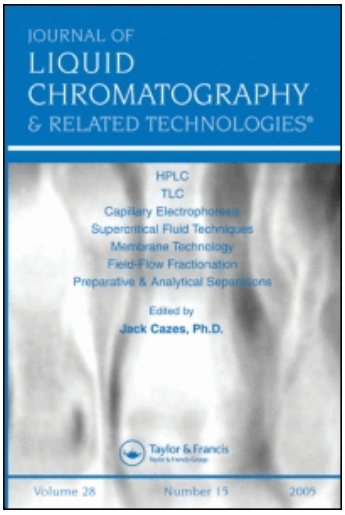

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IDENTIFICATION AND QUANTIFICATION OF MONOAMINERGIC NEUROMODULATORS IN THE SUB-CORTICAL REGION OF CAT VISUAL CORTEX BY MICROBORE HPLC-ED AND PROTEIN ASSAY

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ABSTRACT

Removal of retinal input from a restricted region of adult visual cortex leads to a substantial reorganisation of the retinotopy within the deprived zone. To investigate the role of the total (intra- and extracellular) concentration of the monoaminergic neuromodulators in the sub-cortical region of cat visual cortex, which is possibly involved in this reorganisation mechanism, a method for identification and quantification of noradrenaline, dopamine, serotonin, and their major metabolites, 3,4-dihydroxyphenylacetic acid, 4-hydroxy-3-methoxyphenylacetic acid, 5-hydroxyindole-3-

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acetic acid in small amounts of brain extracts has been developed and validated. Control or retinal lesion cats were killed with pentobarbital; the brains were quickly frozen, and 200 μm cryostat sections were cut.

Under visual control through a surgical microscope, the cortical tissue was first separated from the underlying white matter. Three pieces of tissue measuring 2 x 4 mm^2, containing the six cortical layers, were sampled out from the different cortical regions subserving different parts of the visual field (central and peripheral portion of area 17). After homogenising these samples in 80 μL, 0.01M HCl which included 0.01% cysteine as antioxidant, 10 μL of the supernatant was injected directly onto the microbore HPLC system, separated on a microbore column (150 x 1 mm i.d.; ODS), and the components detected electrochemically at a potential of +0.75V. The flow rate of mobile phase through the column was 44 μL/min.

The specificity, recovery, analytical precision, calibration curves, and detection limits for each neurotransmitter were determined. In order to express the total neuromodulator concentration as pg/μg protein, the pellets obtained after centrifugation were used for protein determination. A modified protein assay with non-linear regression equation data processing is also described.

INTRODUCTION

Noradrenaline (NA), dopamine (DA), and serotonin (5-hydroxytryptamine, 5-HT) are well known neurotransmitters and form three different diffuse modulatory systems in the CNS of mammals.[1] They are assumed to modulate cellular functions associated with long-term plastic properties of neuronal circuits.[2-4] These neuromodulators, released from presynaptic terminals, do not produce fast synaptic actions, but are able to modulate the reactivity of target neurones to stimulation by other afferents.

To investigate whether these monoaminergic neuromodulators are involved in molecular mechanism of cortical reorganization, which responds to partial sensory deprivation, and understand the possible role of monoaminergic neuromodulators in cortical plasticity,[5] a proper method for quantification of NA, 5-HT, dopamine (DA), adrenaline (AD) and their metabolites: 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxyphenylacetic acid (HVA), 5-hydroxyindole-3-acetic acid (5-HIAA) is needed. In our laboratory, an HPLC method[6] has been developed for direct injection of the supernatants of brain homogenates onto a conventional column and determination of these monoamines in the hypothalamus of the chicken brain. However, in the visual cortex, NA, 5-HT and DA

are present only in fibres and not in cell bodies. For instance, NA is released from terminals of fibres originating in the nucleus locus coeruleus of the brainstem,[7] whereas serotonin-containing fibres originate in the midbrain raphe nuclei.[8]

DA originates in the tegmental ventral area (VMT)[9] and its fibres also project to the visual cortex.[10] The monamines concentration in these fibres is much lower than that in the brain areas which contain monoamine neurones. Therefore, our previously described method[6] and other methods[11-13] which use a conventional column can not be used.

Furthermore, we need to compare these neuromodulators in sensory-deprived and normal cortex. The tissue amount, which could be sampled from the different cortical regions subserving different parts of the visual field (central and peripheral portion of area 17), are extremely limited. A suitable method to measure monoamine level from monoamine fibres in the sub-cortical region is unavailable

Instead of weighing frozen[14] or wet[15] tissue, a protein assay[6] has been used for accurate measurement of the tissue mass in each sample and the result expressed as pg neuromodulator/μg protein. This protein assay, a modification of the Bradford method, is based on the shift of the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 from 465 nm to 595 nm when binding to protein occurs[16]. Beer's Law may be applied for accurate quantification of protein by selecting an appropriate ratio of dye volume to sample concentration. However, over a broader range of protein concentration, the dye-binding method gives an accurate, but not entirely linear response. The low levels of monoamines in visual cortex demanded larger tissue samples containing several hundred μg of protein in each sample. This resulted in difficulties in getting accurate quantification of the protein concentration using a linear equation.

One part of the present study produced a special sampling procedure for preparation of a very small amount of tissue (2 mm thicker cortex contains the 6 layers of cell) in cat visual cortex for directly injecting onto a HPLC column. A second part of this study modified a microbore HPLC method, which used a 1x100 mm ODS microbore column with a small volume flow-cell electrochemical detector[14, 17, 18] to obtain enough sensitivity for our application. In the third part of this study, a nonlinear regression equation data processing to calculate the protein concentration in brain tissue samples was described.

EXPERIMENTAL

Chemicals

HPLC-grade acetonitrile (ACN), disodium ethylenediaminetetraacetate (EDTA), tetrahydrofuran (THF), ortho-phosphoric acid (H_3PO_4 85%), and hydrochloric acid (HCl) were obtained from Merck (Belgolabo, Overyse,

Belgium), monobasic sodium phosphate, diethylamine and 1-octanesulfonic acid from Aldrich (Sigma-Aldrich, Bornem, Belgium), tri-sodium citrate dihydrate from Vel (Leuven, Belgium). The protein assay dye reagent was purchased from Bio-Rad Laboratories (Bio-Rad Laboratories, Nazareth, Belgium), while all the following chemicals DA, NA, DOPAC, AD, HVA, 5-HIAA, 5-HT, L-cysteine, and chicken egg albumin (ovalbumin) were obtained from Sigma (Sigma-Aldrich, Bornem, Belgium).

Stock solution of monoamines and internal standards were prepared in 0.01M HCl containing 0.01% L-cysteine at a concentration of 1 mg/mL and stored at –70°C. Working solutions were freshly prepared by dilution of the stock solution in 0.01M HCl containing 0.01% L-cysteine.

Tissue Preparation and Extraction

Two normal cats were used for the determination of total monoaminergic neuromodulator concentration in the visual cortex and sacrificed with an overdose of Nembutal (60 mg/kg, i.v.). The brain was rapidly removed and immediately frozen by immersion in liquid nitrogen-cooled isopentane and stored at –70°C. 200 μm brain sections were cut on a cryostat. From three sections, 2 x 4 mm^2 cortical tissue in each section from the peripheral or central portion of area 17, containing all 6 cortical layers, were dissected under the microscope using a surgical blade (as shown in Fig. 1), and expelled together into a 1.5 mL conical tube, containing 80 μL 0.01M HCl. The tissue was homogenised by grinding for 30 sec. at 4°C and the homogenate was centrifuged at 12,000 *g* for 15 min at 4°C. The supernatant was filtrated through a 0.22 μm syringe filter and used for HPLC analysis, where the pellet was used for determination of the protein concentration.

Chromatographic System

The LC system was a BAS 200A Chromatograph (BAS: Bioanalytical System, West Lafayette, IN, USA), equipped with an amperometric detector (BAS). Separation was performed on a SepStik microbore column: 150 x 1 mm I. D., 3 μm ODS (BAS). A flow splitter (BAS) was used to provide low volumetric flow rates required for the microbore column and to recycle the mobile phase without flowing through the column. The split ratio was 1/16. Operating the pump at 0.7 mL/min yielded a microbore column flow rate of approximately 44 μL/min. Automated sample injection was performed by using a BAS Sample-Sentinel (BAS). The injection volume was 10 μL. The microbore column was coupled directly to the amperometric detector cell to minimise the dead volume. The electrochemical detector was equipped with a dual glassy, carbon working electrode with a different sensitivity range. The operating potentials were set at 750 mV ver-

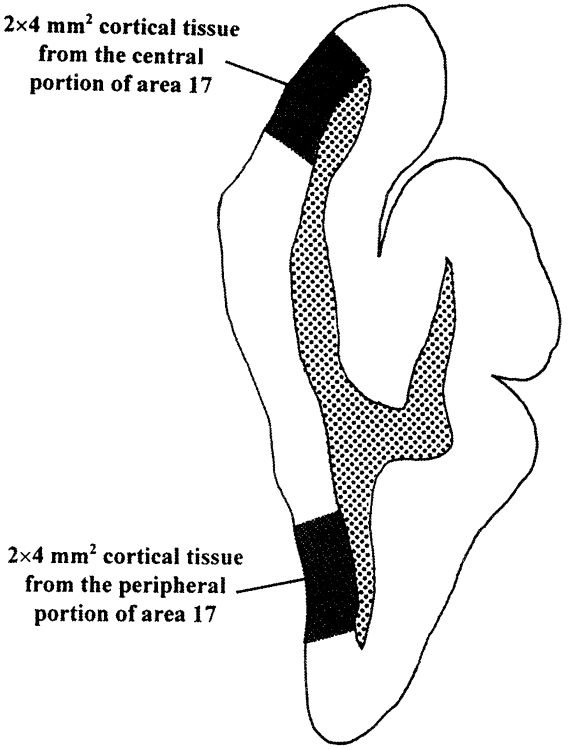

Figure 1. Localisation of sampling in the area 17 of a cat brain section (200 μm thick).

sus an Ag/AgCl reference electrode. The cell volume was reduced by a 16 μm gasket. The chromatographic system was controlled by "BAS control" software and the chromatograms were integrated with "ChromGraphTM" software (BAS).

The isocratic mobile phase consisted of 25 mM sodium citrate, 10 mM monobasic sodium phosphate, 1.8 mM 1-octanesulfonic acid, 10 mg/l EDTA, and 10 mM diethylamine in Milli-Q water, and the pH was adjusted to 3.1 with H_3PO_4. Following filtration through a 0.22 μm filter, ACN and THF were added to a final volume concentration of 5.0% and 0.5%, respectively. The mobile phase was degassed on line with helium gas for 4 min. The whole system was maintained at 35°C.

Protein Assay

The tissue pellet was dissolved in 300 μL 0.1M NaOH and diluted with 700 μL PBS buffer (0.15M NaH_2PO_4, pH=7.4) for determination of the protein con-

tent. A protein standard solution was prepared in 0.1 M PBS buffer containing varying amounts (50-600 μg/mL) of ovalbumin. A 200 μL aliquot of Rad Protein Assay reagent (diluted 1:9 with Milli-Q water) was added to 20 μL of the standard solutions or samples in polystyrene microtiter plates. The absorbance at 620 nm was measured after 5 minutes using an Enzyme Immunoassay Auto Reader. A standard curve was constructed using a four-parameter logistic function curve fit program in SigmaPlot (Jandel Scientific Software, Windows 95). The protein concentration in tissue sample was calculated by the following nonlinear regression equation:

$$Y = (a - b) / [1 + (x/c)^{b}] + d \quad (1)$$

The four parameter logistic functions are also called sigmoids, since they have the characteristics of an elongated S shape. The parameters *a* and *d* are the asymptotic maximum and minimum values, respectively. The parameter *c* is the *x* value at the inflection point. *B* is the slope parameter; for $b > 0$, the slope is negative. Therefore, if $b < 0$ the sigmoid is rising and if $b > 0$ the sigmoid is falling. Values of *a, b, c* and *d* were calculated according to the concentration of a set of standards and their absorbance by the curve fit program. *Y* is the absorbance and *x* is the concentration of protein standard or protein samples. This allowed us to express the results as pg monoamine /μg protein.

Quantitative Method

The concentration of NA, DOPAC, AD, HVA, DA, 5-HIAA, and 5-HT was calculated on the response of standard curves using an external standard method. The recovery or extraction efficiency for each neuromodulator was determined in the following way: a piece of visual cortex tissue was dissected and cut into two portions; one part was spiked with 80 μL of a 2.5 pg/μL (or 5.0, 10.0 pg/μL) standard mixture as the sample, and another part spiked with 80 μL 0.01M HCl as blank brain tissue. After homogenisation and centrifugation, the supernatant was injected into the HPLC for analysis and the pellet was used for protein assay. The recovery was calculated using the following equation:

$$\text{Recovery} = [(Sm - B \times P_S/P_B)/S] \times 100\% \quad (2)$$

where S = the peak area of each monoamine in the standard mixture; B= the peak area of each monoamine in the blank brain tissue; Sm = the peak area of each monoamine in the brain tissue spiking with standard; P_S = the protein content of brain tissue spiked with standard; P_B = the protein content of blank brain tissue.

RESULTS AND DISCUSSIONS

Specificity

Representative chromatograms of a standard mixture and a brain homogenate are shown in Fig. 2 and 3, respectively. The identities of the peaks were confirmed by their retention time. Each analysis was completed within 15 minutes. NA, AD, DOPAC, DA, 5-HIAA, HVA, and 5-HT were eluted from the column with the peak number 1 to 7, respectively.

All components in Fig. 3 were well resolved and identical with those in Fig. 2. Each peak of these seven monoamines was verified by addition of a standard mixture, which increased its height, but did not change its shape. Two chromatographic columns of the same characteristics but with a different batch number demonstrated comparable chromatographic profiles.

In addition, a flow splitter is used to convert a conventional pump to the microbore regime. The splitter consists of a tee and a restrictor in the form of a capillary. After a hundred-samples were injected, or recycling mobile phase overnight, the resistance in the system increased as a result of the particles and the small amount protein deposited on the top of the microbore column. The flow rate of mobile phase in microbore column will be decreased by the changing of split ratio. Therefore, a standard mixture must be run between each ten samples used to calibrate the retention time delay.

Extraction Efficiency

Intra-day accuracy was assessed from the relative recoveries, which were determined by spiking with 2.5, 5.0, and 10.0 pg/μL standard (two samples at each concentration) and calculating concentrations as described in section 2.5. The results are shown in Table 1. For such recovery rate assessments, the standard mixture was spiked before the homogenisation step. Therefore, the influences for extraction efficiency by all sample preparation steps (homogenisation, centrifugation and filtration) have been included. Concentration differences in the amounts of endogenous monoamine in the blank samples as a result of different amounts of tissue in samples and blanks, was calibrated by item P_S/P_B in equation 2.

Calibration Curves

Representative calibration curves of NA, AD, DOPAC, DA, 5-HIAA, HVA, and 5-HT gave relevant regression lines as shown in Table 2. The concentration range of the standard curve was 0.5 - 20.0 pg/μL.

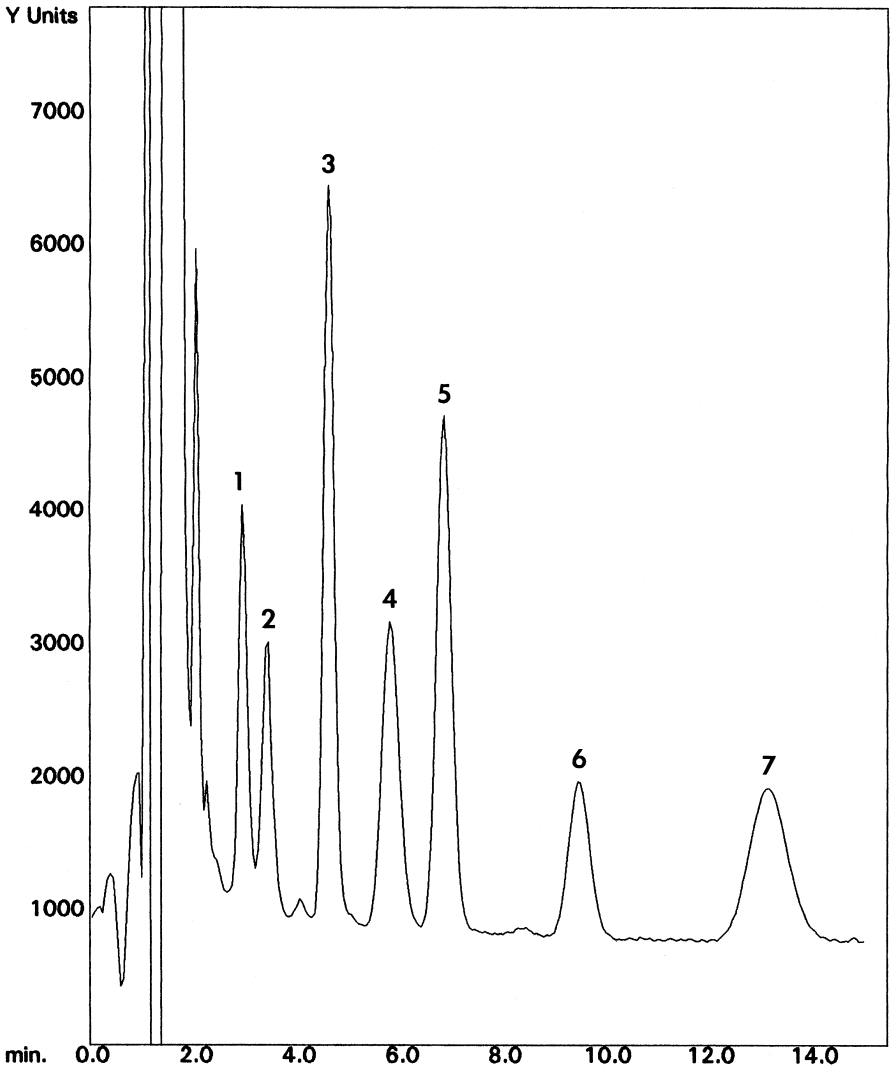

Figure 2. Chromatogram of a standard mixture of monoaminergic neuromodulators (20 pg/μL): (1). NA; (2). AD; (3). DOPAC; (4). DA; (5). 5-HIAA; (6). HVA; (7). 5-HT. Sensitivity range: 2.0 nAFS.

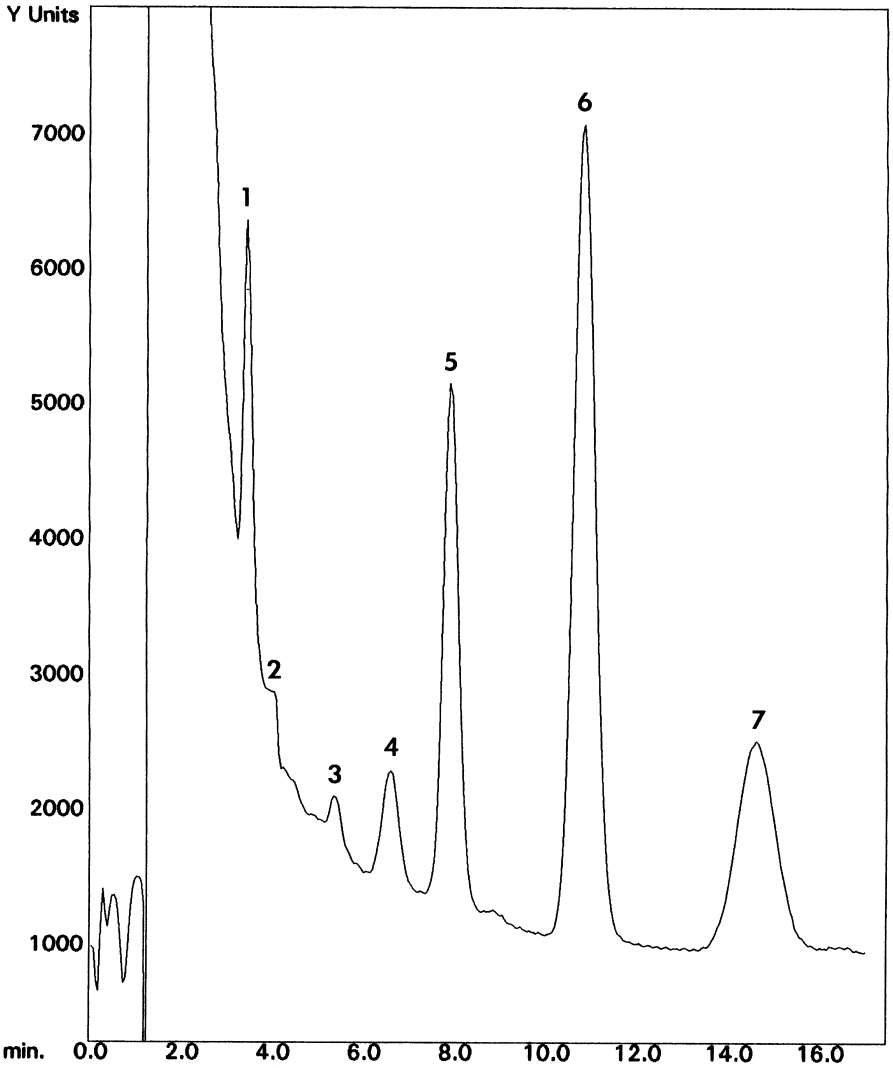

Figure 3. Chromatogram of a brain homogenate of the central portion of normal cat area 17. Peaks as in Fig. 1. Sensitivity range: 2.0 nAFS.

Precision

Precision was studied from replicate sets of standards with known concentrations at levels corresponding to the lowest, middle, and highest concentration

Table 1. Recovery of Monoaminergic Modulators in Brain Tissue

Monoamine	Mean Recovery%	R.S.D.%
NA	91.4	5.06
AD	-	-
DOPAC	105.7	9.4
DA	107.4	16.2
5HIAA	99.41	13.2
HVA	108.3	13.9
5HT	94.0	17.6

*The level of AD in the visual cortex of cat brain are too low to be measured.

values of the calibrating range, and assessed from the relative standard deviation (R.S.D %) as shown in Table 3.

Detection Limit

The lowest concentration values of each monoaminergic summarised in Table 2 were measured with signal-to-noise ratio of 4 and an injection volume of 10 μL.

Protein Determination

Two representative Sigrid standard curves (Sigma plot) of protein assay with different concentration ranges are shown in Fig. 4A and 4B, respectively. Curve 4A is almost in the linear part of the S-shape curve within the low protein concentration range, whereas curve 4B is in the up-stretched region within high concentration range. This method has not only been used in the work to estimate

Table 2. Calibration Curves and Detection Limit

Monoamine	Regression Equation	Correlation Coefficients	Detection Limit (pg/μl, S/N=4)
NA	$y = 11.66x - 1.839$	0.9968	0.1
AD	$y = 8.506x + 7.017$	0.9985	0.2
DOPAC	$y = 24.47x + 0.09968$	0.9995	0.1
DA	$y = 16.92x + 2.263$	0.9993	0.2
5HIAA	$y = 25.25x - 1.342$	0.9997	0.3
HVA	$y = 11.00x + 0.9398$	0.9996	0.3
5HT	$y = 17.73x + 6.948$	0.9996	0.5

Table 3. Analytical Precision - Relative Standard Deviation (R.S.D.%) of Standard Mixture

	0.5 pg/μL		5.0 pg/μL		20.0 pg/μL	
Monoamine	Peak Area	Retention Time	Peak Area	Retention Time	Peak Area	Retention Time
NA	0.0002	11.09	0.19	0.57	0.16	0.62
AD	0.64	10.66	0.47	3.31	0.81	6.52
DOPAC	0.44	6.81	0.18	0.82	0.24	0.65
DA	0.43	4.52	0.33	0.57	0.34	0.40
5HIAA	0.39	6.30	0.39	1.18	0.34	0.56
HVA	0.44	8.56	0.33	0.95	0.29	0.16
5HT	0.91	4.28	0.55	3.68	0.42	0.97

the amount of tissue in each sample, but has been applied to other work in this laboratory as well.

Table 4 shows the protein assay results, which were obtained for different expressions. The second column in this table shows the protein concentration for the expression of amino acids neurotransmitter in the visual cortex of a cat. The tissue size is about 2×4 mm^2. The third column shows the protein concentration for the expression of total RNA in the visual cortex of cat. The tissue size is less than 2×2 mm^2. These results indicated a very convenient and accurate measurement of protein ranging from 8.0 to 700 μg. Selecting an appropriate linear range of calibration curve and ratio dye volume to sample concentration is not necessary for this method.

Application

The method was used to study the effect of sensory deafferentation on monoaminergic neuromodulators in the visual cortex of adult cats. The result is in the following chapter. The concentration of monoaminergic neuromodulators in the visual cortex of one normal cat is summarised in Table 5.

CONCLUSION

The isocratic microbore HPLC-ED method described in this report is evaluated for quantification of monoaminergic neuromodulors NA, DA, 5-HT, and their major metabolites DOPAC, 5HIAA, HVA in the sub-cortical region of the visual cortex of cats within 15min. It appeared to be more sensitive and allowed the use of a small amount of tissue by only a 10 μL injection. The high stability

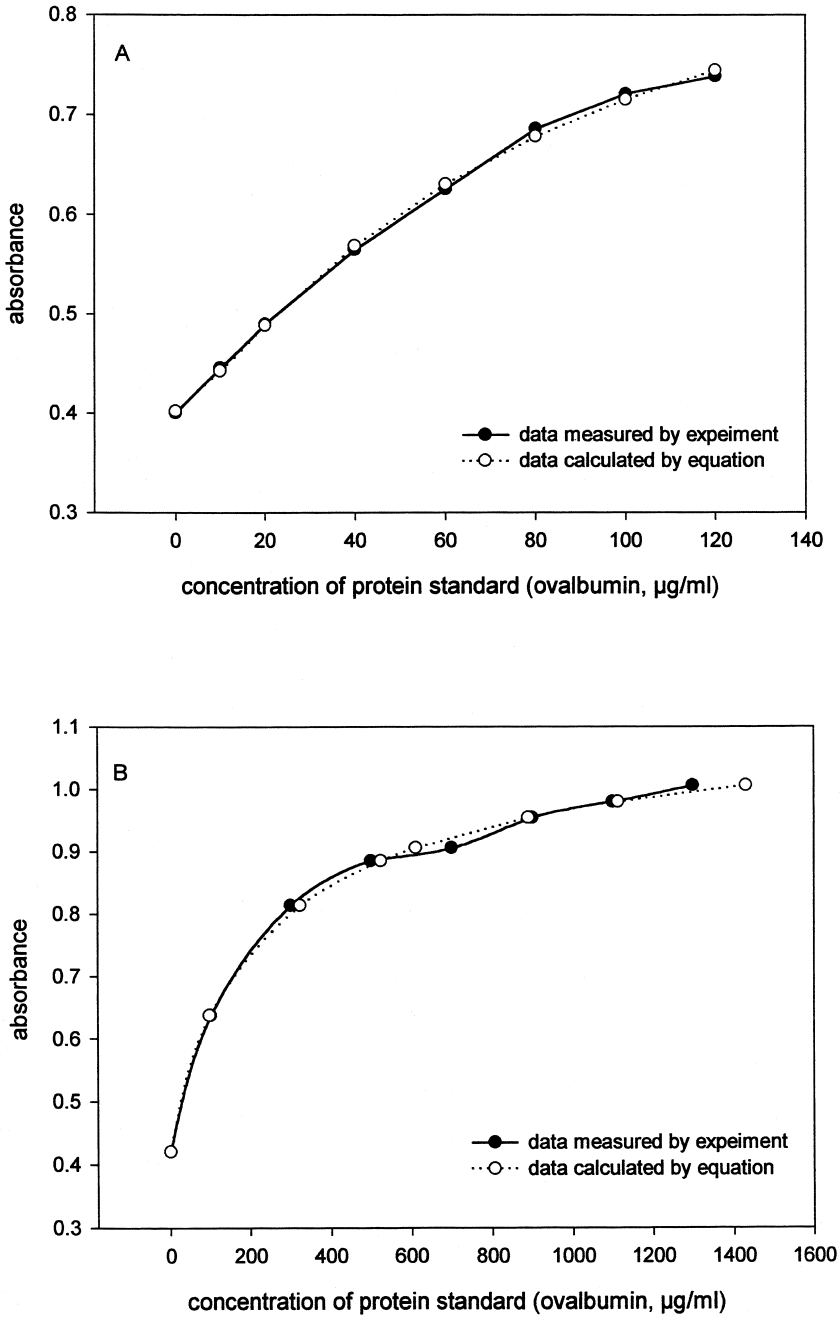

Figure 4. Calibration curves of protein standards. (A). Concentration range: 20-120 μg/mL; nonlinear regression equation: a=0.40; b=1.22; c=79.1; d=0.95. (B). Concentration range: 200-1400 μg/ml; nonlinear regression equation: a=0.42; b=0.78; c=289; d=1.15.

Table 4. Protein Concentrations in Different Tissue Samples

Visual Cortex Tissue Samples in Cat for Measuring Monoaminergic Neuromodulator (μg)*	Visual Cortex Tissue Samples in Cat for Measuring of Amine Acid Neurotransmitter (μg)	Visual Cortex Tissue in Cat for Measuring of Total RNA (μg)
567.36	20.83	8.69
135.93	42.35	12.79
278.79	49.69	8.50
310.19	41.19	8.49
746.01	43.97	11.87
404.17	55.54	8.93
610.04	65.75	11.36
629.80	68.80	
355.34		
302.84		

* In this work.

Table 5. Monoaminergic Neuromodulators in the Central Portion of Visual Cortex in One Normal Cat

Monoamine	Concentration (pg/μg Protein, n=20)
NA	3.31 ± 0.39
AD	0.63 ± 0.10
DOPAC	1.13 ± 0.13
DA	1.23 ± 0.19
5HIAA	2.04 ± 0.21
HVA	2.90 ± 0.25
5HT	6.47 ± 1.18

of this method is applicable to routine analysis of large numbers of samples in Neuroscience research. The mobile phase consumption is 66 mL per 100 samples. In addition, the method used for protein assay is very simple and convenient. The non-linear regression data process allows accurate measurement of protein over an 8 to 700 μg range, without selecting an appropriate ratio of dye volume to sample concentration, or diluting the sample to obtain a suitable standard linear range. It can be used as a method for estimating the tissue mass for any number of research applications.

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REFERENCES

1. Bear, M.F.; Connors, B.W.; Paradiso, M.A. *Neuroscience: Exploring the Brain*; Williams & Wilkins: U.S.A., 1996; 402-431.
2. Foote, S.D.; Morrison, J.H. Annu. Rev. Neurosci. **1987**, *10***,** 67-95.
3. Bear, M.F.; Singer, W. Nature **1986**, *320*, 172-176.
4. Jonsson, G.; Kasamatsu, T. Exp. Brain Res. **1983**, *50*, 449-458.
5. Qu, Y.; Eysel, U.T.; Vandesande, F.; Arckens, L. Neuroscience **2000**, *101*, 863-868.
6. Qu, Y.; Moons, L.; Vandesande, F. J. Chromatogr. B. **1997**, *704* (1-2), 351.
7. Foote, S.L.; Bloom, F.E.; Aston, J.G. Physiol. Rev. **1983**, *63*, 844-914.
8. Azmitia, E.C.; Segal, M. J. Comp. Neurol. **1978**, *179***,** 641-688.
9. Törk, I.; Turner, S. Neurosci. Lett. **1981**, *40*, 1019-1025.
10. Parkinson, D. Neuroscience **1989**, *30*, 171-179.
11. Qu, Y.; Hu, P.; Zhu, P.L. J. Liq. Chromatogr. **1991**, *14* (14), 2755.
12. Magnusson, O.; Nilsson, L.B.; Westerlund, D. J. Chromatogr. **1992**, *582*, 1.
13. Saito, H.; Murai, S.; Abe, E.; Masuda, Y.; Itoh, T. Pharmacol. Biochem. Behav. **1992**, *42*, 351.
14. Cheng, F.C.; Kuo, J.S.; Ying, S.; Lai, J.S.; Ni, D.R.; Chia, L.G. J. Chromatogr. B. **1993**, *615***,** 225-236.
15. Saito, H.; Murai, S.; Abe, E.; Masuda, Y.; Itoh, T. Pharm. Biochem. Behav. **1992**, *42***,** 351-356.
16. Bradford, M.M. Anal. Biochem. **1976**, *72*, 248-256.
17. Huang, T.; Shoup, R.E.; Kissinger, P.T. Current Sep. **1990**, *9*, 139-143.
18. Qu, Y.; Arckens, L.; Vandenbussche, E.; Geeraerts, S.; Vandesande, F. J. Chromatogr. A. **1998**, *798*, 19-26.

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