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Simple and rapid determination of serotonin and catecholamines in biological tissue using high-performance liquid chromatography with electrochemical detection

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Abstract

Using the CNS of *Lymnaea stagnalis* a method is described for the rapid analysis of neurotransmitters and their metabolites using high performance liquid chromatography coupled with electrochemical detection. Tissue samples were homogenised in ice-cold 0.1 M perchloric acid and centrifuged. Using a C_{18} microbore column the mobile phase was maintained at a flow rate of 100 μl/min and consisted of sodium citrate buffer (pH 3.2)–acetonitrile (82.5:17.5, v/v) with 2 mM decane-sulfonic acid sodium salt. The potential was set at +750 mV versus Ag|AgCl reference electrode at a sensitivity of 50 nA full scale deflection. The detection limit for serotonin was 11.86 ng ml^{-1} for a 5 μl injection. Preparation of tissue samples in mobile phase reduced the response to dopamine and serotonin compared with perchloric acid. In addition it was found that the storage of tissue samples at −20 °C caused losses of dopamine and serotonin. As a result of optimising the sample preparation and mobile phase the total time of analysis was substantially reduced resulting in a sample preparation and assay time of 15–20 min.

Keywords: Microbore HPLC; Serotonin; Catecholamines; *Lymnaea stagnalis*

1. Introduction

Serotonin and catecholamines play a major role as neurotransmitters in the control and regulation of the central and peripheral nervous system and their measurement in the brain may be important for monitoring neurodegenerative diseases and psychiatric disorders.

There are many approaches to quantify levels of the monoamine neurotransmitters including fluorescence [1], chemical luminescence [2], voltammetry [3,4] and capillary electrophoresis [5,6], though fluorometric or other photometric methods are limited due to poor fluorescence and low molar absorptivity, respectively. Whilst voltammetric methods are the method of choice for in vivo real time recording, overlapping voltammetric peaks, high concentrations of ascorbate, uric acid and other electroactive matrix compounds in typical biological matrices makes separation techniques particularly attractive.

In recent years, high-performance liquid chromatography (HPLC) with electrochemical detection (ED) has been used widely for the analysis of biological samples [7–9] and is an excellent technique as it allows for the separation and analysis of components within the neurochemical matrix and the selectivity of ED offers advantages in the complex biological matrix. ED has been widely used for the determination of neurochemicals in blood samples, tissue and cerebrospinal fluid and microdialysates [10].

Whilst many investigations have been carried out using chromatography, notably using microbore analytical columns, [10] there appears to be great inconsistency in the methodology between analyses. By providing a more robust and accurate method, comparable data can be obtained between different investigations. The majority of dif-

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ferences occur in the sample preparation, where parameters such as the centrifugation parameters and sample storage conditions vary significantly. The rate of degradation and auto-oxidation is influenced by altering the conditions and preparation of biological samples [11,12]. This has led to inconsistency in data e.g. levels of serotonin that vary from μg/g of tissue [13] to ng/g [7,14] of tissue in the hypothalamus of rat brains. There have been chromatographic methods that have shown retention times of serotonin ranging from approximately 10 min [1,15] to 30 min [7,16].

We have chosen to investigate the serotonin and catecholamine content of the pond snail, *Lymnaea stagnalis*. The main reason for using the CNS of these invertebrates over mammalian systems is due to the ease of reproducible analysis and interpretation. The brain of *Lymnaea* is relatively simple compared to that of a mammal and consists of 11 ganglia and a total of about 25,000 neurones and therefore reproducible identification of various CNS regions can be made. The nervous systems of these animals are well known and contain neurons that are easily accessible. Moreover, much is known on the biology and physiology of these animals [17,18].

This paper describes the methodology, including the sample preparation procedure, by which neurotransmitters and their metabolites can be rapidly measured in very small samples of brain tissue.

2. Experimental

2.1. Chemicals

Noradrenaline (norepinephrine, NA), adrenaline (epinephrine, AD), 3-hydroxytyramine (dopamine, DA), 4-hydroxy-3-methoxyphenyl acetic acid (homovanillic acid, HVA), 5-hydroxyindole-3-acetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC) and LD-octopamine (OA) were obtained from Sigma and used as received. 5-hydroxytryptamine (serotonin, 5-HT) was obtained from ICN Biochemical Inc.

All other chemicals used were obtained from Sigma and used as received. All standards were prepared in class A volumetric glassware.

2.2. Sample preparation

The methodology for the preparation of the tissue samples was adapted from previous methods [19–22].

The CNS was removed from *Lymnaea stagnalis* and pinned out in a silicone elastomer (Sylgard)-lined dish filled with ice-cold HEPES buffer (consisting of 10 mM HEPES, 50 mM NaCl, 1.7 mM KCl, 2 mM $MgCl_2 \cdot 6H_2O$ and 4.0 mM $CaCl_2 \cdot 2H_2O$, buffered to pH 7.9 [19]) to facilitate the dissection of the CNS segments (Fig. 1).

2.2.1. Whole CNS preparation

The CNS was placed in 800 μl of ice cold 0.1 M perchloric acid. Samples were then homogenized and centrifuged at 20,000 × *g* at 4 °C.

2.2.2. Individual sample preparation

The buccal ganglia and associated lateral and ventral buccal nerves and cerebrobuccal connective, cerebral ganglia, pedal ganglia and viscero-parietal-pleural complex were analysed as four separate samples. Each of these tissue samples were homogenised in 200 μl of ice cold 0.1 M perchloric acid and centrifuged at 20,000 × *g* at 4 °C. All samples were run between 15 and 20 min of preparation, and were stored in ice prior to analysis.

2.3. Chromatography

The HPLC system consisted of an Agilent HP1050 pump, autosampler equipped with a 5 μl loop and column heater kept constantly at 25 ± 0.15 °C. LUNA® ODS 3 μm 150 mm × 1.0 mm i.d. analytical column with a 4.0 mm × 2.0 mm i.d. 5 μm guard column (Phenomenex®, Macclesfield, UK) were used. The HPLC system was used in a completely isocratic mode for the determination of neurotransmitters. Epilson® LC amperometric detector (Bioanalytical systems, West Lafayette, IN, USA) was used to control detector voltage and record the current. A 3 mm glassy carbon electrode (Unijet, BAS) served as the working electrode and was used with a Ag|AgCl reference electrode and a stainless steel auxiliary block as the counter electrode. The working electrode was set at a potential of +750 mV versus Ag|AgCl reference electrode. The sensitivity of the detector was maintained at 50 nA full scale deflection. Control and data collection/processing were handled through BAS ChromGraph™ software.

The mobile phase composition was similar to previous methods [23,24]. A stock buffer was prepared as follows: 25 mM sodium dihydrogenorthophosphate, 27 μM disodium ethylene-diamine-tetra-acetate (EDTA), 50 mM sodium citrate, 10 mM of diethylamine, 10 mM sodium chloride and 2 mM of decane-sulfonic acid sodium salt were dissolved in 1 l of deionized, distilled water and buffered to pH 3.2 using concentrated phosphoric acid. To prepare the mobile phase, sodium citrate buffer (pH 3.2) was mixed with UV- grade acetonitrile (CHROMSOLV® for HPLC, Riedel de Haën) in a ratio of 82.5:17.5 (v/v) and filtered through a 0.20 μm membrane filter and degassed under vacuum after mixing.

2.4. Standards and accuracy

Standard solutions were prepared from a 100 μg dm^{-3} stock standard of each analyte and were made up in freshly prepared ice-cold 0.1 M perchloric acid (BDH). Each of the standard solutions was prepared on the day of analysis and stored in ice between injections.

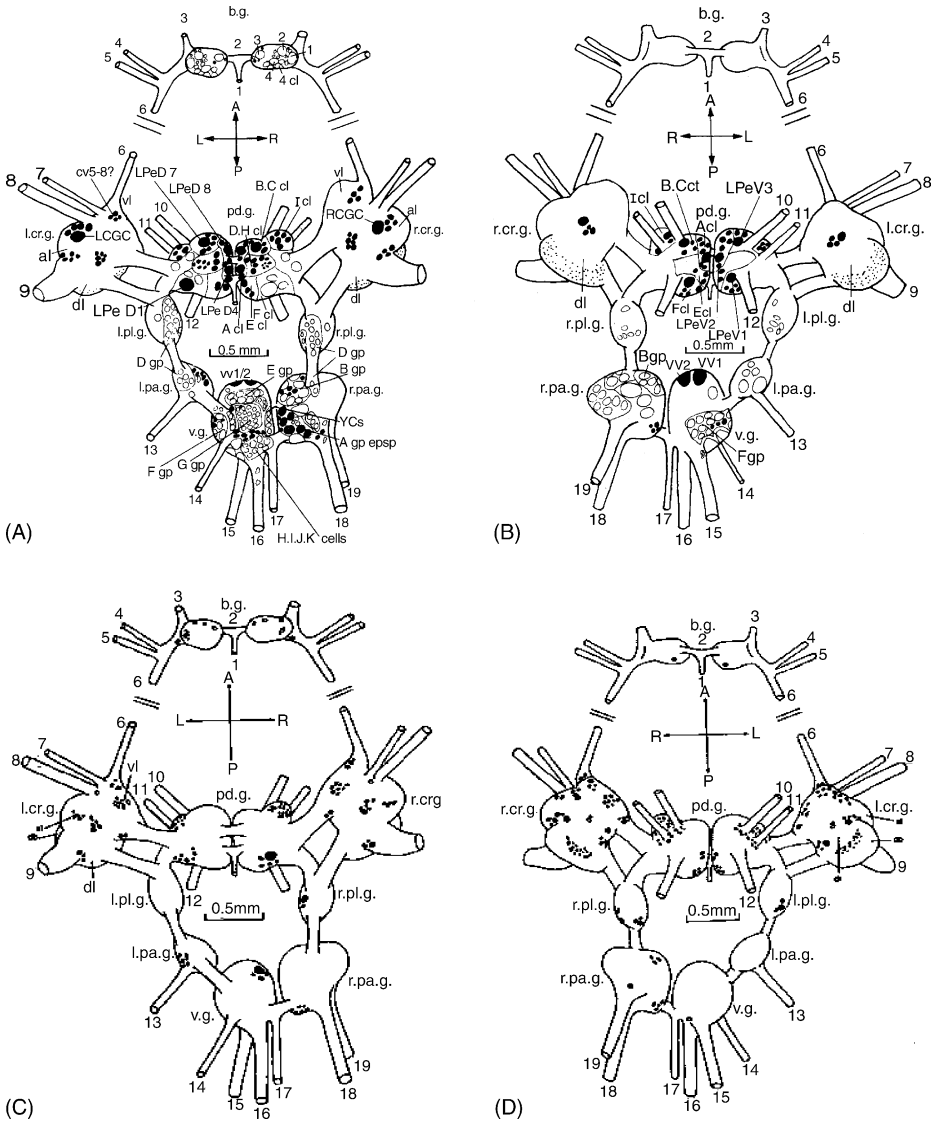

Fig. 1. CNS maps of *Lymnaea stagnalis*. A & B shows a map of the serotonergic neurones present on the dorsal (A) and ventral (B) surfaces of the CNS. C & D show equivalent maps for the distribution of dopaminergic neurones on the dorsal and ventral surfaces of the CNS respectively. Ganglia: buccal (b.g.); cerebral (cr.g.); pedal (pd.g.); pleural (pl.g.); parietal (pa.g.); and visceral (v.g.). L, R and 1, r indicates left and right; A, P indicates anterior and posterior. (From ref. [19,36].)

Spike and recovery data were obtained to account for errors during sample preparation. Recovery factors were calculated using standard IUPAC procedures [25,26].

For each spike and recovery experiment, two snails were used. Recovery measurements were carried out using the whole CNS and the various regions of the CNS. Both CNSs were removed and the bilaterally symmetrical CNS divided sagittaly along its midline. The left part of one snail's CNS was matched with the right side of another snail's CNS and vice-versa. One half of the CNS was placed in 800 μl of ice cold 0.1 M perchloric acid, and the second set was placed into 800 μl of ice cold perchloric acid spiked with 40 μg dm^{-3} of serotonin and dopamine. For individual CNS regions, samples were placed in 200 μl of 0.1 M perchloric acid and spiked with the same concentration of serotonin and dopamine (40 μg dm^{-3}). Spiked solutions were also homogenised without tissue and also centrifuged to quantify total sample losses.

3. Results and discussion

3.1. Effect of sample preparation

3.1.1. Extraction solvent

For chromatography, samples are typically prepared in the mobile phase, to prevent the production of a solvent peak. Fig. 2, shows that by preparing the tissue in the mobile phase there is a significant decrease in the concentration of the analyte measured (Using Student's *t*-tests, $P < 0.01$,

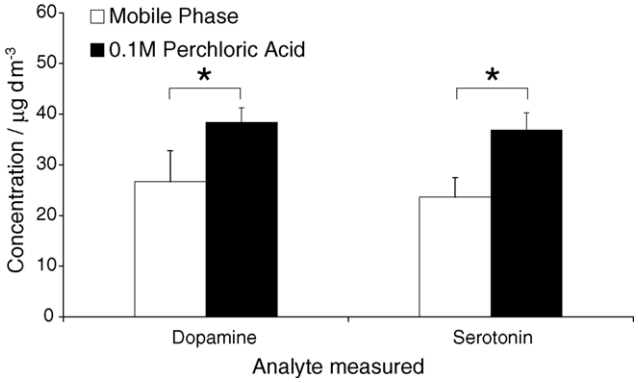

Fig. 2. Effect of sample preparation conditions on the content of serotonin and dopamine. Error bars shown ± one standard deviation of the mean ($n = 6$ and $^{*}P < 0.01$). Both samples centrifuged at 20,000 × g for 5 min at 4 °C.

for dopamine and serotonin responses) compared with using 0.1 M perchloric acid. By preparing samples in buffer, 30.63% loss was observed for dopamine and 35.87% loss for serotonin. This would indicate that the analyte concentrations are enhanced with sample preparation in ice-cold 0.1 M perchloric acid. This accounts for discrepancies in published data, where tissue samples from rat brains have been prepared in 0.1 M phosphate buffer [14] or in methanol/water [13]. This will also cause problems for fluorescence detection, where samples are left to stand for up to 20 min and treated at high temperatures [16] to be derivatised.

By carrying out all sample preparation steps in the mobile phase, enzymes within the sample may not be denatured or inhibited. These enzymes therefore could continue to metabolise the neurotransmitters, and cause inaccurate estimates of the levels of serotonin and dopamine. Losses could also be related to auto-oxidation of serotonin and dopamine. The perchloric acid however will act in denaturing the enzymes, thus preventing any further metabolism or auto-oxidation.

3.1.2. Centrifugation

Tissue samples are often centrifuged prior to analysis. Various centrifugation times and forces have been reported [19,27,28]. For high sample throughput it is important to optimise the time taken for one analysis. All samples were centrifuged at 20,000 × g at 4 °C, for 5, 10, 15, 20, 25 and 30 min. Fig. 3 shows the responses obtained at various times. Statistical evaluation using ANOVA showed that there was no significant effect ($P > 0.75$ for dopamine and $P > 0.90$ for serotonin) of centrifugation time of the final measured concentration of the two analytes. This enables more rapid analysis than some previous investigations. In another investigation into the levels of neurotransmitters in *Lymnaea*, 30 min was used as the centrifugation time (Kemenes et al. [20]). Other investigators have used centrifugation times between 5 and 30 min for tissue samples from other species [7,28–31]. The results from experiments on other species, also indicates that size of the tissue sample has no influence on the centrifugation time.

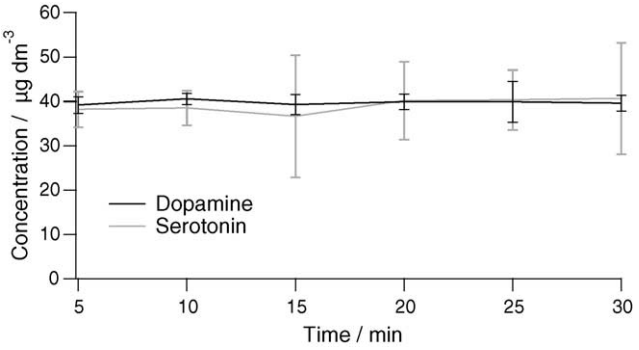

Fig. 3. Varying time taken for the centrifugation of tissue samples. Error bars shown ± one standard deviation of the mean ($n = 5$).

3.2. Chromatography

Quasi-steady state (50 mV s^{-1}) hydrodynamic voltammograms were obtained for 5-HT, 5-HIAA, DA, DOPAC, NA, AD, HVA and OA (20 µg ml^{-1}) in continuous flow between the potential range 0.2–0.9 V and these are shown in Fig. 4. All compounds except adrenaline and HVA oxidise at diffusion-limited rates at a potential of +900 mV. No response for octopamine was obtained, whilst 5-HIAA had the greatest current response. From this result subsequent experiments were performed at a potential of +750 mV versus Ag|AgCl. Lower potentials have been used before [32] without voltammetric characterisation which results in the use of a potential where the diffusion limited current for all compounds is not obtained. This can lead to poor precision and accuracy especially where, as in the case for carbon electrodes, the heterogeneous rate constant can be influenced by adsorption and thus the current response altered.

The majority of methods use complex linear gradients for delivering the mobile phase [33,34]. Some more basic methods have been developed using multiple solvents in the mobile phase. The influence of acetonitrile and tetrahydrofuran (THF) on the capacity factor (k') of various biogenic amines was studied [31] and optimal separation of all analytes was achieved within 15 min (capacity factor of 8.9). We have shown in this paper that a capacity factor less than 10 can also be achieved without using multiple solvents. By using

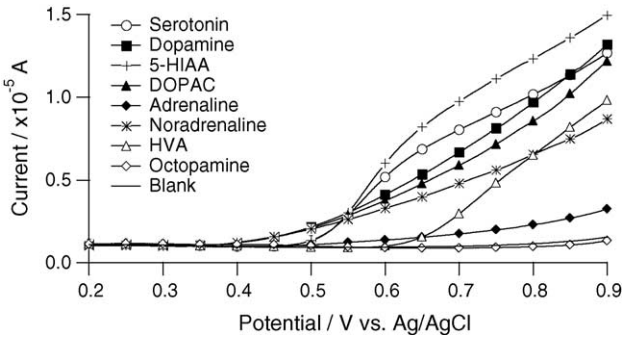

Fig. 4. Quasi-steady state (50 mV s^{-1}) hydrodynamic voltammograms for several biogenic amines and their metabolites using a glassy carbon.

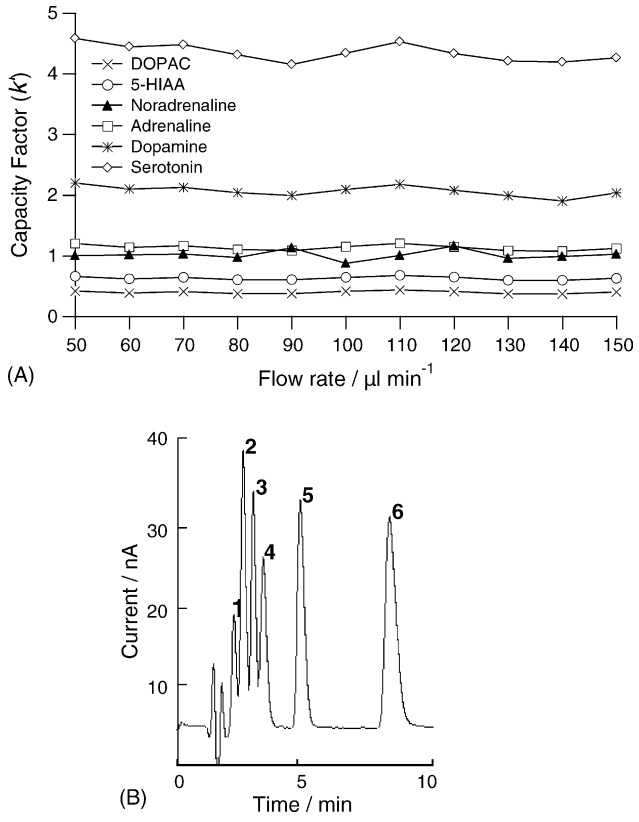

Fig. 5. (A) Capacity factor vs. flow rate using 17.5% acetonitrile, 82.5% buffer with 2 mM decane-sulfonic acid. (B) Chromatogram of standard mixture at 100 μl min^{-1} flow rate. Solutes: (1) DOPAC; (2) 5-HIAA, (3) noradrenalin; (4) adrenaline; (5) dopamine and (6) serotonin.

an elevated level of acetonitrile (17.5%), separation of all major components can be achieved. Separations are obtained with a capacity factor of less than 5 (retention time approximately 10 min). Fig. 5A, shows that the greatest difference in the capacity factor occurs at a flow rate of 100 μl min^{-1}. At this point there is sufficient separation of noradrenaline and adrenaline (resolution is 1.69 using 1 pg of each analyte) are observed (Fig. 5B). Taylor dispersion of the analytes of interest has a great influence on their quantification, and thus affects the capacity factors of the compounds.

The retention times of DOPAC, 5-HIAA, NA, AD, DA and 5-HT are 2.6, 2.9, 3.3, 3.8, 5.2 and 8.7 min, respectively. Excellent resolution ($R > 3$) between serotonin, dopamine and adrenaline is observed, with adequate resolution ($R \geq 1.5$) between DOPAC, 5-HIAA, noradrenaline and adrenaline.

Using a mobile phase consisting of 17.5% acetonitrile: 82.5% citric buffer with 2 mM decane-sulfonic acid sodium salt, it was possible to separate all of the major analytes within 10 min. This short multi-analyte analysis time for neurochemicals provides an ideal basis for a rapid assay. Others report analysis times typically from ∼15 min [16,31] to 40 min [15]. The use of electrochemical detection for the analysis of the neurochemicals allows it to be possible to carry out the separation under an isocratic HPLC system.

Temperature control during a chromatographic analysis is widely overlooked. Temperature control was held consistently at 25 ± 0.15 °C using a column heater. This is essential as a change in a few degrees can affect a number of factors, notably diffusion coefficients (using Stokes–Einstein equation and published viscosity coefficient data [35], it is a simple matter to show that diffusion coefficients in water typically vary by ±2.5% per degree in the vicinity of 25 °C. This would thus increase the band width by 5% per degree), retention time, redox kinetics and background noise, which may significantly compromise quantification of the signal [10].

3.3. Linearity, detection limits and reproducibility

We have used spike and recovery as a means of accounting for sample preparation error. No loss was observed when sample preparation steps were carried out without the use of the tissue samples. In the presence of the whole CNS 100% recovery was observed from the 40 ng ml^{-1} standards of serotonin and dopamine. When using small tissue sections, such as the buccal ganglia from the CNS, the relative recovery was reduced by half. Table 1 shows the percent loss in sample preparation. The recovery is based on the sample weight and the majority of loss occurs during tissue homogenisation. Losses of approximately 50% were observed for both serotonin and dopamine in the buccal ganglia. Approximately 15% losses for dopamine and serotonin were also observed in the viscero–parietal–pleural complex. The loss seems to be correlated to the number of either serotonergic or dopaminergic cells in each of the ganglia. There are typically only three

Table 1
Mean recovery rates of the main neurotransmitters

CNS region	Mean recovery rates (%)	
	Serotonin	Dopamine
Centrifugation only	100.45 ± 0.30[a]	100.56 ± 0.37[a]
Homogenisation and centrifugation	99.89 ± 0.95	99.76 ± 1.31
Whole CNS	102.40 ± 2.76[a]	101.85 ± 2.94[a]
Buccal ganglia and associated lateral and ventral buccal nerves and cerebrobuccal connective	53.01 ± 2.00	52.82 ± 5.08
Cerebral ganglia	98.74 ± 2.63	104.32 ± 8.04[a]
Pedal ganglia	105.30 ± 7.73[a]	101.87 ± 1.40[a]
Viscero-parietal-pleural complex	83.38 ± 5.62	85.68 ± 2.14

Error bars shown ± one standard deviation of the mean ($n = 6$). Same conditions as shown in Fig. 5b.

[a] Recovery results are greater than 100%, but are within the 2% of instrumental error, and therefore are assumed to be 100% recovery.

cells in the buccal ganglia [19] that are known to contain serotonin and 15 cells that contain dopamine [36], thus variability in the levels of serotonin within an individual neuron can significantly alter the response from the spike and recovery data. The greatest number of dopaminergic and serotonergic neurons are present in the pedal ganglia, where no losses were observed.

For each analyte, calibration curves were obtained individually. As the results from the tissue samples varied over two orders of magnitude for some neurotransmitters, two separate calibration curves were required for many of the chemicals to cover the expected range of the tissue samples. From the calibration curve, linear regression analysis was carried out to obtain the correlation coefficient (R^2) and the limit of detection (LOD, based on the three standard deviations of the *y*-intercept using least-squares regression). Table 2 showed the calibration range, limit of detection and relative correlation for all components.

These calibration curves would allow quantitation of serotonin and dopamine in both the whole CNS and in the various CNS regions including the buccal ganglia, which contain only two serotonergic neurons.

The absolute limits of detection are 2.36 ng ml^{-1} for DOPAC, 1.19 ng ml^{-1} for 5-HIAA, 1.47 ng ml^{-1} for AD, 0.47 ng ml^{-1} for NA, 4.20 ng ml^{-1} for DA and 11.86 ng ml^{-1} for serotonin at the detector sensitivity of 50 nA full scale deflection. The LOD of the standards are generally very good for the measurement of tissue samples, but the results obtained for the responses from DOPAC and serotonin were fit for purpose but not exceptional. All the standard calibration curves have high correlation coefficients. All the responses have been obtained at a sensitivity of 50 nA full scale deflection, indicating that the detector still has a few orders of magnitude left for low concentration measurements. Using the conventional HPLC-EC technique, 30 fmol limit of detection can be obtained for biogenic amines [10]. When using microdialysis, results of approximately 0.1–1.0 fmol of serotonin from 5 μl of dialysate have been reported [12].

3.4. Sample storage

A number of investigations have looked at the stability of neurotransmitters and their metabolites in various media. These compounds are known to be unstable in solution and thus for automated systems, with samples left for analysis over a long time period (e.g., 15–20 h), there is ample opportunity for significant degradation to occur. There have also been some investigations in which tissue sample supernatants have been stored at −20 °C prior to analysis [14] and also at 0 to 5 °C for up to 3 days [7] or in a refrigerated autosampler until analysis [37]. More surprisingly, standard solutions have also been stored at −20 °C [14,34]. This would be a problem if internal standards were used to assess the relative recovery of monoamines, as the rate of degradation of different compounds can vary significantly.

Stability has been shown to be improved by the addition of antioxidants such as L-cysteine and ascorbic acid (though these are electroactive and further complicate an already complex matrix [12]) or by using a combination of the antioxidant sodium bisulfite and/or metal chelating agent disodium edetate [11].

When studying the effect of storing tissue sample supernatants at −20 °C, it was found there were significant losses in stability of serotonin and dopamine ($P < 0.0001$ using ANOVA) after 1 week, with losses of 42.9% and 92.8% observed for dopamine and serotonin, respectively. Fig. 6 shows the responses of dopamine and serotonin from supernatant after 1 day and a week. From Tukey's tests, the majority of degradation occurred over the first day for dopamine ($P < 0.0001$, loss of 30.4%), with little degradation between a day and a week ($P < 0.01$, with a loss of 18.1%).

For serotonin, the extent and rate of degradation was far greater, with the majority of degradation once again occurring within the first day ($P < 0.0001$, loss of 82.7%). There was no significant degradation observed between 1 day and a week (58.16% losses observed for serotonin), suggesting that the entire sample is degraded during the first day.

Table 2
Calibration range, limit of detection, R^2 values from a linear regression analysis of the peak area for the standards ($n = 6$)

Standard	Calibration range (ng ml^{-1})	R^2	Limit of detection (ng ml^{-1})
5-HIAA	50–1	0.997	1.19
DOPAC	100–1	0.992	7.85
	10–1	0.939	2.36
Adrenaline	100–1	0.993	2.58
	10–1	0.982	1.47
Noradrenaline	100–1	0.992	2.63
	10–1	0.998	0.47
Dopamine	500–50	0.989	37.61
	100–10	0.996	4.20
Serotonin	500–50	0.995	33.62
	100–10	0.984	11.86

Each of the standards is individually analysed. All conditions similar to that in Fig. 5b.

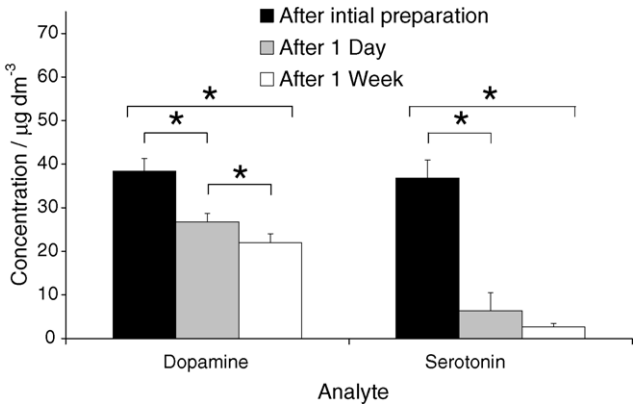

Fig. 6. Effect of sample storage at −20 °C. After initial run, sample is stored for a day to assess degradation, and then a reassessed after a week. Error bars shown ± one standard deviation of the mean ($n = 6$, $^{*}P < 0.0001$).

These results would indicate that ideally analysis should be carried out once the sample preparation steps have been performed. Therefore, if samples have to be stored, then storage at −80 °C is required [11].

3.5. Analysis of biological tissue samples

Fig. 7 shows the typical data obtained using the sample preparation techniques and chromatographic method for the determination of CNS samples from the pond snail, *Lymnaea stagnalis*. Resolved neurochemical peaks can be observed in the sample containing the whole CNS and the buccal ganglia. There is baseline resolution of serotonin, dopamine, DOPAC, 5-HIAA and noradrenaline in the whole CNS sample. The results from Fig. 7A correspond to approximately 170 nmol of serotonin and 158 nmol of dopamine present in the whole CNS of the pond snail.

Some resolution is lost from the buccal ganglia sample as 5-HIAA and DOPAC elute on the solvent front peak. But the responses of serotonin and 5-HIAA correspond to the content of neurotransmitter from just three neurons. This accounts for concentrations of 4.50 ± 2.80 pmol of serotonin, 840 ± 330 fmol of 5-HIAA, 2.40 ± 0.99 pmol of dopamine, 190 ± 140 fmol of DOPAC and 1.10 ± 1.10 fmol of adrenaline per neuron in the buccal ganglia ($n = 15$, ±one standard deviation of the mean).

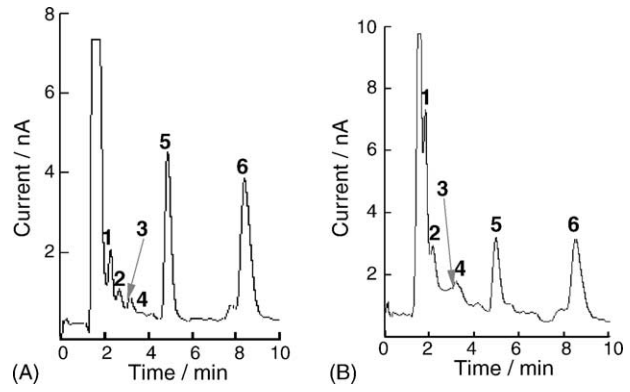

Fig. 7. Chromatographs obtained from tissue samples. (A) Whole CNS sample prepared in 800 μl of 0.1 M perchloric acid. (B) Buccal ganglia sample prepared in 200 μl of 0.1 M perchloric acid. Solutes: (1) DOPAC; (2) 5-HIAA, (3) noradrenalin; (4) adrenaline; (5) dopamine and (6) serotonin.

4. Conclusion

We have developed a rapid protocol for sample preparation and assay of major neurotransmitters and their metabolites in tissue samples. Each sample takes approximately 15–20 min to analyse from start to finish. By carrying out analysis of all samples on the day they are prepared, the method overcomes sample degradation from auto-oxidation. Using ice-cold 0.1 M perchloric acid in preference to buffer solutions enhances the response and thus may prevent the possibility of any further metabolism. By storing samples at −20 °C, sufficient degradation of the sample occurs to provide inaccurate responses.

This assay has been able to show accuracy and sensitivity to measure the content of serotonin and its metabolite 5-HIAA from just two neurones in the buccal ganglia of the pond snail *Lymnaea stagnalis*.

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