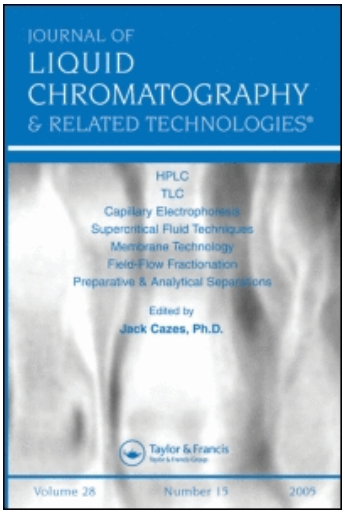

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Simultaneous Determination of Norepinephrine, Dopamine, and Serotonin in Hippocampal Microdialysis Samples Using Normal Bore High Performance Liquid Chromatography: Effects of Dopamine Receptor Agonist Stimulation and Euthanasia

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SIMULTANEOUS DETERMINATION OF NOREPINEPHRINE, DOPAMINE, AND SEROTONIN IN HIPPOCAMPAL MICRODIALYSIS SAMPLES USING NORMAL BORE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: EFFECTS OF DOPAMINE RECEPTOR AGONIST STIMULATION AND EUTHANASIA

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ABSTRACT

A sensitive and selective conventional chromatographic method has been developed for the simultaneous measurement of norepinephrine, dopamine, and serotonin from in vivo rat brain microdialysis samples. Ion-pair reversed-phase high performance liquid chromatography was used in combination with coulometric electrochemical detection to generate femtogram-level chromatograms reliably and routinely. Analysis is completed within 10 minutes making it ideally suited for the in vivo study of acute pharmacological manipulation in the rat brain to monitor the effects of drugs on and the potential interactions between monoaminergic systems. System performance was evaluated using in vivo microdialysis perfusate samples obtained from the right ventral hippocampus of the anesthetized rat before and after administration of the D_2/D_1 receptor agonist apomorphine as well as post-euthanasia. Basal levels and pharmacological effects are reported and discussed.

INTRODUCTION

In order to improve the understanding of normal brain function, it is especially important to study the in vivo changes in regional extracellular neurotransmitter release and metabolism. Microdialysis is now a routine sampling technique for the collection of many compounds within the extracellular space in many tissues [1]. It has found particular popularity in combination with HPLC and electrochemical detection for the examination of small changes in neurotransmitter levels in brain tissue [2,3]. Only recently with improvements in analytical equipment, techniques, and the introduction of high efficiency microdialysis probes have the low-level analyses of biogenic amines in microdialysis samples been possible [3-6]. However the majority of current established chromatographic methods typically allow the analysis of only one particular neurotransmitter/system per sample [7-11].

Rather than investigating individual neurotransmitters as single extracellular biochemical events, a deeper understanding would result from the study of several neuronal systems simultaneously. Consequently the important interplay and interdependency between the distinct monoaminergic systems could be better defined not only intra-regionally [12] but between brain regions as well [13], as implied by evidence for inter-regional neuronal networks [14]. Accordingly, the effect of a drug on normal brain activity could be more accurately assessed as the understanding of normal brain function is further refined.

A suitable analytical method must be completed well within the time constraints imposed by sampling and sensitive enough to allow depletion studies in regions containing low levels of neurotransmitters, without the need for artificially elevating basal levels of the monoamines by either increased calcium levels [15-17] in artificial CSF (aCSF) or by localized introduction of reuptake blockers [18, 19] by way of the aCSF. In this way the analysis can be performed under as near normal physiological conditions as possible. Presented here is a new, highly sensitive, stable and selective conventional-HPLC system for the simultaneous analysis of NE, DA, and 5HT from rat brain microdialysis samples.

The hippocampus was chosen to validate the method since the analysis of the low-level of monoamines found there represents a significant challenge. One specific objective was to verify and establish the presence of NE, 5HT and particularly DA in the ventral hippocampus. A second objective was to examine levels of these monoamines following a drug intervention designated to decrease their release. The non-specific D_2/D_1 receptor agonist apomorphine was chosen to reduce DA levels, based, in part, on its known effects in the striatum [20]. The consequence of its action following peripheral administration will be discussed.

METHODS

Chemicals And Reagents

The purest grades of chemicals available were obtained and used in all solutions and mobile phases. Disodium ethylenediaminetetraacetate (EDTA) and phosphoric acid were purchased from Fisher Scientific Co. (Fairlawn, NJ, U.S.A.). Sodium dihydrogen phosphate, acetonitrile, and methanol were purchased from EM Science (Gibbstown, NJ, U.S.A.), while triethylamine (TEA) was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Perchloric acid (PCA) was purchased from J.T. Baker Inc. (Phillipsburg, NJ, U.S.A.) and ethanol from Pharmco (Dayton, NJ, U.S.A.). The monoamines norepinephrine (NE), epinephrine (E), dopamine (DA) and serotonin (5HT), and R(-)-apomorphine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Apomorphine solutions were prepared in 0.1μM ascorbate (Gibco Laboratories, Chagrin Falls, OH, U.S.A.) to ensure stability. Sodium dodecyl sulfate (SDS) was obtained from American Bioanalytical (Natick, MA, U.S.A.). All aCSF components (sodium chloride, calcium chloride, magnesium chloride, potassium chloride, and sodium dihydrogen phosphate) were procured from Fluka Chemical Corp. (Ronkonkoma, NY, U.S.A.).

Water Treatment

Milli-Q water (18 MΩ) was passed over a C18 solid-phase extraction cartridge (Sep-pak, Millipore Corp., Milford, MA, U.S.A.) prior to use in every aqueous stock solution, dilution, and mobile phase preparation introduced onto the HPLC-ECD system.

Standard Stock Solutions

Individual stock solutions of the monoamines (5 mM or 1 mg/ml) were prepared in 0.2 M PCA, 100 μM EDTA and stored at 4 °C.

Microdialysis Probe Preparation

The 3 mm loop-type regenerated cellulose probe (ESA Inc., Bedford, MA, U.S.A.) was perfused with 70% ethanol overnight (0.3 μl/min) by a microdialysis syringe pump (Model 22, ESA Inc.) to remove the storage solution. On the day of implantation, the probe was flushed with treated Milli-Q water at 5 μl/min for 30 minutes. Artificial CSF [15, lacking ascorbate] was then perfused through the probe (1.5 μl/min) and immersed in a fresh monoamine standard mixture (20 nM) diluted from 1mg/ml stocks with aCSF. In vitro recovery was determined daily prior to implantation with flow at 1.5 μl/min at 25 °C, to verify probe consistency and system stability. In vitro recoveries were not used to determine extracellular fluid (ECF) levels as this would underestimate the actual ECF concentrations [21-23].

Microdialysis

Male Sprague-Dawley rats (275-350 g) were anesthetized with urethane (2 g/kg i.p.) and stereotactically implanted with a precalibrated 3 mm probe into the right ventral

hippocampus (coordinates from bregma: AP -5.8, LR 4.8, and DV -7.5 mm from the dura surface). The probe was perfused with aCSF at 1.5 µl/min. Samples were collected every 20 minutes into a tube containing 5 µl 0.2 M PCA/100 µM EDTA to minimize monoamine decomposition. Body temperature was monitored rectally and was maintained at 37 °C by a heating pad. After allowing a period of 2h for stabilization of 'injury-mediated' neurotransmitter release resulting from probe implantation [2, 23], basal samples (defined as three consecutive samples in which monoamine levels varied by less than 10%) were then collected. Animals then received either physiological saline or apomorphine in 0.1µM L-ascorbic acid (0.5 mg/kg) administered subcutaneously (sub. cut.). Samples post-euthanasia (induced by anesthetic overdose) were also collected to verify that monoamine release increased upon death.

Microdialysis Probe Storage And Reuse

The storage conditions of the probes were dependent upon the length of time between experiments. If the probe was to be used the next day then at the completion of an experiment the syringe was washed and filled with water while the probe was washed with and immersed in water. The flow was maintained at 0.3 µl/min overnight. If the probe was not to be used the following day then it was rinsed with water followed by 70% ethanol and stored in that solution for a period of up to 3 weeks. If the latency period exceeded 3 weeks, the probe was washed with water and then filled with 5% glycerol prior to storage.

HPLC Analysis

The chromatographic system was modified to optimize performance for electrochemical detection. A low stroke volume (10 µl) dual piston pump (Model 580) equipped with PEEK (polyetheretherketone) tubing was fitted with two in-line serial

PEEK pulse dampeners. An inert Rheodyne 9125 injector with fixed 20 µl PEEK sample loop was used to introduce perfusates onto an ESA HR-80 column (3µ, C18, 8.0 cm x 4.6 mm i.d.). The analytes were detected on a Coulochem II electrochemical detector equipped with a dual electrode analysis cell (Model 5014 Microdialysis Cell: E_1 -175 mV; E_2 +175 mV vs Pd reference electrode) and a Model 5020 Guard Cell (E_{GC} +300 mV), all from ESA Inc. The mobile phase, pumped at 1.0 ml/min, consisted of 75 mM NaH_2PO_4, 1.5 mM SDS, 100 µl/l TEA, 20 µM EDTA, in 15% acetonitrile and 13% methanol. The pH of the aqueous phase was adjusted to 5.6 before addition of organic modifiers. Final concentrations and amounts are reported. The separation was affected at 25 °C. Hydrodynamic voltammograms were generated for optimization of the electrode potentials.

RESULTS

The optimal applied potentials, according to the hydrodynamic voltammograms, were determined to be 75 mV for DA, 100 mV for NE, and 125 mV for both E and 5HT. A standard chromatogram representing 800 fg injected on column for NE, DA, and 5HT is shown in Figure I. The analytical run time was less than 10 minutes and the retention times with day-to-day variability were 2.83 ± 0.07, 5.20 ± 0.16, and 9.16 ± 0.38 minutes for NE, DA, and 5HT respectively. The on-column limit of detection at s/n=4 was estimated from external standards to be approximately 400 fg for NE and DA, and 700 fg for 5HT.

The in vitro recovery of these monoamines using the 3mm 'loop-type' probe at 1.5 µl/min and 25 °C was 25-30%. No interferences were derived from the aCSF. However, as a matter of observance it is reported that small non-interfering chromatographic artifacts occasionally appeared in the baseline after the NE and DA peaks and appeared to be associated with the potassium and magnesium components in the

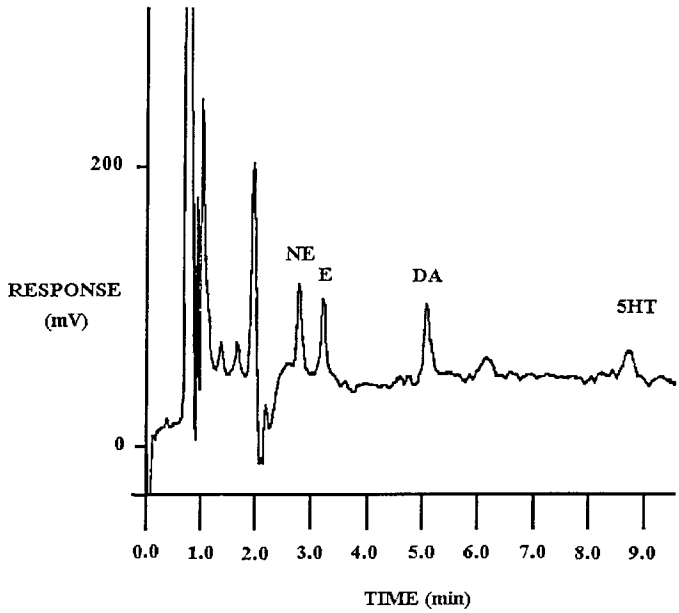

FIGURE I

A low-level standard (800 fg of each NE, E, DA, and 5HT) was injected onto the column with chromatographic conditions as described in the text to generate the above chromatogram. The detector current range is 2 nAFS with a 5 second filter time constant.

aCSF. A basal sample from the right ventral hippocampus of the anesthetized rat is shown in Figure II. In that chromatogram, the on column amounts represent 2.1, 0.49, and 0.95 pg of NE, DA, and 5HT, respectively. The overall basal levels of monoamines per 20 minute collection (30 µl microdialysis sample) in the rat right ventral hippocampus were calculated to be 4.3 ± 0.78, 2.2 ± 0.94, and 8.1 ± 3.58 pg of NE, DA, and 5HT, respectively (n=17). Treatment with the D_2/D_1 receptor agonist apomorphine induced a maximal decrease (56%) in the level of DA in the hippocampal ECF after 100 min with concurrent decreases in both NE (34%) and 5HT (40%) (n=7) (Figure III). Marked increases (typically greater than 15 fold) in all three monoamines were

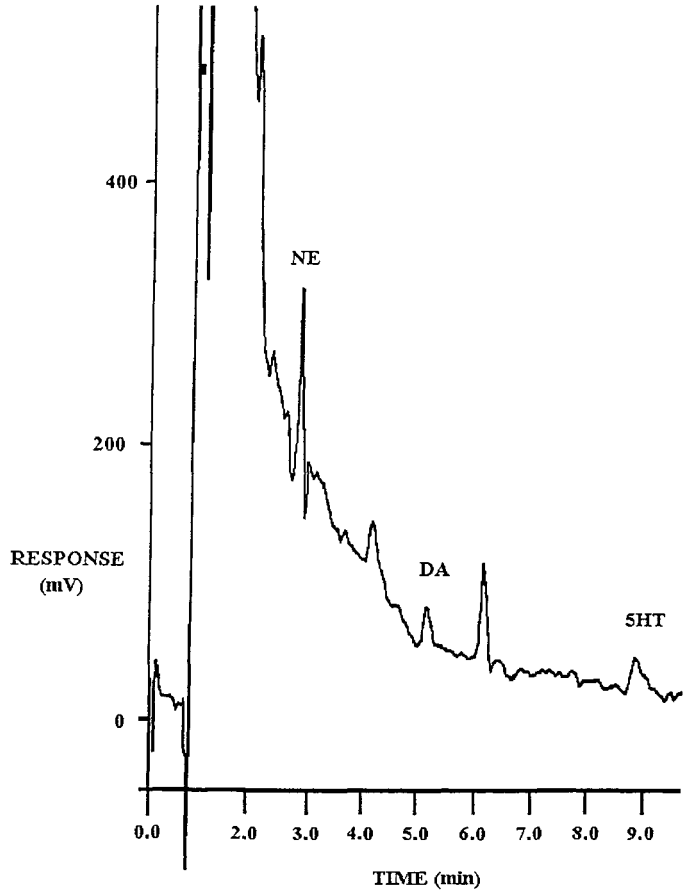

FIGURE II

This chromatogram represents a 20 µl injection of a 30 µl basal microdialysis sample (plus 5 µL PCA) from the right ventral hippocampus of the anesthetized rat. The on-column amount of each NE, DA, and 5HT shown is 2.1, 0.49, and 0.95 pg respectively. Chromatographic conditions are described in the text. The detector current range is 2 nAFS with a 5 second filter time constant.

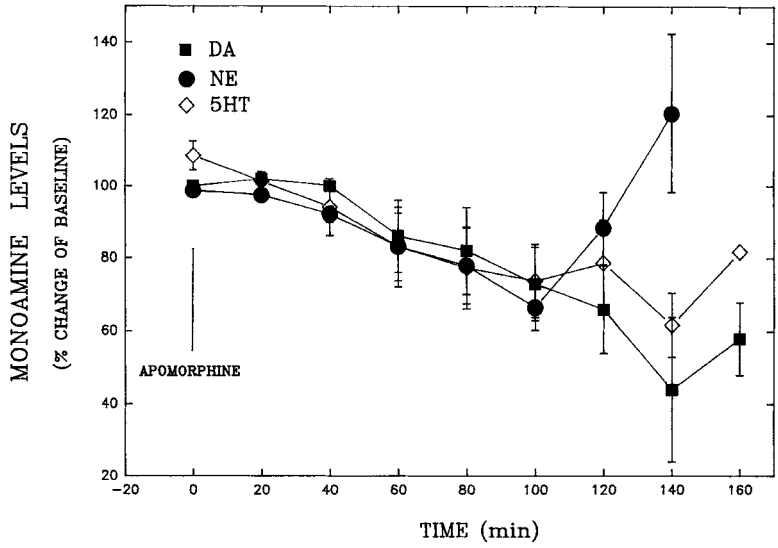

FIGURE III.

The effect of peripherally administered apomorphine (0.5 mg/kg sub. cut.) on the hippocampal levels of NE, DA, and 5HT in the anesthetized rat. The drug was introduced at t=0 min. Changes in the concentration of the monoamines are expressed as a percentage of the mean of the basal levels. Mean values are plotted with the s.e.m. indicated by vertical bars.

observed after administration of an overdose of anesthetic for euthanasia (n=3). The i.p. administration of saline (control) had no significant effect on any of the monoamines measured, except for 5HT which tended to slowly decrease with time (data not shown). Although E standards were analyzed, no equivalent peak was measured in any of the hippocampal samples, even post-mortem. Additionally, no unknown sample components were found to co-elute with any of the monoamines in the microdialysis samples.

All data are reported uncorrected in regard to the in vitro recovery. Probes were typically reused in 10-20 animal experiments before inefficient in vitro recovery compromised analytical sensitivity.

DISCUSSION

Chromatography

Reversed-phase chromatography has long been employed for the general analysis of biogenic amines. However, the chromatographic system presented here was specifically designed to measure the monoamines NE, E, DA, and 5HT only, from microdialysis samples within the time constraint imposed by sampling. Monoamines were selectively retained as potential interferences present in the perfusate (such as DOPAC, HVA, and 5HIAA) were intentionally placed in the void.

Alkylsulfate ion-pair agent forms a strong ion-pair complex with monoamines, at this pH [24]. The relatively high level of SDS in this mobile phase was required to enhance selectivity and separation in the front of the chromatogram. However the strong retaining effects of this long chain ion-pair agent on the late-eluting 5HT had to be moderated by a high content of strong organic modifier, particularly acetonitrile. Methanol was also included to refine the retention of the monoamines but also to provide greater solubility for the buffer and ion-pair reagents. Thus pH, column selection, choice and level of ion pair agent, and organic modifier percentage were manipulated to exert control over metabolite and monoamine-SDS pair placement and retention time.

The fairly clean chromatogram and system stability that results is due in large part to the filtration through the 6000kD molecular weight cutoff membrane of the dialysis probe. However several other components were added to the mobile phase to further enhance the stability of the system as well as peak symmetry, system sensitivity. The amine modifier TEA was included in the mobile phase to reduce tailing problems associated with secondary-retention effects [25]. The resultant improvement in peak symmetry provided better peak resolution and quantitation. The inert PEEK components substituted for stainless steel pieces in places along the fluid path improved system performance. However free metals from such apparent sources as

column frits, column walls, and irreplaceable stainless steel tubing were chelated by EDTA in the mobile phase. Additionally, EDTA stabilizes the catecholamine structure [26].

Increased background currents and random contamination peaks were also limited by the use of ultrapure water in mobile phase preparations. Commercial water purification systems effectively produce water of quality 18.2 $M\Omega/cm^3$, yet the quantity of trace organic material from bacterial sources is unknown. These organic compounds are not removed by the traditional purification cartridges but are easily eliminated by passage over a C18 solid phase extraction cartridge. This 'polished' water was used in all solutions introduced onto the HPLC system to ensure that the HPLC system was kept clean and metal-free.

Finally, the buffer concentration was maintained at 75 mM to provide sufficient buffering capacity to the mobile phase as well as to serve as an adequate electrolyte for electrochemical detection to provide a stable baseline.

Detection

The electrochemical detector employed here was comprised of low noise potentiostats and a high-efficiency porous graphite analytical cell, designed specifically for the analysis of microdialysis perfusates. The application of fairly low potentials and the use of the guard cell resulted in extremely low background currents (typically below 3 nA) and ultimately better sensitivity. This HPLC-ECD system coupled with the use of high efficiency microdialysis probes yielded excellent selectivity and sensitivity for monoamines in the in vivo samples for a period of greater than 1 year.

Monoamine Analysis

The monoamine metabolites were deliberately excluded from the assay. In the past, levels of the metabolites were used to estimate monoamine release (especially when

measurement of the parent monoamine itself was difficult), but their levels are now thought to be more a reflection of intraneuronal metabolism rather than an accurate indicator of neurotransmitter release per se [27-29]. Although we are not the first to measure a monoamine with the exclusion of its metabolites [30], we have developed this assay to measure three monoamine neurotransmitters simultaneously and with high temporal resolution.

Basal monoamine levels of NE from the rat hippocampus were comparable to those found elsewhere [11, 31]. To date, the role of DA in the hippocampus has remained obscure. In the past, support for the presence of DA in the hippocampus has mainly come from evidence of DA innervation [32], tissue levels [33], and release from synaptosomes [34]. Previously basal hippocampal ECF DA levels have usually only been detected in the presence of the reuptake blocker nomifensine [19], probably due to analytical system constraints. However, only recently has DA been quantitated in the hippocampus using in vivo microdialysis without reuptake blockade [35]. With our system, not only were hippocampal basal levels of DA routinely observed and measured but ECF levels of NE and 5HT could also be examined concurrently. Levels of 5HT were only slightly higher than previously published [36] but this may be due, in part, to differences in animal models and diffusion efficiencies across the dialysis membranes.

The sensitivity of the system allowed measurements of diminished release of all three monoamines following pharmacological intervention. As in the striatum [20], peripheral administration of the D2/D1 agonist apomorphine lowered the ECF levels of DA, however the magnitude and the time course were different. Since apomorphine is known to act at both D2 and D1 receptors, studies are ongoing in an attempt to qualify which receptor subtype was affected. It is unclear at present why apomorphine also affected ECF levels of NE and 5HT in the hippocampus. It is unlikely that apomorphine is acting directly on noradrenergic receptors. Another question is whether DA is acting as a neurotransmitter per se, or whether it just constitutes a NE precursor pool which happens to be co-released when the NE neuron is depolarized. Future research might include lesioning studies and the use of enzyme inhibitors to better characterize the source and function of DA in this region.

As has been previously reported for DA in the striatum [37], death caused a major increase (greater than 15 fold) in hippocampal ECF levels of NE, DA, and 5HT, suggesting that each may be neuronal in origin. Since the epinephrine standard was chromatographically resolved from the other monoamines using this method, and no peak was found to correspond to its capacity factor, it was concluded that E was either not present in this region of the brain or that its level was below the limit of detection of this system.

Although possibly present in the microdialysis sample and selectively retained with the other monoamines, 3MT was not observed in the chromatogram. Firstly, the potential applied to the working electrode was too low for the oxidation of this amine so that it was intentionally screened from the chromatogram. Secondly, if similar to the striatum the extracellular level of 3MT is approximately 1/3 that of DA [38, 39] then the level would probably be below the detection limit as currently established. Also present in the microdialysis sample and absent from the chromatogram were the amino acids. Even though most need to be derivatized for detection by ECD [10], those amino acids that are inherently electroactive (such as tyrosine and tryptophan) are not observed since the applied potential was not optimum for their oxidation. In this way these compounds were also intentionally screened from the analysis.

This method is presently being employed to study the possible interaction between these monoamines in the hippocampus before and after administration of known receptor agonists. Since there were no late eluting compounds, this method could be used for on-line analysis if desired. Although adequate for the majority of current analyses, the sensitivity might be further improved by substituting a 3 mm i.d. column in place of the 4.6 mm column, with only minor adjustment in the flow rate.

CONCLUSION

This analytical system delivers routine and sensitive measurement of monoamines in vivo microdialysis samples. The achievement of femtogram level sensitivity is

possible without having to employ microbore methods. This method successfully demonstrates detection of basal NE, DA and 5HT in the hippocampus without the addition of reuptake blockers or increased calcium in the aCSF. Additionally, because of its conventional nature, this system is easy to use, stable, and appropriate for routine analysis. Therefore, it might also be useful in the pharmaceutical industry or in clinical laboratory, for the analysis of monoamines in other biological matrices such as plasma.

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