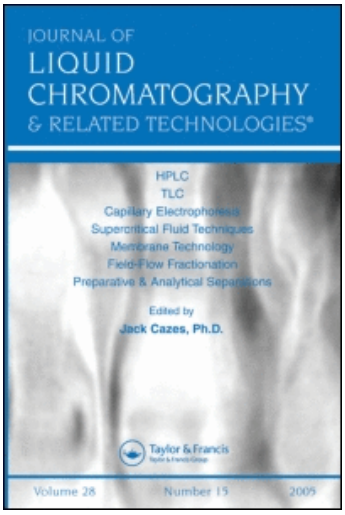


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# DETERMINATION OF BIOGENIC AMINES IN RAT BRAIN DIALYSATES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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## ABSTRACT

A general problem found in the analysis of samples from brain microdialysis is the existence of some substances which can interfere with the dopamine. In our chromatographic conditions, we found an interfering peak with the following mobile phases: acetic acid / sodium acetate 1M (with 5% methanol and 1mM EDTA), and citric acid / sodium phosphate (with 5% methanol and 2mM EDTA). The solution for this problem was to employ a mobile phase consisting of $KH_2PO_4$ (with 1mM EDTA and 5% methanol), with an ion-pair agent of sodium salt of 1-octanesulfonic acid. The interfering peak was identified as the uric acid (resulting from the cellular metabolism) based on its chromatographic properties.

## INTRODUCTION

High Performance Liquid Chromatography with Electrochemical Detection (HPLC-ECD) has been widely used for the determination of various neurochemical substrates. Two kinds of detector systems are currently used for the electrochemical detectors: a single-electrode amperometric detector and a dual- or multiple-electrode coulometric detector. For the simultaneous determination of dopamine (DA) and their metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in brain tissue and microdialysis samples, the amperometric detection system is the most used. This system is simple and inexpensive, but it presents some difficulties in clearly resolving the early eluting DA and its metabolites: 1) these substances interfere with the large solvent front and with other substances; 2) their low levels. On the other hand, coulometric detection systems are relatively expensive, but a series of two coulometric electrodes provide the capability to screen out interfering peaks.[1] This potential ability of the coulometric detection system is based on a reversible oxidation/reduction of the electroactive substrates in the electrodes and it may allow the improvement of their separation without increasing in the chromatographic analysis time.



Many methods have been developed for the analysis of the catechol derivatives, including the substances implicated in the metabolism of the dopamine.[2,3] Several papers have already described the HPLC method using the coulometric detector for the simultaneous determination of catecholamines and their metabolites in brain samples.[4,5,6] However, these methods are not satisfactory with respect to the analysis time and/or the selectivity for the simultaneous determination of other related substrates, including uric acid.

Many of those methods used buffered solutions with an acidic pH (in the range 3-5) for the separations of those compounds under isocratic reversed-phase conditions.[7] The use of ion-pairing agents (hexane, heptane, and octane sulfonic acid) allows a better separation between the different catechol derivatives, even with respect to the indol derivatives, improving their separation from the solvent and reducing the effect of other interfering compounds.[8]

Microdialysis permits the *in vivo* monitoring of drugs and endogenous compounds in the Central Nervous System (CNS).[9] The release of the neurotransmitters is measured in awaked freely-moving animals by the implantation of dialysis membranes in several brain regions in order to allow the exchange between the perfusion medium and the extraneuronal fluid. This technique presents some advantages with respect to other techniques previously used, as the push-pull perfusion and the implantation of electrodes into the brain because the tisular damage is very small, recovery of substances is high. The

major advantages of the microdialysis technique are that no sample preparation is needed and that the samples are ready for direct injection into the chromatograph.

In the analysis of samples from striatal microdialysis we found a peak which interferes with the DA when we utilized the following mobile phases: acetic acid / sodium acetate 1M (with 5% methanol and 1mM EDTA) and citric acid / sodium phosphate (with 5% methanol and 2mM EDTA). In the present paper, we report a rapid HPLC method coupled to a coulometric detection with the use of an ion-par agent to resolve the chromatographic interference consisting of the co-elution of the uric acid (produced from the cellular metabolism of N) with the dopamine released from the neurons of the rat brain striatum.



## EXPERIMENTAL

### Chemicals and Reagents

Methanol HPLC grade was supplied by Merck. Water was obtained from a MilliQ system (Millipore). Pure substances of DA, DOPAC, HVA, 5HIAA, and uric acid were obtained from Sigma (St. Louis, MO, USA). The stock solutions of pure substances (10 mg/mL) were kept at 4°C during a long period of time. The other reagents ($KH_2PO$, EDTA, citric acid, sodium acetate, acetic acid, and the sodium salt of 1-octanesulfonic acid SOS) were analytical grade and they were supplied by Sigma (St. Louis, MO, USA).

### Microdialysis

Male Sprague-Dawley rats (250-300 g) were anaesthetized with chloral hydrate (400 mg/g, i.p.) and placed in an stereotaxic apparatus (Narishige). The probe (CMA/12, 0.5 mm internal diameter, 3 mm length) was stereotaxically placed within the left striatum (coordinates from bregma: A +2, L +3, and V +6) through a hole drilled in the skull. A standard Ringer medium (147 mM NaCl, 3.4 mM $CaCl_2$, 4 mM KCl) was perfused through the probe at 2 μL/min, and the samples were collected every 15 min.

The microdialysis samples were used for the determination of the levels of DA, DOPAC, and HVA. The typical *in vitro* probe recovery was found to be approximately 15 % for DA and 25 % for its metabolites.

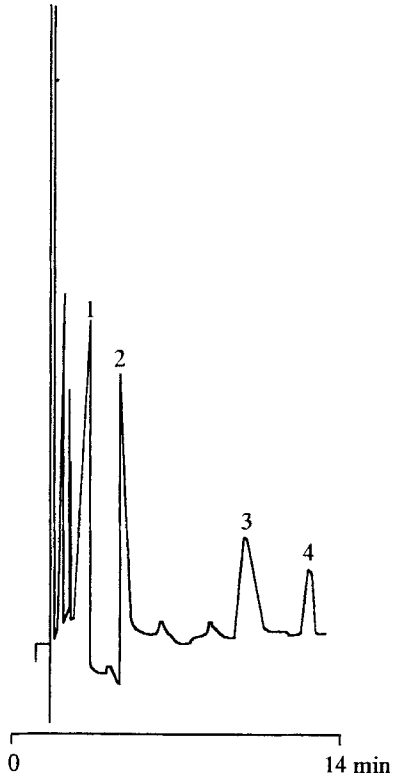





**Figure 1**. Representative chromatogram of dialysate of rat striatum obtained using eluent consisting of acetic/acetate buffer 1 mM EDTA and 5% methanol at pH 5.0 and flow rate of 1 mL/min. Peaks: 1= DA + uric acid, 2= DOPAC, 3= 5-HIAA, 4= 5-HT.

## Chromatographic Conditions

The HPLC system was an HP Series 1050 Pump equipped with an injection valve, Rheodyne 7125. The separation of the DA and its acidic metabolites (DOPAC and HVA) was made under isocratic conditions, using reversed-phase columns Spherisorb ODS-1 (with particles of 10 $\mu$m).

The ESA model 5100A Coulochem electrochemical detector (ESA Inc., Bedford, MA) was used with a model 5011 dual electrode analytical cell. The potential used for the analysis was +400 mV.

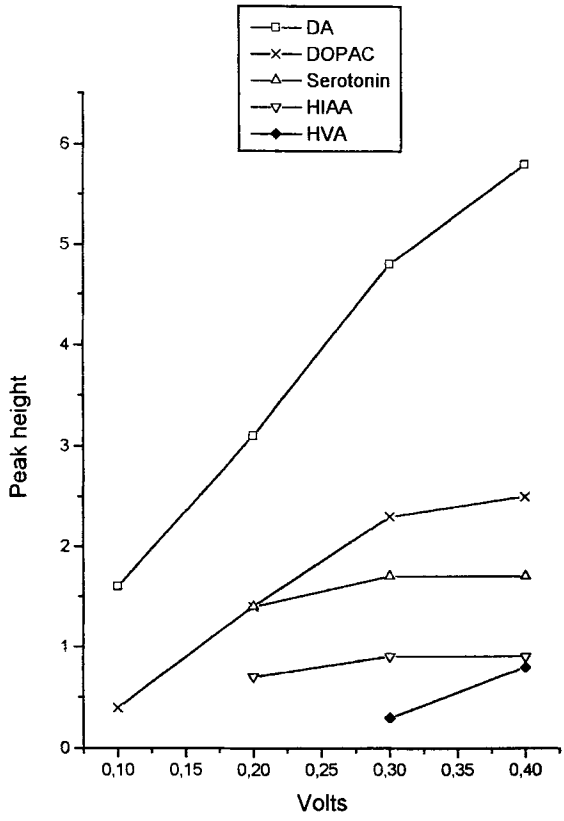



**Figure 2.** Hydrodynamic voltamnograms of 1 ng/injection of DA (1); DOPAC (2), 5-HT (3), 5-HIAA (4) and HVA (5) with acetic/acetate mobile phase at pH 4.0. Flow rate 1.5 mL/min.

For the hydrodynamic voltammograms, the oxidation potentials assayed were in the range of +100 to +500 mV. The output signal from the detector was registered by an Omniscribe Series D5000 Recorder (Houston Instruments). The mobile phases assayed were the following:

1. A buffer of acetic acid / sodium acetate 1M with 5% methanol and 1mM EDTA, at a flow rate of 1 mL/min. The assayed pHs were 4.0, 4.5, and 5.0.

2. A buffered solution of citric acid / sodium phosphate with 5% methanol and 2mM EDTA. The flow rate was 1 mL/min. The assayed pHs were 3.5, 4.0, 5.0, 6.0, and 6.5.

3. A solution of potassium phosphate ($KH_2PO_4$) with 1mM EDTA, using as ion-pair agent the SOS. The different conditions were as follows: a pH range 3-4, a % of methanol between 5-10, and a flow rate between 1 and 2 mL/min.

The parameters used to evaluate the retention of the different compounds were the Retention Time (RT) and the Capacity Factor (K′). In order to calculate the parameter of retention K′, we used the RT obtained in the same chromatographic conditions.

## RESULTS

A representative chromatogram obtained from a dialysate of striatum using the acetic-acetate eluent is shown in Figure 1. It is observed that the DA concentration is about 20-30 ng/20 μL of sample, while the metabolite concentrations are about 3 ng/20 μL.

The amount of DA is higher than expected, which made us think that other substances present in the sample could co-elute with DA. The uric acid produced from the cellular metabolism of N could be the substance which produces the interfence with the DA peak.

The use of the eluent consisting of acetic-acetate buffered solution at pH 5.0 permitted the resolution of all analytes of interest (DA, 5-HT, and metabolites) in 18-20 min, with 5-HT eluting as the final component (Table 1). However, the uric acid appears with a RT coincident with the DA (6.8 min). In chromatograms obtained from external standard mixtures of DA and uric acid it appears as a unique peak, which is the sum of both substances.

The optimal potential for the analysis, according to the hydrodynamic voltammograms (Fig. 2), was determined to be +0.4 V for DA and all the metabolites. Substances with a catechol or 5-hydroxyindol structure generate detector signals at relatively low oxidation potentials, while the o-methylated derivatives (HVA) require potentials of at least +0.4 V in order to obtain measurable detector signals.[10]

The retention times (RT) for the different external standards (DA, DOPAC, HVA, 5-HT, 5-HIAA, and uric acid) in different analytical conditions are shown in the Table 1.

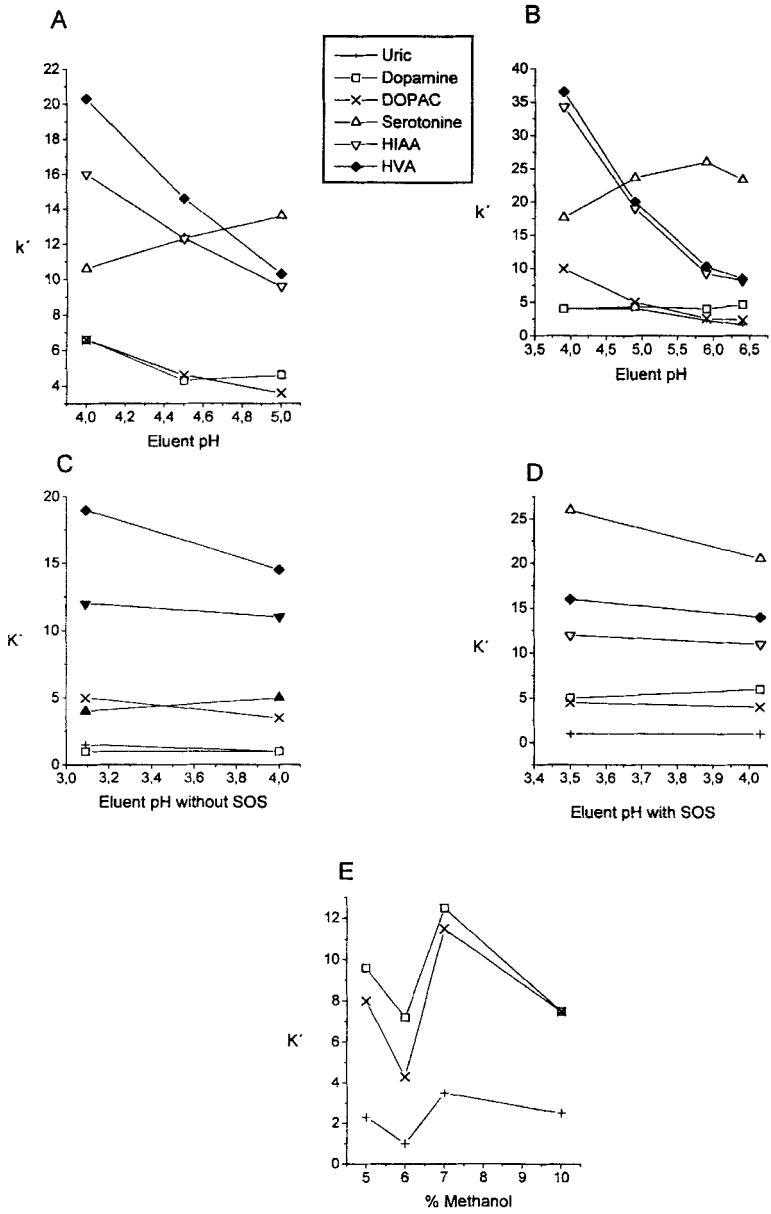
The retention parameters K′ calculated for the different substances and chromatographic conditions are shown in the Figure 3. The results are expressed as the mean of 4-6 determinations.

**Table 1**

**Retention Data for Uric Acid, Biogenic Amines, and Acidic Metabolites Using Different Eluents in Different Conditions**

| | | Retention Time (Min.) | | | | | |
|---|---|---|---|---|---|---|---|
| | **pH** | **Uric Acid** | **DA** | **DOPAC** | **5-HT** | **5-HIIAA** | **HVA** |
| Acetic/Acetate 1M | 4.0 | — | 9.2 | 9.2 | 14 | 20.4 | 25.6 |
| F=1mL/min | 4.5 | — | 6.4 | 6.8 | 16.4 | 16 | 18.8 |
| 1mM EDTA | 5.0 | — | 6.8 | 5.6 | 17.6 | 12.8 | 13.6 |
| 5% Methanol | | | | | | | |
| Citric/Phosphate | 3.4 | 6 | 5.2 | 14.4 | — | — | — |
| F=1mL/min | 4.0 | 6 | 6 | 13.2 | 22.4 | 42.4 | 45.2 |
| 2mM EDTA | 5.0 | 6 | 6.4 | 7.2 | 29.6 | 24 | 25.2 |
| 0% Methanol | 6.0 | 4 | 6 | 4.4 | 25.2 | 12.4 | 13.6 |
| | 6.0 | 4 | 6 | 4.4 | 25.2 | 12.4 | 13.6 |
| | 6.4 | 3.2 | 6.8 | 4 | 29.2 | 11 | 11.4 |
| | **Conditions** | | | | | | |
| $KH_2PO_4$ | without SOS, pH 3.0 | 2 | 1.6 | 4.8 | 4 | 10.4 | 16 |
| F=2 mL/min | with SOS, pH 3.5 | 1.6 | 4.8 | 4.4 | 16.8 | 10.4 | 13.6 |
| 1mM EDTA | without SOS, pH 4.0 | 1.6 | 1.6 | 3.6 | 4.8 | 9.6 | 12.4 |
| 5% Methanol | with SOS, pH 4.0 | 1.6 | 5.2 | 4 | 17.2 | 9.6 | 12 |
| | **% Methanol** | | | | | | |
| $KH_2PO_4$ | 5% | 4 | 12.8 | 10.8 | — | — | — |
| F= 1 mL/min | 6% | 3.6 | 12.6 | 9.6 | — | — | — |
| 1mM EDTA | 7% | 3.6 | 10.8 | 10 | — | — | — |
| 1mM SOS | 10% | 2.8 | 6.8 | 6.8 | — | — | — |

Figure 3 illustrates the effect of the pH change and the different mobile phases on the retention of the substances analyzed. As we can see, K′ is pH-dependent for all the substances. The values of K′ decrease when the dissociation of the solutes increase (low pH). For the acidic compounds, the pH of the mobile phase was low enough to suppress the dissociation of the molecules and to obtain longer retention times and better separation.



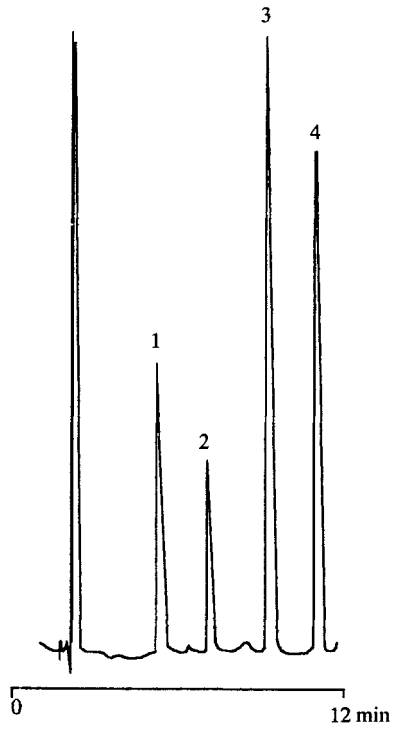



**Figure 4.** Standard chromatogram (1 ng/injection of each 1= DA, 2= DOPAC, 3= 5-HIAA and 4= HVA) obtained using the mobile phase $KH_2PO_4$ with SOS, 5% methanol and pH 4.0. Flow rate 2 mL/min.

The mobile phase consisting of citric/phosphate at pH 6.0 or 6.4 permitted the separation of DA and uric acid, but interferences between other substances have been observed, besides a run time too long. A standard chromatogram showing the separation of 1 ng of each substance, using the eluent consisting of potassium phosfate solution containing SOS, is shown in the Figure 4. The compounds were resolved and identified in base of RT, as well as because their electrochemical behaviour.

**Figure 3 (left).** Retention data of DA, DOPAC, 5-HIAA, 5-HT, HVA and uric acid with (A) acetic/acetate eluent in the pH range of 4.0-5.0, (B) citrate/phosphate eluent in the pH range 3.5-6.5, (C) $KH_2PO_4$ eluent without SOS in the range of 3.0-4.5, (D) $KH_2PO_4$ eluent with SOS, and (E) with methanol in the range of 5-10% methanol.

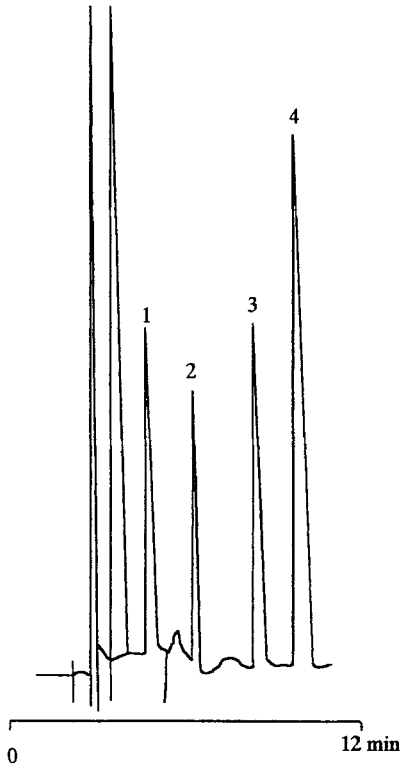



**Figure 5**. Representative chromatogram of dialysate of rat striatum obtained using the mobile phase $KH_2PO_4$ with SOS, 5% methanol and pH 4.0. Flow rate 2 mL/min. Peaks: 1= DA; 2= DOPAC; 3= 5-HIAA; 4= HVA.

At pH 4.0 and with 5% of methanol, all compounds were well resolved, without interferences and the analysis was completed in less than 18 min. The on-column limit of detection was estimated from external standards to be approximately 100 pg/ 20 µl.

An increase in the concentration of methanol lead to a decrease in K´values for all compounds without affecting the order of elution. Optimal resolution was achieved with a methanol concentration of 5%. The presence or absence of SOS in the eluent interfered with the retention of amines, especially DA and 5-HT, and uric acid (Table 1, Fig. 3). Figure 5 shows a typical chromatogram from the rat brain striatum obtained using $KH_2PO$ with SOS, 5% methanol and pH 4.0. The two monoamine neurotransmitters (DA and 5-HT) and the three major

metabolites (DOPAC, 5-HIAA and HVA) were well separated from each other and from tissue-derivated interferences. No solvent front peak interfered with the separation of the early eluting peaks, being apparent the uric acid peak.

## DISCUSSION

A very common problem present in the analysis of samples from microdialysis is the existence of an interfering compound, which co-elutes with DA. Because of the high levels of this compound and the very low levels of endogenous DA, the problem is practically impossible to resolve with mobile phases without ion-pair agents. This problem is not present in the case of post-mortem samples.[5] In our chromatographic conditions, we found that interference with the mobile phases of acetic acid / sodium acetate 1M (with 5% methanol and 1mM EDTA), and citric acid / sodium phosphate (with 5% methanol and 2mM EDTA). We resolved this problem with the use of a mobile phase consisting of $KH_2PO_4$ with 1mM EDTA, using as ion-pair agent the SOS.

The interfering peak was the uric acid from the cellular metabolism and it was identified by its chromatographic behaviour. That was also demonstrated by the use of the pure substance in the same chromatographic conditions.

Reversed-phase chromatography has long been employed for the general analysis of biogenic amines.[11] However, the chromatographic method shown here was specifically designed to measure DA, DOPAC, 5-HIAA, and HVA from microdialysis samples within the time constraint imposed by sampling. DA was selectively separated from potential interferences present in the perfusate output (such as uric acid) using as ion-pairing agent the SOS.

Alkylsulfate ion-pair agents form a strong ion-pair complex with monoamines. The relatively high level of SOS in the mobile phase was required to enhance the selectivity and separation from the solvent front. However, the strong retaining effects of the long chain ion-pair agent on the late-eluting HVA had to be moderated by the high content of a strong organic modifier (methanol). As expected, methanol enhanced the elution of all compounds, whereas SOS increased the retention of amines only.[10]

Thus, pH choice and level of ion-pair agent, and organic modifier percentage were manipulated in order to exert control over metabolite and catecholamine-SOS pair placement and retention time. Additionally, EDTA in the mobile phase stabilized the catecholamine structure and it avoided the possible interference of inorganic ions.[11]

## REFERENCES

1. H. Saito, S. Murai, E. Abe, Y. Masuda, T. Itoh, "Rapid and Simultaneous Assay of Monoamine Neurotransmitters and their Metabolites in Discrete Brain Areas of Mice by HPLC with Coulometric Detection," Pharmacol. Biochem. Behav., **42**, 351-356 (1992).

2. R. Durán, M. Aldegunde, J. Marco, "Simple HPLC-EC Method for the Simultaneous Determination of Biogenic Amines and their Main Metabolites in Small Rat Brain Regions," Anal. Lett., **18(B17)**, 2173-2181 (1985).

3. I. J. Kopin, "Catecholamine Metabolism: Basic Aspects and Clinical Significance, Pharmacol. Rev., **37**, 333-364 (1985).

4. A. Colzi, A. Musolino, A. Iuliano, F. Fornai, U. Bonuccelli, G. U. Corsini, "Identification and Determination of 3,4-Dihydroxyphenylacetaldehyde, the Dopamine Metabolite,"in In Vivo Dialysate from Rat Striatum, J. Neurochem., **66(4)**,1510-1517 (1996).

5. I. C. Kilpatrick, M. W. Jones, O. T. A. Phillipson, "Semiautomated Analysis Method for Catecholamines, Indoleamines and Some Prominent Metabolites in Microdissected Regions of the Nervous System: An Isocratic HPLC Technique Employing Cuolometric Detection and Minimal Sample Preparation," J. Neurochem., **46(6)**, 1865-1876 (1986).

6. G. Archilli, C. Perego, F. Ponzio, "Application of the Dual-Cell Coulometric Detector: A Method for Assaying Monoamines and Their Metabolites," Anal. Biochem., **148**, 1-9 (1985).

7. Y. Bednar, P. Södersten, G. Ali Qureshi, "A Simple HPLC Method in Monitoring Catecholamines, Tryptophan and Their Metabolites in CSF of Rats: A Role of Dopamine and Noradrenaline in Food Intake," J. Liq. Chromatogr., **15(17)**, 3087-3096 (1992).

8. M. G. Mazzacoratti, D. Amado, E. Cavalheiro, "A. HPLC Determination of Norepinephrine, 5-Hydroxytyramine, and 5-Hydroxytryptamine in Rat Brain Using Sodium Dodecyl Sulphate as Ion-pair," Braz. J. Med. Biol. Res., **23(3-4)**, 255-262 (1990).

9. S. Sarre, Y. Michotte, "High-Performance Liquid Chromatography with Electrochemical Detection for the Determination of Levodopa, Catecholamines, and their Metabolites in Rat Brain Dialysates," J. Chromatogr., **575**, 207-212 (1992).

10. A. Jussofie, J. Löjewski, C. Hiemke, "Simultaneous Automated Determination of Catecholamines, Serotonin and Their Metabolites in Brain Tissue by HPLC and Electrochemical Detection," J. Liq. Chromatogr., **16(2)**, 447-463 (1993).

11. K. Cox Gariepy, B. Bailey, J. Yu, T. Maher, Y. N. Acworth, "Simultaneous Determination of Norepinephrine, Dopamine, and Serotonin in Hippocampal Microdialysis Samples Using Normal Bore High Performance Liquid Chromatography: Effects of Dopamine Receptor Against Stimulation and Euthanasia," J. Liq. Chromatogr., **17(7)**, 1541-1556 (1994).

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