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In vivo on-line HPLC-microdialysis: simultaneous detection of monoamines and their metabolites in awake freely-moving rats[1]

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Abstract

An on-line microbore high performance liquid chromatographic–electrochemical (HPLC–EC) detection method has been developed for the simultaneous measurement of dopamine (3,4-dihydroxyphenethylamine, DA), its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid, HVA), as well as serotonin (5-hydroxytryptamine, 5-HT) and its metabolise 5-hydroxyindole-3-acetic acid (5-HIAA) in the striatum of awake and freely moving rats. Our method was capable of detecting DOPAC, 5-HIAA, HVA, DA and 5-HT at retention time of 3, 5, 6.5, 9, and 24 min, respectively, in repeated on-line microdialysis sampling. Analysis was performed using a 150 × 1 mm 5 μm C18 microbore column attached directly to a thin-layer radial flow electrochemical cell (UniJet®) comprising a 6 mm glassy carbon working electrode. In order to accommodate signal outputs (due to a varying level in the neurochemicals under investigation), an amperometric detector was equipped with a sensitivity programmable controlling software for automatically switching sensitivity inattentively and repeatedly for each collection cycle.

Keywords: In vivo microdialysis; Electrochemical detection; Microbore column; Dopamine; Serotonin; Metabolites, Rat striatum; Freely moving animal

1. Introduction

On-line microdialysis [1] provides the advantage of dynamically following relevant extracellular chemical events in an intact tissue in awake and freely moving animals instead of their assessment in post-mortem tissue obtained long after neurochemical changes have occurred. Numerous re-

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ports have shown effects of several centrally acting serotonergic drugs modulating brain DA release and vice versa [2,3]. In this context, simultaneous measurement of DA, 5-HT and their metabolites are desired in order to assess the neuropharmacodynamic effects of drugs on the central dopaminergic and serotonergic systems.

However, there are very few examples of simultaneous in vivo detection of 5-HT, DA and their metabolites in various areas of the rat brain reported to date, due primarily to very low levels of extracellular DA and 5-HT as compared to their metabolites, DOPAC, HVA, and 5-HIAA, respectively (Table 1) [3–8]. Therefore, a high degree of resolution is required for the baseline separation of DA and 5-HT within a reasonable elusion time from their metabolites present in a relatively much higher concentrations in the same sample. At the same time, a high level of sensitivity is needed in order to detect rather a low basal concentration of striatal 5-HT [9]. A number of factors including a low striatal concentration of 5-HT and low probe recovery, has probably limited the number of laboratories attempting to measure the simultaneous detection of 5-HT and DA.

Using the recently introduced UniJet® amperometric cell system [10,11] coupled to a microbore column (Fig. 1), our present experiment design was capable of detecting striatal DA, 5-HT and their metabolites in a 15 μl microdialysate sample within a 30-min run time. Mircodialysates were collected from awake and freely moving rats, and analyzed on-line by HPLC method. An automated computer program was used to change the sensitivity range of the amperometric detector switchable for the detection of DA, 5-HT and their metabolites in the repeated samplings (Fig. 2).

2. Materials and methods

2.1. Animal preparation

Adult female (250–350 g) Sprague-Dawley rats (Taconic Farms, Germantown, New York) were housed at a controlled temperature (70–75°F) under 12: 12 h dark/light cycle with food and water supplied ad libitum. At least 4 days prior to study, rats were anesthetized with chloral hydrate (400 mg kg^{-1} intraperitoneal) and siliconized guide cannulae (CMA/Microdialysis, Sweden) were stereotaxically implanted in right corpus striatum. The coordinates were 0.5 mm anteroposterior to bregma, 2.5 mm lateral and 2.5 mm dorsoventral from aura as described by Paxinos and Watson [12]. On the day of the experiment, microdialysis probe (CMA/12, 4 mm, 20000 dalton cut-off membrane) was positioned within the guide cannula. Artificial cerebral spinal fluid (155.0 mM $Na+$, 1.1 mM Ca^{+2}, 2.9 mM K^{+}, 132.76 mM $C1^{-}$ and 0.83 mM Mg^{+2}) [8,13] was perfused through the probe at a flow rate of 0.5 μ1 min^{-1} using CMA/100 microinfusion pump.

Table 1
Simultaneous determination of DA, 5-HT, and their metabolites (DOPAC, HVA and 5-HIAA, respectively) in the rat striatal region using in vivo microdialysis technique

Concentrations (ng ml^{-1})					
DOPAC	DA	HVA	5-HT	5-HIAA	References
117.7	0.8	91.1	0.026	70.74	[3]
—	—	—	0.00033	0.33	[4]
50	0.2	—	—	—	[5]
—	0.2	—	0.04	—	[6]
130.0	—	123.9	—	428.68	[7]
281.9	1.1	253.6	0.58	177.3	[8]

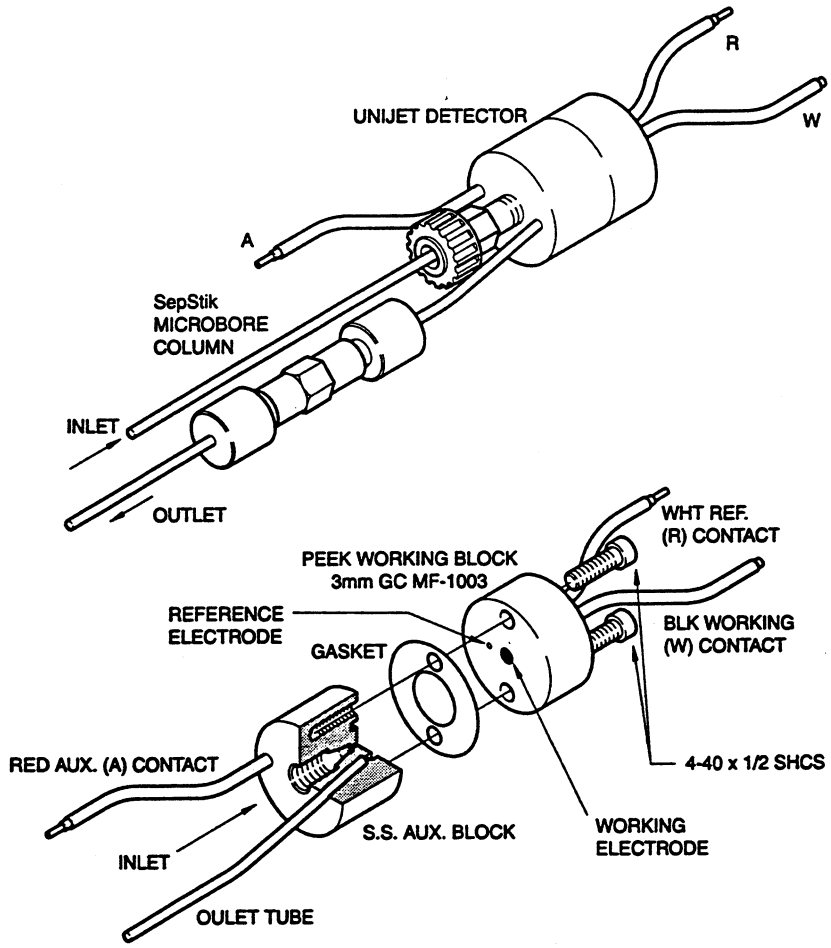

Fig. 1. UniJet® amperometric detector cell for microbore liquid chromatography (reproduced from C.E. Bohs et al. [11]).

2.2. Separation of DA, 5-HT, DOPAC, HVA and 5-HIAA, and data collection

Two hours after probe insertion, 15 μl dialysate was collected and injected on-line (CMA/160 on-line injector) every 30 min onto a 150 × 1 mm 5 μm C18 microbore column (BAS, Bioanalytical Systems, Indiana) directly attached to a 15 × 1 mm C18 pre-column (Opti-Guard®, Optimize Technologies, Oregon) with no dead volume in between. A radial flow thin-layer cell (UniJet® system, BAS) with 6 mm glassy carbon working electrode (MC-1016, BAS) was set at 750 mV (versus Ag/AgC1 reference electrode). Buffer solution containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1.0 mM sodium octyl sulfate, 17 mM sodium chloride, and 50 mM sodium phosphate monobasic (pH 4), was filtered (0.2 μm), and mixed with pre-filtered (0.2 μm) acetonitrile at 13.28:1 ratio (equivalent to 7% v/v acetonitrile in the final mixture). The mobile phase was driven by a dual-piston pump (PM-80, BAS) at a flow rate of 1.1 ml min^{-1} maintaining back pressure at ~2800 lb in^{-2}. The net flow rate calibrated from microbore column was 0.12 ml min^{-1} obtained by using a flow-splitting technique [14]. A 100 × 3.2 mm, 3 μm, C18 column (BAS) was used in parallel to microbore as a pressure dampening device via a three-way tee before the sample collecting loop.

Data was collected on-line using ChromGraph® (BAS) software as well as on a dual-pen strip chart recorder with the voltage set at 1 V and 20 mV. The voltage setting on the chart recorder was kept the same for each rat throughout the experiment. A preprogrammed sensitivity schedule (Table 2) was exercised through a data acquisition module (DA-5, BAS) on an amperometric detector (LC-4C, BAS) inattentively during each collecting cycle.

A baseline level, defined as three consecutive peak areas differing by $\leqslant 10\%$, was usually obtained 2–3 h after probe insertion. The concentrations (as nmol 15 μl^{-1} sample from striatum) of biogenic amines and their metabolites in dialysates were calculated by determining each peak area relative to the standard mixture. The identity of chromatographic peaks resulting from the analysis of brain dialysate was confirmed by their retention times with those from authentic standard samples.

2.3. In vitro recovery experiments

The percent recovery (i.e. the ratio between the concentration in the perfusate and the concentration outside the dialysis membrane) was estimated by a method similar to that of Kalen et al. [4]. Briefly, dialysis loops were perfused in vitro in a bath containing Ringer's solution and known concentrations of the analyses (0.25, 1.25, or 6.25 pmol/100 µl of DA and 5-HT, and 2.5, 12.5 and 62.5 pmol/100 ml for DOPAC, HVA, and 5-HIAA) at 37°C. Ringer's solution was passed through the probes at a constant flow rate of 2 pi min^{-1} for 3 h, and fractions collected every hour for analysis of analyse content. Three perfusates were collected from each solution, utilizing at least three different dialysis membranes. The amount of analyse in the perfusate was compared with the amount outside the dialysis probe and expressed as percent recovery. The in vitro recovery of the monoamines and their metabolites measured were in the range 10–15%.

3. Results and discussion

3.1. Effects of acetonitrile and ion-pairing agent

Although the effects of organic solvent and ion-paring agents on reversed-phase chromatography have been well known, fine-tuning the amount of both ingredients in our experiment was found to be critical. The effects of acetonitrile composition on the retention time of the analyses (Section 2) are displayed in Fig. 3. In general, too high a concentration of acetonitrile gave inade-

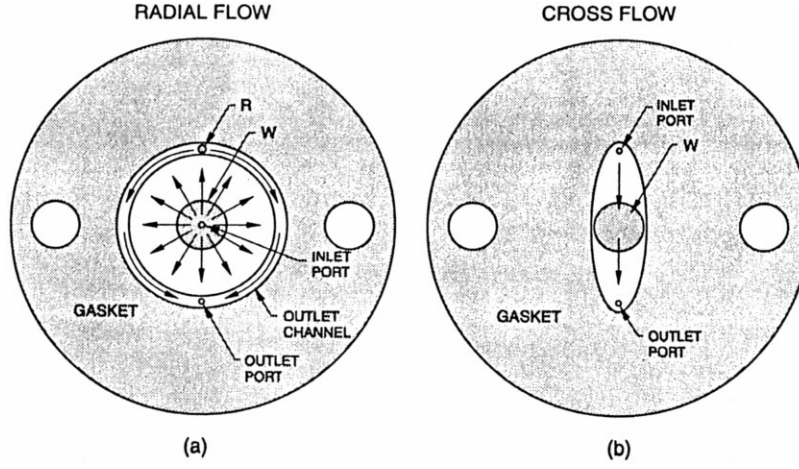

Fig. 2. (a) The flow pattern of the UniJet® radial flow thin-layer cell; (b) the classic cross flow arrangement. The letters, 'W' and'R' stand for'working' and 'reference' electrodes, respectively (reproduced from C.E. Bohs et al. [11]).

Table 2
ChromGraph® software program schedule for amperometric detector sensitivity

Time (min)	Applied potential (mV)	Sensitivity range	Signal noise filter (Hz)
0.00	750	+0.2 μA	0.03
13.50	750	+50 nA	0.10
13.75	750	+50 nA	0.05
14.00	750	+50 nA	0.02
14.50	750	+50 nA	0.02
28.00	750	+0.2 μA	0.03
28.50	750	+0.2 μA	0.03
29.00	750	+0.2 μA	0.03

quate resolution by either unacceptably decreasing the separation between DA and HVA or causing 5-HIAA to coelute with HVA. However, a rather low concentrations of acetonitrile ($<6.5\%$v/v) moved the 5-HT peak beyond a 30-min retention time range (Fig. 3), and made integration of peak area unreliable due to peak broadening and tailing. Mobile phase composition of acetonitrile in the range of 6.5–7% was optimum to obtain a baseline separation and reasonable retention time for DA and their metabolites.

Changes in the concentration of ion pairing reagent sodium octyl sulfate is also known to alter the retention times of biogenic amines such as DA and 5-HT [15]. Therefore, we investigated the effects of different concentrations of sodium octyl sulfate in the mobile phase containing 7% v/v acetonitrile in order to obtain a baseline separation of DA from HVA, as well as a reasonable retention time (less than a 30-min sample run) for 5-HT in the same microdialysate. We observed a ± 1 and ± 3 min shift in the retention times of DA and 5-HT, respectively, with a change in the concentration of sodium octyl sulfate by 50 mg l^{-1} (Table 3). This also allowed to adjust the mobile phase composition to maintain baseline separation of DA and HVA.

Thus, by manipulating the amount of sodium octyl sulfate and acetonitrile in the mobile phase, we were able to obtain DA, HVA, DOPAC and 5-HIAA peaks well resolved from each other in the standard (Fig. 4) as well as in the brain microdialysates (Figs. 5 and 6), keeping the serotonin peak well within a 30-min run-cycle. Any attempt to obtain a further baseline resolution between 5-HIAA and HVA was found to be impractical, as it delayed the 5-HT elusion time (Fig. 3), thus jeopardizing the 30-min on-line collection interval. It is noteworthy that to obtain a meaningful result in neuropharmacodynamic investigations, the effects of a drug are monitored at frequent and relatively short intervals. This ensures a high temporal resolution of the analyses under study.

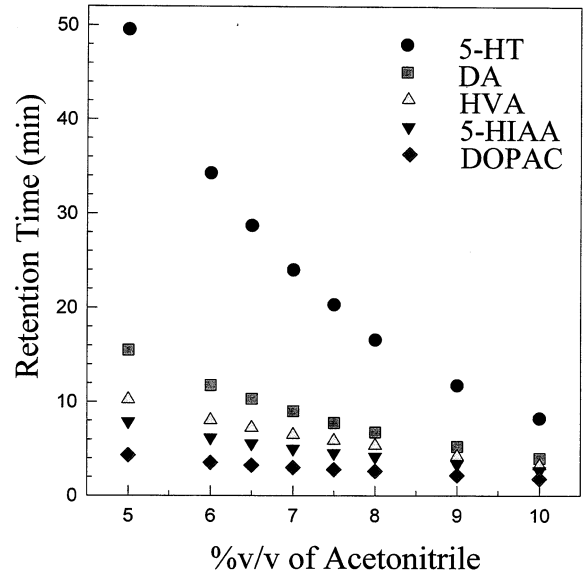

Fig. 3. Effects of acetonitrile on the retention times of DA, 5-HT, and their metabolites DOPAC, HVA, and 5-HIAA, respectively. Column: 150×1 mm 5 μm C18 microbore column (BAS) directly attached to a $1\ 5 \times 1$ mm C18 precolumn (Opti-Guard®, Optimize Technologies); mobile phase: 0.1 mM ethylenediaminetetraacetic acid, 1.0 mM sodium octyl sulfate, 17 mM sodium chloride, and 50 mM sodium phosphate monobasic (pH 4); net flow rate: 0.12 ml min^{-1} (see text for details).

Table 3
Effects of sodium octyl sulfate on the retention times of dopamine and serotonin

Sodium octyl sulfate	Retention time of DA	Retention time of 5-HT
250	7.77	21.08
300	8.92	24.07
350	10.03	27.08
400	11.23	30.23

A gradient analysis was also tried for shortening the retention time of 5-HT. However, this approach had limitations, such as inconsistent retention time and irreproducible peak area, which was experienced in our early method development stage. In addition, changing composition of the mobile phase during a gradient run is known to cause an increased and drifting background when using EC detection [16]. Additionally, an increased baseline noise generated from pump pulses by passing the transducer may not be ruled out in our experiments.

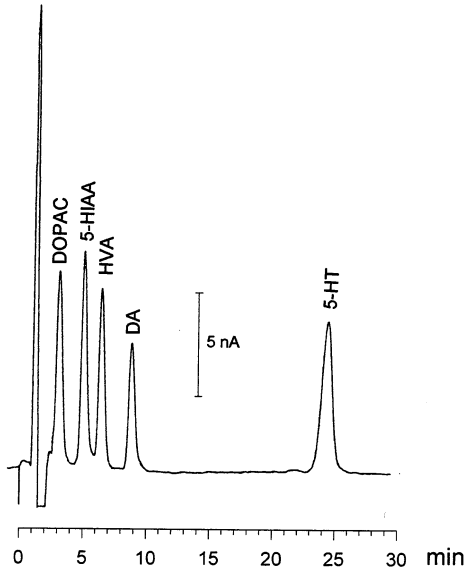

Fig. 4. Typical chromatogram of a standard containing the mixture DOPAC (230 ng/ml), 5-HIAA (220 ng ml^{-1}), HVA (200 ng ml^{-1}), DA (150) ng ml^{-1}, and 5-HT (1750 ng ml^{-1} with retention times at 3, 5, 6.5, 9, and 24 min, respectively, using mobile phase containing 0.1 mM EDTA, 1.0 mM sodium octyl sulfate, 17 mM sodium chloride, 50 mM sodium phosphate monobasic (pH 4) and 7% v/v acetonitrile.

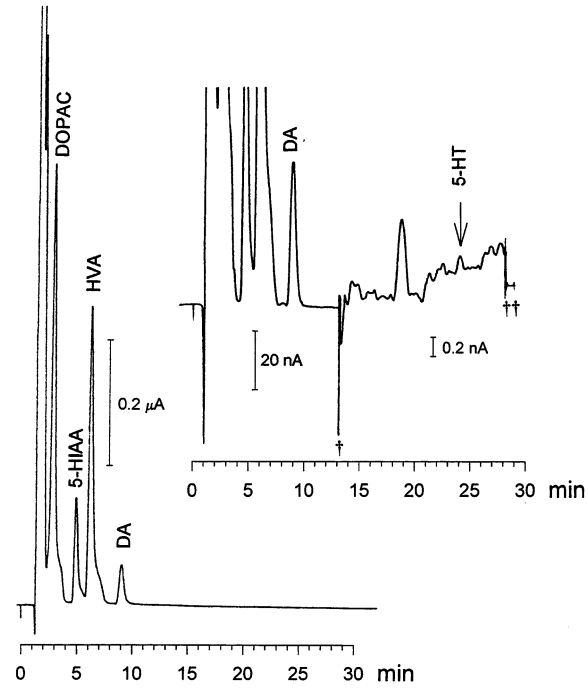

Fig. 5. Chromatogram of DA and 5-HT collected from a 15 μl rat striatal microdialysate in a 30 min run using the same mobile phase in Fig. 4. The scheduled times for changing sensitivity are indicated by '†' and '††' at time 13.5 and 28 min, respectively. Sensitivity was changed from 0.2 μA 0.03 Hz to 50 nA 0.02 Hz, and returned to the initial setting after 5-HT was eluted. The 5-HT peak reflects its typical basal level at amplified sensitivity range.

Recently, Cheng and coworkers [8] have also reported a simultaneous detection of DA, 5-HT and their metabolites in the rat striatal microdialysates. However, we were unable to obtain satisfactory results in our on-line microdialysis analysis using their experimental conditions. It should be noted that these investigators used a homemade glass-lined microbore column that may have analytical conditions different than the commercially used columns. In addition, they used a fraction collector (apparently, without any refrigeration device) for their sampling rather than using an on-line injection. In the assay of biogenic amines, the microdialysates are preferably analyzed on line or collected into a refrigerated fraction collector, and analyzed thereafter. In

the absence of an on line injection, the refrigeration prevents degradation of biogenic amines. Alternatively, acidification of dialysate may be used to retard the process of degradation, however, this may destroy other analyses, and may also be detrimental to chromatographic analysis [17]. It is also noteworthy that collection of dialysate over a fixed interval prior to analysis is known to change the nature of the dialysis sample [17]. Thus, in order to obtain a high temporal resolution, an on-line injection is generally preferred, as it provides analysis of sample without any physical and/or chemical manipulation.

3.2. Chromatographic characterization

Fig. 4 shows typical chromatogram of a standard mixture containing DA, DOPAC, HVA, 5-HIAA and 5-HT. The analysis was completed within 25 min. The detection limits (at signal-to-noise ratio 3–5) of DA and 5-HT was approximately 0.5 to 1 fmol injected onto the column in the present assay. A typical chromatogram of the biogenic amines from microdialysate of the rat striatum is shown in Fig. 5. The retention time of each peak corresponding to DA, DOPAC, HVA, 5-HIAA and 5-HT (in the dialysate sample) was identical to that in Fig. 4, respectively. Furthermore, basal concentrations (average of five rats) in a 15 μL dialysate collected from rat striatum were 1.88 ng mL^{-1} for DA, 568.5 ng mL^{-1} for DOPAC, 507.2 ng mL^{-1} for HVA, 390.1 ng mL^{-1} for 5-HIAA and 1.1 ng mL^{-1} for 5-HT. These values are either in agreement with or higher than those reported by other investigators [3–8]. As expected (see below), microbore LC with the UniJet® radial flow cell electrode provides high sensitivity and low detection limit to enable simultaneous detection of basal 5-HT along with DA and its metabolites.

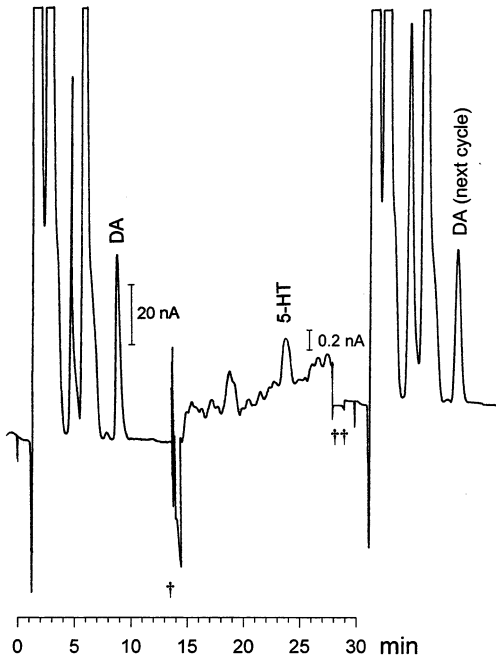

Fig. 6. The chromatogram depicts the beginning of the next collection cycle after the sensitivity returned to initial setting at 28 min (indicated by '††'). The 5-HT peak represents a 275% increase of the basal peak height (corresponding to the retention time 24 min in Figs. 4 and 5) obtained 1 h after the treatment of fluoxetine (3 mg kg^{-1} i.p.); confirming the authenticity of the striatal 5-HT. The mobile phase used is described in Section 2.

The 5-HT peak shown in Fig. 5 (inset) reflects the typical basal concentration of 5-HT at amplified sensitivity level. After the basal level was established, 3 mg kg^{-1} of fluoxetine (a selective 5-HT uptake inhibitor) was administered intraperitoneally to confirm the retention time of 5-HT [5]. It is to be noted that 3-methoxytyramine (3-MT), a DA metabolise, has been reported to occasionally coelute with serotonin [3]. However, administration of fluoxetine has been shown to bring a rapid and significant increase in 5-HT concentration (in dialysate from rat striatum), while the DA and 3-MT concentrations remain unchanged [3]. Due to basal extracellular 5-HT levels in the striatum being low [8], various laboratories have given fluoxetine systemically or directly into the striatum via the perfusate. This results in an increase in striatal 5-HT levels, helping to confirm 5-HT peak identity in the brain dialysates [18,19]. Fig. 6 is a representative chromatograph obtained one hour after the treatment of fluoxetine administration, and indicates a three-fold increase in the peak area of the chromatogram at 24 min compared to that of its basal level. This substantiates the authenticity of the 5-HT chromatogram in the brain dialysate. Fur-

thermore, the increased peak area was statistically significant ($P < 0.01$, average of five samples after post drug treatment compared to average of pre-drug baseline, Student's paired t-test).

Analytical accuracy and precision of the assays were tested using standard mixtures of various concentrations. The intra-assay variability was assessed with three replicates of each dilution at 1 h intervals and expressed as a percentage of relative standard deviation (RSD).

In the standard mixture containing 2 pg–10 ng of each analyse, all analyses exhibited an acceptable $\leqslant 5\%$ RSD, and the response measured from the height of corresponding peak was linear.

The inter-assay variability was assessed with the standard mixture containing ~ 100 pg of each analyse during 5 consecutive working days. The coefficient of variation values were again satisfactory (RSD $\leqslant 5$–7%).

3.3. Microbore columns and in vivo detection of S-HT and DA

Liquid chromatography is a diluting process, and in order to increase sensitivity of analyses, one needs to reduce the amount of the dilution of the sample by the mobile phase. Small-bore columns (1–2 mm diameter) have been developed for this purpose, and have gained relatively widespread use for in vivo brain microdialysis as well as in pharmacokinetic studies [17,20]. The 1 mm i.d. microbore columns have a 20-fold smaller cross section than the conventional 4.6 mm i.d. columns [21]. This characteristic of microbore column provides a corresponding increase in sample concentration [22] as well as a decrease in band broadening [23]. Indeed, the use of microbore columns has been reported to produce a tremendous increase (up to 2000%) in the sensitivity [21] compared to the normal HPLC columns. Overall, microbore columns require smaller sample volume (5–10 μl) for analysis of neurochemicals in microdialysates. However, any system dead volume in a microbore column strongly influences the overall performance of LC system with respect to sample diffusion, and consequent peak broadening [24]. Recently, Bohs et al. [11] introduced a UniJet® electrochemical cell to minimize the dead volume (< 1 μl) for microbore column. The cell serves as an end fitting to microbore column eliminating the use of connecting tube and adapter. The microbore column, with filter frits welded directly inside the ends of the column, extends almost all the way to the center of the working electrode thus minimizing the dead volume. In addition, the UniJet® system creates a radial flow pattern (Fig. 2(a)) of the eluents as opposed to the cross flow pattern (Fig. 2(b)) in typical electrochemical cells. As a result, the UniJet® system not only provides a less dilution of sample (due to its smaller internal volume), but also exhibits a two–three-fold higher sensitivity due to its unique radial flow pattern [11].

Although the combination of microbore and thin-layer amperometric cell is used for increasing the sensitivity of HPLC detection, we did not obtain a satisfactory 5-HT peak height (when DOPAC, 5-HIAA, and HVA had to be simultaneously analyzed) from a 5–10 μ1 of dialysate. Therefore, in order to acquire a better signal response, we, collected a 15 μl sample size that was well within the 20-μ1 maximal recommended sample size on microbore column.

Typically, microdialysate samples are collected in small fractions (5–10 μl), making their filtration impractical and unfeasible for on-line analysis. However, injecting unfiltered samples on a 1×100 mm microbore column packed with 3 μm particles led to clogging of the column. Use of a guard column only remedied the problem temporarily (25–30 microdialysate sample injections from approximately four to five rats), and was thus not very practical from a cost and time point of view. Kissinger and Shoup [25] have also reported that clogging occur with smaller particle size microbore columns. The clogging was minimized to a great extent by using a 1×150 mm column with larger particle size (5 μm).

The efficiency loss due to the larger particle size was largely compensated by increasing the column length from 100 to 150 mm [25]. The life span of the column was not tested in our laboratory or others in brain microdialysis experiments. However, fitted with a guard column, we were able to inject 90–100 dialysate samples (from approximately ten rats) without any appreciable column

clogging, consequent increase in HPLC pressure, and/or change in the retention time of the analyses.

3.4. Automatic sensitivity switch

As mentioned earlier, extracellular DA, 5-HT and their metabolites, DOPAC, HVA, and 5-HIAA have to be detected at various sensitivity levels due to their different basal levels in the rat striatum. Although this can be achieved by using a dual-electrode cell splitting signals to two amperometric detectors with individual sensitivity settings, the extra detector can be eliminated if sensitivity range can be automatically changed on a single detector during the run. In our experiment, the amperometric detector (LC-4C, BAS) equipped with sensitivity-controlling software was capable of automatically reprogramming sensitivity, as well as applied potential and noise filter at designated times. A schedule for modifying sensitivity was written (Table 2) for this purpose. At each command line, the program autozeros the detector, and executes the desired conditions for detection at an appropriate time. It should be noted that any change in the sensitivity and detection conditions is best applicable only when the chromatograph has a minimal or relatively low output during an ongoing analytical measurement. This minimizes the baseline drifting while zeroing the detector. Figs. 5 and 6 show the sensitivity changed at scheduled 13.5 min (indicated by '†') from 0.2 μA to 50 nA with the chromatograph reflecting a relatively low signal output. In order to stabilize the baseline after the sensitivity was switched, an extra command line was added at time 14.5 min to further zero the output. The whole process occurred in ~ 2 min, and the 5-HT peak was visibly brought out thereafter. The range of the noise filter was also manipulated automatically from the initial 0.03 Hz to 0.1 Hz during the beginning of sensitivity modifying course for a quicker response. After 5-HT was eluted, a command line at time 28 min was executed to bring sensitivity back to the initial (0.2 μA) setting (Figs. 5 and 6) preparing the system for the next sample collection.

4. Conclusions

A sensitive electrochemical detection method has been developed for the simultaneous detection of extracellular striatal DA, 5-HT, and their metabolites using an on-line microdialysis technique in awake, freely moving rats. The convenience of the technique provides full automation for on-line collection and eliminates the cost for an extra amperometric detector.

The sensitivity-programmable amperometric detector, along with the controlling software, was found to be capable of switching sensitivity inattentively, thereby, keeping the eluents within a proper sensitivity range for their detection and measurement in repeated on-line collection cycles throughout the experimental run.

Our method permits the use of the same rats as their own control for all the neurochemicals under investigation. This allows a better assessment with regard to the modulations of one neurotransmitter by the other (i.e. DA by 5-HT or vice versa). In addition, simultaneous detection of different neurotransmitters in the same sample reduces the number of probes, animals and manpower considerably compared to the analysis of these neurochemicals in a group of separate rats.

This method is currently being used in our laboratory to study the neuropharmacodynamic effects of steroidal and non-steroidal estrogenic compounds in the central DA and 5-HT systems.

Acknowledgements

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