0274	R^2 = 0.9989

2.2. Method validation results

2.2.1. Linearity/calibration curve

The calibration curve constructed was evaluated by means of its linear regression value. Linearity was excellent over the respective calibration ranges (Table 1), with corresponding coefficient of determination (R^2) consistently greater than 0.99 for all the analytes.

2.2.2. Quantification and detection limits (sensitivity)

The lower limit of detection (LLOD) and the lower limit of quantification (LLOQ) were determined to be 2.5 ng/ml and 5.0 ng/ml for all the analytes respectively.

2.2.3. Precision and accuracy

The precision and accuracy results of the four concentrations tested are provided in Table 2. Both the precision and accuracy results were within the acceptable criteria ranges set by the method validation parameters in point 2.1. Throughout these four concentrations, the %RSD for both the intra-day and inter-day precision was below 4 %. The accuracy of all concentration levels for all of the analytes tested was between 90.11 and 109.16 %.

2.2.4. Recovery

The mean absolute recovery for each analyte, measured in triplicate for all four concentrations tested, were constantly above 90%, with the mean recovery indicated in Table 3.

2.2.5. Stability

In Table 4, the mean percentage stability results for the all the analytes tested in the eight standards and the two brain tissue

Table 2: Accuracy and precision results in solution A

Analyte concentration (ng/ml)	Intra-day (n=6)			Inter-day (n=6)		
	Measured concentration (ng/ml) (mean±SD)	Precision (%RSD)	Accuracy (%)	Measured concentration (ng/ml) (mean±SD)	Precision (%RSD)	Accuracy (%)
Norepinephrine						
5 (LLOQ)	5.06 ± 0.16	3.25	101.17	4.83 ± 0.10	1.98	96.57
10	10.54 ± 0.11	1.07	105.42	9.86 ± 0.15	1.48	98.63
75	74.86 ± 0.74	0.98	99.81	76.53 ± 0.54	0.71	102.04
200	199.09 ± 2.04	1.03	99.55	202.80 ± 0.45	0.22	101.40
MHPG						
5 (LLOQ)	4.92 ± 0.15	3.05	98.31	4.86 ± 0.09	1.92	97.28
10	10.44 ± 0.22	2.13	104.36	9.65 ± 0.09	0.93	96.49
75	75.30 ± 0.45	0.60	100.40	75.80 ± 0.60	0.80	101.07
200	201.36 ± 1.64	0.81	100.68	199.70 ± 0.91	0.45	99.85
Epinephrine						
5 (LLOQ)	5.38 ± 0.16	3.04	107.62	4.50 ± 0.19	4.15	89.99
10	10.89 ± 0.28	2.60	108.86	9.05 ± 0.10	1.12	90.46
75	76.27 ± 0.40	0.52	101.70	73.77 ± 0.88	1.20	98.36
200	198.36 ± 1.76	0.89	99.18	203.45 ± 0.58	0.29	101.73
Dopamine						
5 (LLOQ)	5.05 ± 0.17	3.27	101.07	4.61 ± 0.09	2.04	92.19
10	10.82 ± 0.31	2.88	108.18	9.36 ± 0.15	1.60	93.58
75	75.80 ± 1.43	1.89	101.06	71.96 ± 0.51	0.71	95.59
200	198.50 ± 1.89	0.95	99.25	201.15 ± 1.59	0.79	100.58
Dopac						
5 (LLOQ)	4.85 ± 0.17	3.56	97.07	4.66 ± 0.11	2.44	93.14
10	10.93 ± 0.37	3.40	109.26	9.36 ± 0.18	1.92	93.55
75	75.53 ± 0.75	0.99	100.71	74.57 ± 0.89	1.19	99.43
200	199.91 ± 1.58	0.79	99.96	201.41 ± 0.77	0.38	100.70
3-MT						
5 (LLOQ)	5.46 ± 0.08	1.44	109.16	4.51 ± 0.18	4.04	90.11
10	9.80 ± 0.23	2.39	97.96	10.29 ± 0.19	1.85	102.90
75	79.62 ± 0.81	1.01	106.17	73.49 ± 1.44	1.96	97.99
200	198.29 ± 3.20	1.61	99.14	201.35 ± 1.98	0.98	100.68
HVA						
5 (LLOQ)	5.05 ± 0.17	3.30	100.99	4.64 ± 0.09	1.98	92.81
10	10.81 ± 0.36	3.33	108.08	9.51 ± 0.27	2.82	95.13
75	78.03 ± 1.43	1.84	104.03	71.87 ± 1.61	2.24	95.82
200	200.37 ± 2.05	1.02	100.19	196.14 ± 1.15	0.59	98.07
Serotonin						
5 (LLOQ)	5.32 ± 0.21	3.86	106.40	4.65 ± 0.22	4.82	93.00
10	10.83 ± 0.20	1.81	108.32	9.48 ± 0.21	2.21	94.76
75	76.68 ± 0.81	1.05	102.24	72.01 ± 0.59	0.82	98.74
200	201.10 ± 2.97	1.47	100.55	196.20 ± 0.97	0.49	98.10
5-HIAA						
5 (LLOQ)	5.43 ± 0.13	2.35	108.56	4.96 ± 0.11	2.30	99.13
10	10.21 ± 0.34	3.37	102.11	9.40 ± 0.13	1.40	93.99
75	76.43 ± 0.83	1.08	101.91	71.80 ± 0.81	1.12	95.73
200	203.01 ± 2.61	1.28	101.51	189.99 ± 2.24	1.18	95.00

samples are demonstrated. All the monoamines and their metabolites were stable when tested at 24 h and 48 h stored at room temperature in the auto-sampler tray for both the standards and the two rat brain tissue samples. Epinephrine however was below the limit of detection in the brain tissue samples and therefore not detected.

Table 3: Percentage recovery results

Monoamines	Concentration (ng/ml)	% Recovery (mean ± SD)
Norepinephrine	5.0 (LLOQ)	96.86 ± 2.59
	10.0	98.14 ± 2.36
	75.0	100.69 ± 0.10
	200.0	97.49 ± 3.01
MHPG	5.0 (LLOQ)	96.32 ± 2.50
	10.0	98.44 ± 0.23
	75.0	96.48 ± 6.51
	200.0	97.35 ± 6.12
Epinephrine	5.0 (LLOQ)	99.14 ± 1.72
	10.0	98.54 ± 2.40
	75.0	98.24 ± 1.29
	200.0	99.65 ± 1.16
Dopamine	5.0 (LLOQ)	96.04 ± 0.73
	10.0	97.22 ± 0.73
	75.0	101.13 ± 0.30
	200.0	97.52 ± 1.26
Dopac	5.0 (LLOQ)	98.16 ± 2.72
	10.0	101.77 ± 1.88
	75.0	101.03 ± 1.35
	200.0	99.33 ± 0.80
3-MT	5.0 (LLOQ)	98.54 ± 0.72
	10.0	97.86 ± 1.85
	75.0	100.61 ± 1.38
	200.0	99.77 ± 0.22
HVA	5.0 (LLOQ)	98.76 ± 2.32
	10.0	98.28 ± 3.20
	75.0	100.86 ± 1.62
	200.0	98.79 ± 0.78
Serotonin	5.0 (LLOQ)	97.52 ± 1.33
	10.0	99.87 ± 1.06
	75.0	97.85 ± 1.20
	200.0	97.44 ± 3.26
5-HIAA	5.0 (LLOQ)	98.55 ± 0.87
	10.0	95.87 ± 1.53
	75.0	94.50 ± 0.08
	200.0	96.08 ± 5.57

2.3. Chromatographic results

Table 5 present the relative retention times of all the monoamines, their metabolites and the internal standard for this method.

Table 4: Stability results for the eight standards

% Stability (mean±SD) of monoamines analytes at room temperature after 24 h and 48 h		
Time	**24 h***	**48 h***
Norepinephrine	99.89 ± 1.46	99.85 ± 1.86
MHPG	99.81 ± 0.57	99.73 ± 1.35
Epinephrine	99.09 ± 1.66	98.68 ± 1.90
Dopamine	99.03 ± 1.89	98.85 ± 2.18
Dopac	99.32 ± 1.16	99.18 ± 1.65
3-MT	99.34 ± 1.28	99.23 ± 2.46
HVA	98.12 ± 2.27	97.71 ± 2.70
Serotonin	99.26 ± 1.76	99.12 ± 1.79
5-HIAA	99.05 ± 1.05	98.72 ± 0.84
Stability results for the two brain tissue samples		
Time	**24 hours***	**48 hours***
Norepinephrine	99.78 ± 0.08	97.23 ± 0.07
MHPG	98.01 ± 0.27	93.46 ± 3.57
Epinephrine	Not detected in brain tissue samples	
Dopamine	99.59 ± 0.26	98.98 ± 0.45
Dopac	99.20 ± 0.16	98.62 ± 0.36
3-MT	99.89 ± 1.02	95.86 ± 1.28
HVA	99.11 ± 0.77	96.23 ± 2.39
Serotonin	99.11 ± 0.34	97.48 ± 1.14
5-HIAA	99.75 ± 1.81	95.21 ± 0.29

*Compared to 100% at t = 0, mean ±SD.

The following three chromatographic figures are representative of a blank sample containing only the internal standard, 5-HMT (Fig. 2), a 5 ng/ml standard (Fig. 3) and a 200 ng/ml standard (Fig. 4).

Table 5: The relative retention times of the monoamines and their metabolites and the internal standard are presented

Analytes	Relative retention times (minutes)
1. Norepinephrine	± 6.378
2. MHPG	± 7.414
3. Epinephrine	± 8.148
4. Dopac	± 10.833
5. Dopamine	± 13.091
6. 5-HIAA	± 21.535
7. HVA	± 25.632
8. 3-MT	± 30.653
9. Serotonin	± 35.929
10. Internal standard (5-HMT)	± 46.413

2.4. Application

The validated method was applied to three different rat brain tissue areas: hippocampus, pre-frontal cortex and striatum. The samples from the different rat brain tissue areas were removed and prepared according to section 4.2.6, and analysed. The internal standard method was used to calculate the concentration of each analyte in the brain tissue samples (CrawfordScientific 2014).

The following three chromatographic figures represent the different rat brain tissue areas that were analysed. The first represents a 69 mg hippocampus (Fig. 5), the second an 85 mg pre-frontal cortex (Fig. 6) and the third a 30 mg striatum (Fig. 7).

In Table 6, the concentrations of the monoamine analytes analysed in the three different brain tissue areas (Figs. 5 to 7) are shown. The results were expressed in ng/g wet weight tissue. Epinephrine levels were below the limit of detection of the method in all three brain tissue samples and MHPG in the striatum.

3. Discussion

A specific HPLC method, coupled to a coulometric electrochemical detection method, was developed for the assay of monoamines

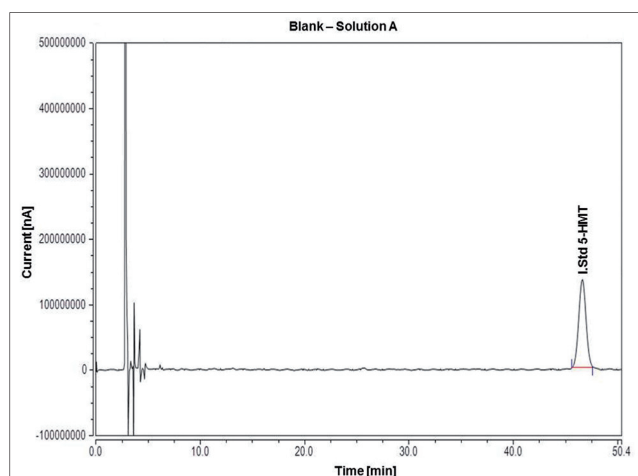

Fig. 2: Blank sample with internal standard (5-HMT)

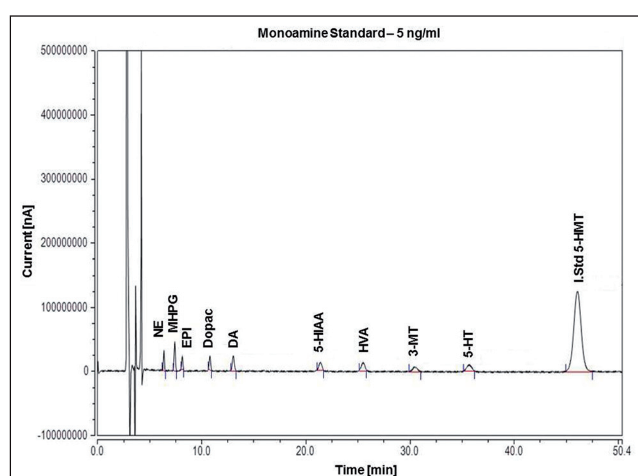

Fig. 3: Monoamine standard – 5 ng/ml

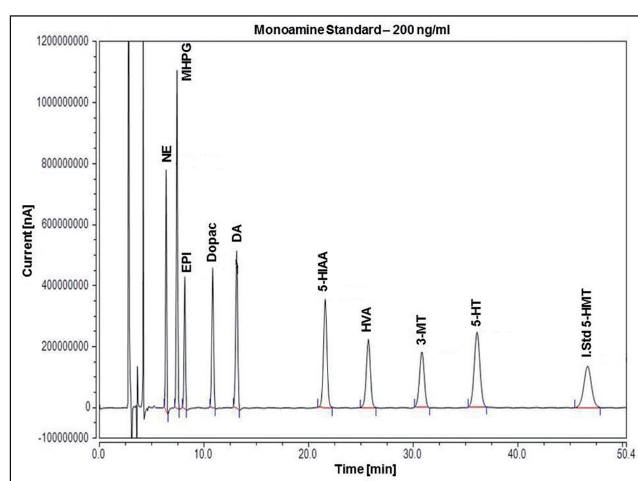

Fig. 4: Monoamine standard – 200 ng/ml

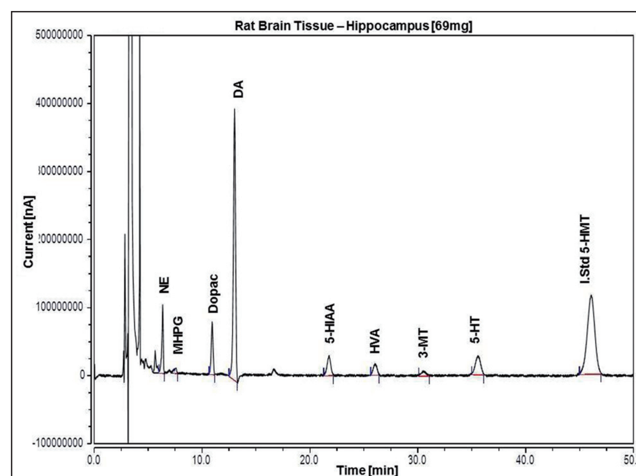

Fig. 5: Rat brain tissue – hippocampus [69 mg]

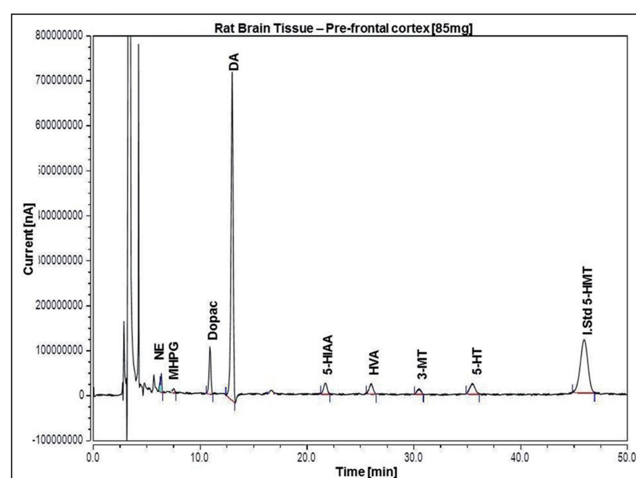

Fig. 6: Rat brain tissue – pre-frontal cortex [85mg]

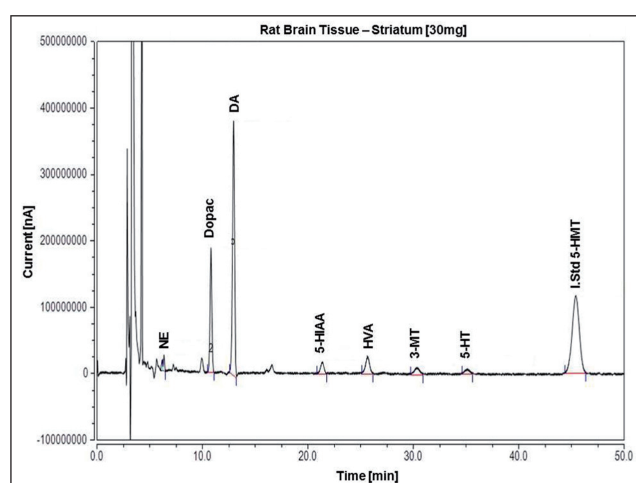

Fig. 7: Rat brain tissue – striatum [30mg]

and their metabolites in rat brain tissue samples. This method was validated for linearity, sensitivity, precision, accuracy, recovery and stability. The linearity for all respective analytes' calibration ranges was excellent and the coefficient of determination (r^2) was consistently above 0.95. The LLOQ and LLOD were 5.0 ng/ml and 2.5 ng/ml, respectively, for the method. The % recovery was acceptable for all the analytes at all the concentrations measured, and the % stability was also acceptable for both 24 and 48 h testing for the standards and the rat brain tissue samples. The method was found to be selective and specific for norepinephrine, dopamine, serotonin and their metabolites in rat brain tissue samples. In the rat brain samples, epinephrine was below the limit of detection. This method can also be used to determine the analytes in cerebrospinal fluid (CSF) samples (Marais et al. 2006) of laboratory animals, however method development and validation must be done in future studies with CSF samples.

Table 6: Rat brain tissue results in ng/g wet weight tissue

Rat brain tissue area	Hippocampus (69 mg)	Pre-frontal cortex (85 mg)	Striatum (30 mg)
Analyte			
Norepinephrine	337.86	33.00	30.84
MHPG	13.96	29.41	BLOD
Epinephrine	BLOD	BLOD	BLOD
Dopamine	2196.43	3243.68	4712.20
Dopac	390.51	463.18	2675.72
3-MT	20.85	28.29	24.88
HVA	64.51	123.77	508.56
Serotonin (5-HT)	253.57	163.92	27.23
5-HIAA	170.28	96.55	179.43

(BLOD - below limit of detection).

We propose that this method can be applied to drug and behaviour studies in laboratory animals (for example rats and mice) to determine the effect on the two monoamine pathways in rodent brain tissue samples. The quantitative analysis of monoamines in CNS samples can provide important information in a variety of studies focusing on CNS related disorders.

4. Experimental

4.1. Chemicals, reagents, materials and instrumentation

4.1.1. Chemicals and reagents

L-Noradrenaline hydrochloride, 3-methoxy-4-hydroxyphenylglycol hemipiperazinium salt, epinephrine bitartrate salt, 3-hydroxythyramine hydrochloride, 3,4-dihydroxyphenylacetic acid, homovanillic acid, serotonincreatinine sulphate, 5-hydroxyindole-3-acetic acid and the 5-hydroxy-Nω-methyltryptamine oxalate (internal standard) were obtained from Sigma-Aldrich Pty (Ltd) (Johannesburg, South Africa). Chemicals used for the mobile phase were HPLC grade deionised water and HPLC grade acetonitrile (ACN); sodium formate; 1-heptanesulphonic acid sodium salt; ethylenediaminetetra-acetic acid disodium salt; orthophosphoric acid (H_3PO_4, 85%). Chemicals used for the sample preparation were ethylenediaminetetra-acetic acid disodium salt; sodium metabisulphite; perchloric acid (60%); potassium acetate. All the chemicals were obtained from Merck (Pty) Ltd (Johannesburg, South Africa).

4.1.2. Materials

The analytical HPLC column used was a Venusil ASB C8 (purchased from Bonna-Agela Technologies, USA), 4.6 x 250 mm, a particle size of 5 µm, pore size of 150 Å and a surface area of 200 m^2/g.

4.1.3. Instrumentation

An Agilent 1200 series HPLC (Agilent Technologies Inc., Santa Clara, CA USA), equipped with an isocratic pump and autosampler, coupled to an ESA Coulochem III Electrochemical detector with a coulometric flow cell (Model 5011A High Analytical Cell and Guard cell 5020) and Chromeleon® Chromatography Management System version 6.8 (obtained from Thermo Fisher Scientific, Waltham, MA USA).

4.2. Methods (standards, mobile phase and sample preparation)

4.2.1. Solution A

Solution A (consisting of 0.1 M perchloric acid; 0.5 mM sodium metabisulphite and 0.3 mM Ethylenediaminetetraacetic acid disodium salt) was used as the preparation solvent for all samples. The purpose of this solution was to keep the monoamines protected from auto-oxidation and for the precipitation of proteins in the biological samples, thus keeping the samples stable for longer (Willemsen et al. 2007; Burtis et al. 2012).

4.2.2. Preparation of standard solutions

Standard stock solution was prepared with a concentration of 100 µg/ml for each of the following analytes; norepinephrine (NE), 3-methoxy-4-hydroxyphenylglycol (MHPG), epinephrine (EPI), dopamine DA), 3,4-dihrydroxyphenylacetic acid (Dopac), 3-methoxytyramine (3-MT), homovanillic acid (HVA), serotonin (5-HT) and 5-hydroxyindole-3-acetic acid (5-HIAA), using solution A as the solvent. From this stock solution, a range (5 ng/ml to 200 ng/ml) of concentrations were prepared to setup a standard calibration curve.

4.2.3. Preparation of the internal standard solution

Standard stock solution of the internal standard, 5-hydroxy-Nω-methyltryptamine oxalate (5-HMT), with a concentration of 100 µg/ml using solution A as the solvent was prepared. The working internal standard solution, with a final concentration of 1500 ng/ml, with an appropriate dilution from the internal standard stock solution using solution A as solvent was prepared.

4.2.4. Mobile phase preparation

A mobile phase consisting of 0.1 M sodium formate buffer, 5 mM sodium 1-heptanesulfonate, 0.17 mM ethylenediaminetetra- acetic acid disodium salt and v/v 5% acetonitrile was prepared. The pH of the mobile phase was set at ± pH 4.00 with ortho-phosphoric acid. The mobile phase was filtered through a 0.22 µm nylon filter before use (Agela Technologies).

4.2.5. HPLC Instrument and electrochemical detector settings

HPLC conditions are summarized in Table 7.

Table 7: Instrumentation settings

HPLC instrument settings	
Flow rate	1.0 ml/min
Injection volume	20 µl
Run time	50 minutes
Electrochemical detector settings	
Cell potential settings	Test electrode 1 (E1):-150 mV (to eliminate background noise)
	Test electrode 2 (E2): +650 mV (to analyse the monoamines)
	Guard Cell (E^{GC}): +350 mV
	Detection range: 500 nA
	Filter: 0.5 seconds
	Offset: 0%
	Signal output: 0.1 V
Data collection rate	20 Hz

4.2.6. Sample preparation of brain tissue samples (Harvey et al. 2006)

Brain tissue samples of different brain areas (hippocampus, pre-frontal cortex and striatum), collected from laboratory animals, male Sprague–Dawley rats, via dissection, were transferred to 1.5 ml Eppendorf tubes, immediately snap frozen with liquid nitrogen and stored at -80 ºC until the day of analysis. On the day of analysis, the brain tissue sample was weighed and 1 ml of solution A was added to the tube. The tissue in the tube was then ruptured by sonication (2 x 12 s, at an amplitude of 14 µ (Keller et al. 1976). The tube was placed in ice for 20 min to complete perchlorate precipitation of proteins and extraction of monoamines from the brain tissue. The sample was then centrifuged at 4 ºC for 25 min at 20 817 rcf. The supernatant fluid (tissue extract) was removed and pipetted into 2 ml amber Eppendorf tube. The pH of the sample was adjusted to 5.0 with the addition of 1 drop of 10 M potassium acetate. An aliquot of 200 µl of the tissue extract, or standard, was pipetted into 1.5 ml Eppendorf tube and 20 µl of the internal standard, 5-HMT, was added to the sample. The rest of the extracted brain tissue sample was stored at -80 °C. The final sample was vortexed and centrifuged for 5 min at 20 817 rcf and transferred to HPLC vial insert.

Conflict of interest: The authors have no conflict of interest to declare.

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