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# Microbore liquid chromatography with dual electrochemical detection for the determination of serotonin and 5-hydroxyindoleacetic acid in rat brain dialysates

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ABSTRACT

A microbore liquid chromatographic assay with dual electrochemical detection is described for the determination of serotonin and its metabolite 5-hydroxyindoleacetic acid in rat brain dialysates. The concentration of serotonin in these samples is usually in the low nanomolar range (fmol per 20 μl range). To optimize separation and detection, several adaptations were made to the system with respect to the injection valve, flow-rate of the pump, connections between injector, column and detector, and cell volume of the detector. These aspects are discussed, as well as the procedure developed for optimal peak identification of serotonin and correct estimation of 5-hydroxyindoleacetic acid. The assay allows the measurement of basal serotonin release without the use of a re-uptake inhibitor added to the perfusion fluid.

## INTRODUCTION

Microdialysis is a well established technique for studying the dynamic chemistry of the central nervous system. The technique has been widely used to monitor pharmacological action on dopamine (DA). Only recently has it been used to examine serotonin (5-HT) [1–3].

The analysis of dialysate samples necessitates routine detection of sub-picogram amounts of neurotransmitters in very small volumes of perfusate, usually collected at 20-min intervals. The shorter sampling intervals required for behavioural studies result in smaller sample volumes and lower analyte concentrations, making the analysis of dialysate samples more challenging.

Typically, liquid chromatographic assays with

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electrochemical detection (LC–ED) use conventional columns (4.6 mm I.D.) with 5-$\mu$m or 3-$\mu$m packings for the determination of neurotransmitter concentrations, *e.g.* DA and 5-HT, in dialysates [4–7]. The basal release of 5-HT is very difficult to record owing to its rapid metabolic inactivation and re-uptake.

There is a trend to reduce the dimensions of the analytical column in these assays [8,9]. With smaller columns, the components of small (microdialysis) samples are diluted less and thus become easier to detect. In this respect microbore chromatography is interesting because of its high mass sensitivity. Microbore chromatography using 1 mm I.D. columns and amperometric detection shows a twenty-fold increase in sensitivity compared with the use of 4.6 mm I.D. columns [10]. Other advantages from using microbore columns include multiple analyses of samples of larger volume, and a reduction in the consumption of mobile phase. However, this increase in sensitivity makes the assay more difficult. Several precautions must be taken to maintain optimal separation and detection.

This paper describes a microbore LC system for the determination of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in rat brain dialysates collected from rat hippocampus. Adaptations to the system are discussed, as well as practical aspects for maintaining the retention time stability and the sensitivity. Also, estimation of the high 5-HIAA concentrations, usually present in at least a 100-fold higher concentration than 5-HT, and peak identification are confirmed using dual ED.

## EXPERIMENTAL

### *Chemicals and reagents*

5-HT, 5-HIAA, 5-hydroxytryptophan (5-HTP) and 1-octanesulphonic acid sodium salt were purchased from Janssen Chimica (Beerse, Belgium). Dihydroxyphenylacetic acid (DOPAC) and 5-hydroxytryptophol (5-OHTrypt) were supplied by Sigma (St. Louis, MO, USA). Stock solutions (0.01% w/v, maintained at 4°C) of the standards were prepared in 0.01 *M* hydrochloric acid containing 0.1% $Na_2S_2O_5$ (w/v) and 0.01% $Na_2EDTA$ (w/v) (antioxidant mixture). Further solutions were made daily in 0.1 *M* acetic acid.

The perfusion fluid contained 147 m*M* $Na^+$, 4 m*M* $K^+$, 2.2 m*M* $Ca^{2+}$ and 156 m*M* $Cl^-$.

All solutions (perfusion fluid, antioxidant mixture, 0.1 *M* acetic acid, buffer) were filtered through a 0.2-$\mu$m membrane filter.

### *Chromatographic system*

The LC system consisted of a Gilson 302 pump (Gilson, Villiers le Bel, France) operating at a normal flow-rate of 0.8 ml/min with a flow splitter kit for SepStik microbore columns (Bioanalytical Systems, West Lafayette, IN, USA) [11]. Separation was performed on a SepStik microbore column: 100 mm × 1 mm I.D., 3-$\mu$m ODS (Bioanalytical Systems). The flow-rate through the column was 66 $\mu$l/min. The SepStik column, equivalent to 1.5 mm O.D. tubing, was coupled directly to a low dispersion injection valve: Rheodyne 9125 with PEEK stator (Cotati, CA, USA). Dual LC 4B electrochemical detectors (Bioanalytical Systems) were equipped with a dual glassy carbon working electrode positioned in parallel. The operating potentials were 600 and 525 mV *versus* an Ag/AgCl reference electrode. The cell volume was reduced by using a 12-$\mu$m gasket. The (manual) injection volume was 10 $\mu$l. The chromatograms were integrated with a dual-channel integration computer program (Integration Pack, Kontron, Milan, Italy).

### *Chromatographic conditions*

The mobile phase was 95% acetate–citrate buffer (0.1 *M* sodium acetate, 20 m*M* citric acid 1-hydrate, 0.1 m*M* octanesulphonic acid, 0.1 m*M* $Na_2EDTA$ and 1 m*M* dibutylamine, pH 4.0) and 5% methanol. The flow-rate was 0.8 ml/min, yielding a microbore flow-rate of 66 $\mu$l/min using the splitter.

Microdialysis experiments require confirmation that the 5-HT peak measured is not contaminated with co-eluting electroactive substances present in the dialysate. Therefore, the following four-step procedure was undertaken when analysing the samples.

(1) Freshly made calibration standards were injected, with working electrode 1 (W1) of the detector set at 600 mV, range 0.2 nA/V, and working electrode 2 (W2) set at 525 mV, range 0.2 nA/V. The standard solutions contained 5-HTP, 5-HT, 5-OHTrypt and DOPAC in concentrations of 1, 2.5 and 5 pg per 10 μl, and 5-HIAA in concentrations of 100, 250 and 500 pg per 10 μl. The regression lines and the ratio of the peak heights for 5-HT obtained from W1 and W2 were calculated.

(2) In the first three dialysates, 5-HT was measured under the same conditions as in step 1. If the ratio of the values from W1 and W2 differed by more than 20% with respect to the mean ratio obtained in step 1, then the 5-HT peak was contaminated and the experiment was interrupted. If the ratio was the same as in step 1, the experiment was continued.

(3) When further analysis was continued, the range of W2 was set to 10 nA/V. At 0.2 nA/V the current signal for 5-HIAA was higher than 1 V so that integration was faulty because the peak signal was cut off.

(4) Finally a calibration curve for 5-HIAA was repeated at 10 nA/V at W2.

*Microdialysis*

The microdialysis samples were collected from the hippocampus (an area in the central nervous system with an important serotonergic innervation) of freely moving rats. Male albino Wistar rats (250 g) were anaesthetized with a mixture of ketamine and diazepam (50 mg/kg and 5 mg/kg) and placed on an stereotactic frame. An intracranial guide cannula (CMA, Stockholm, Sweden) was placed 3 mm above the hippocampus. The rats were allowed to recover from surgery for 24 h. The guide was then replaced by the probe. CMA 10 microdialysis probes with 0.52 mm O.D. and a membrane length of 3 mm were used. The molecular weight cut-off of the membrane is 20 000. The probes were connected to a microinjection pump (CMA 100) and perfused at a flow-rate of 1 μl/min. The collection of dialysates was started at least 8 h after probe insertion. The sampling time was 20 min. The dialysates were collected in plastic vials containing 5 μl of the filtered antioxidant mixture. A 10-μl aliquot of the sample was injected into the chromatograph.

## RESULTS AND DISCUSSION

In 1986 Wages *et al.* [12] discussed the advantages of microbore LC for the determination of DA in perfusates. However, the first assays for 5-HT in these samples used conventional LC. In 1988 Kalén *et al.* [1] described an LC system coupled to fluorimetric detection for which the detection limit for 5-HT was 2 pg (9 fmol) injected on the column. Other assays have been described, but usually a compensatory change is made to the microdialysis experiment to be able to measure basal release of 5-HT. Sharp *et al.* [13] added a re-uptake inhibitor (1 μ*M* citalopram) to the perfusion fluid to elevate 5-HT to measurable levels for more reliable detection. Schwartz *et al.* [8] used a perfusion fluid with a $Ca^{2+}$ concentration (3.4 m*M*) that is about three times higher than in the extracellular space. As neurotransmitter release is $Ca^{2+}$-dependent, this concentration stimulates the release of 5-HT and higher basal values are detected. Auerbach *et al.* [14] opted for a longer sampling time (30 min), which yielded larger sample volumes for injection. They also used a larger probe membrane of 4 mm for a higher recovery of 5-HT.

Our system uses a 3-mm probe and a $Ca^{2+}$ concentration of 2.2 m*M*. There is no need for a re-uptake inhibitor for measuring basal concentrations of 5-HT *in vivo*. We perfused the probe at 1 μl/min, which enhanced the recovery (microdialysis experiments usually use 2 μl/min). The sampling period was kept at 20 min; however, it would be possible to reduce this to 10 min.

Fig. 1 is a dual-channel chromatogram of a standard solution of 2.5 pg per 10 μl (250 pg per 10 μl for 5-HIAA), which shows a good separation of 5-HT and 5-HIAA from the other compounds, 5-HTP, 5-OHTrypt and DOPAC, that can be present in the dialysate. Fig. 2 shows a typical dual-channel chromatogram of a dialysate obtained from hippocampus at the two ranges (W1 = 0.2 nA/V and W2 = 10 nA/V).

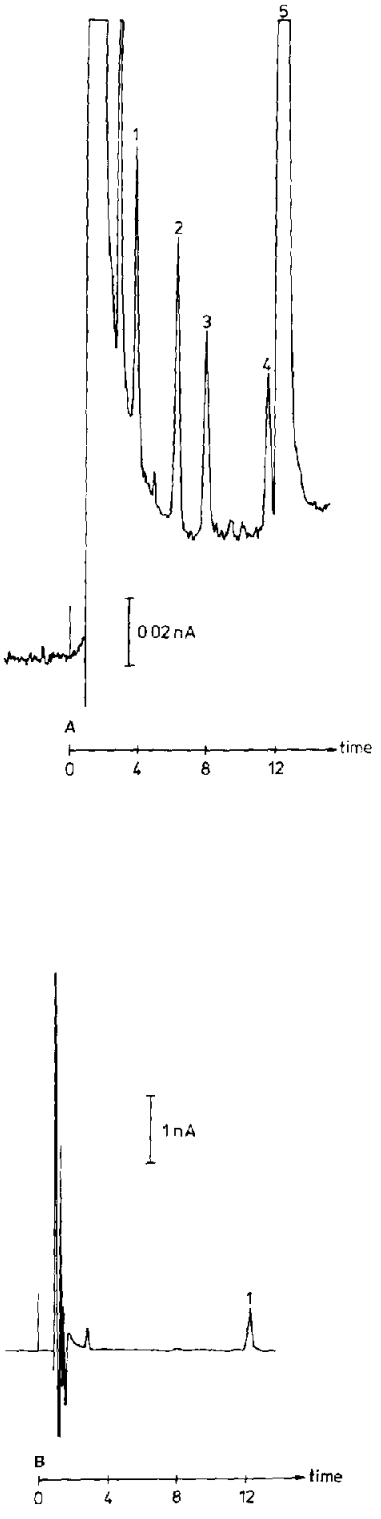


Fig. 1. Dual-channel chromatogram of a standard solution of 2.5 pg per 10 μl (250 pg per 10 μl for 5-HIAA). (A) W1 set at 0.2 nA/V; peaks: 1 = 5-HTP; 2 = DOPAC; 3 = 5-HT; 4 = 5-OHTrypt; 5 = 5-HIAA. (B) W2 set at 10 nA/V; peak 1 = 5-HIAA.

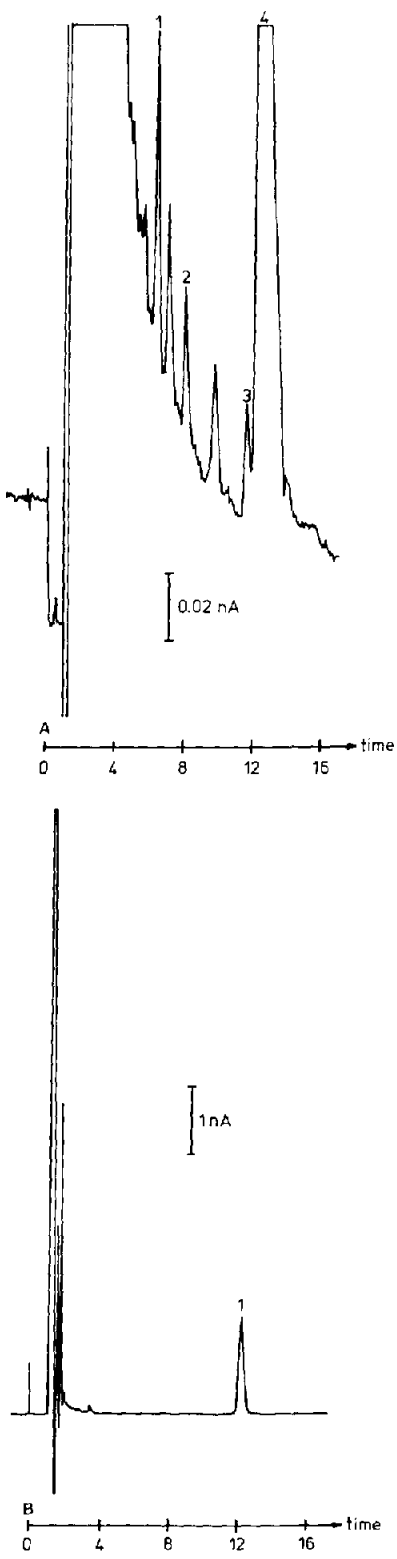


Fig. 2. Dual-channel chromatogram of a dialysate obtained from hippocampus. (A) W1 set at 0.2 nA/V; peaks: 1 = DOPAC; 2 = 5-HT; 3 = 5-OHTrypt; 4 = 5-HIAA. (B) W2 set at 10 nA/V; peak 1 = 5-HIAA.

A system suitability test was carried out by injecting a standard solution of the compounds of 2.5 pg per 10 μl (250 pg per 10 μl for 5-HIAA) six times into the chromatographic system. Coefficients of variation (C.V.) were calculated for the capacity factors and the peak areas. The linearity was also investigated (see step 1 of chromatographic conditions) in the range 1–5 pg per 10 μl (100–500 pg per 10 μl for 5-HIAA). Results are shown in Table I.

Detection limits are shown in Table II, and are taken as the amount corresponding to a signal-to-noise ratio of 2. In comparison with former attempts to measure 5-HT in these microdialysis conditions with conventional LC, we observed an increase in sensitivity of more than thirty-fold.

Several adaptations were made to the system to obtain such conditions. A splitter connected to a conventional pump was preferred to a microbore pump. In the latter, pistons, seals and connecting volumes are smaller, so that each stroke delivers a smaller volume. However, these designs are susceptible to air bubbles and mechanical limitations, are more difficult to purge and generally require more frequent maintenance.

However, it remains essential that micro-systems are kept free of particles. Clogging of the column is a common problem in miniaturized systems. Therefore, the splitter kit has an inlet filter before the tee and all the solutions are filtered over a 0.2-μm membrane filter before use.

Because the microbore column can be directly connected to the low-dispersion injection valve and/or the electrochemical detector cell without additional connecting tubings or unions, the system dead-volume is minimized and high sensitivity is obtained.

The volume of the dual electrochemical cell was reduced to less than 1 μl. This, together with the low flow-rates through the cell, results in a more stable baseline because there is less turbulence inside the cell. Similarly, lower cell volumes and lower flow-rates enhance the peak response within the detector.

There were two reasons for using the dual potentiostat detector. First, selective detection is necessary because the dialysate sample contains a number of electroactive substances that may elute very close to or with 5-HT. In step 1 we determined the ratio of the peak heights at maximal (600 mV) and half-maximal (525 mV) oxidation potentials, characteristic for 5-HT. These potentials were chosen from the voltammogram shown in Fig. 3. The mean ratio of peak heights for the standards was 2.7 ± 0.7 (data from six experiments). This was compared with ratios obtained from *in vivo* dialysates. In this way, the purity of the 5-HT peak could be established. The second reason is that the metabolite 5-HIAA is known to be present in the central nervous sys-

TABLE I

SYSTEM SUITABILITY TEST FOR THE VARIOUS COMPOUNDS

Coefficients of variation (C.V.) for the capacity factors ($k'$) [$= (t_R - t_0)/t_0$, where $t_R$ and $t_0$ are the elution times of retarded and unretarded solutes, respectively] or peak areas (area) are given for a standard solution of the various compounds of 2.5 pg per 10 μl (250 pg per 10 μl for 5-HIAA) injected six times. Linearity is expressed by the correlation coefficient in the range 1–5 pg per 10 μl (100–500 pg per 10 μl for 5-HIAA).

| Compound | C.V. (%) | | Correlation coefficient |
|---|---|---|---|
| | $k'$ | Area | |
| 5-HTP | 0.1 | 1.6 | 0.9994 |
| DOPAC | 0.2 | 1.9 | 0.9998 |
| 5-HT | 0.3 | 3.1 | 0.9996 |
| 5-OHTrypt | 0.5 | 2.7 | 0.9987 |
| 5-HIAA | 0.8 | 4.7 | 0.9910 |

TABLE II

LIMITS OF DETECTION (LOD) FOR THE VARIOUS COMPOUNDS AT 0.2 nA/V AND 600 mV

| Compound | $k'$ | LOD (fmol injected) |
|---|---|---|
| 5-HTP | 3.7 | 0.4 |
| DOPAC | 6.7 | 0.5 |
| 5-HT | 8.7 | 0.5 |
| 5-OHTrypt | 13.6 | 0.9 |
| 5-HIAA | 14.7 | 0.7 |

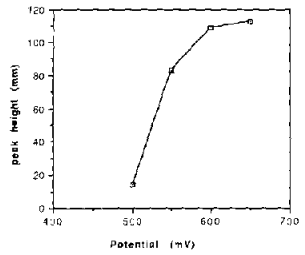


Fig. 3. Voltammogram for 5-HT registered between 650 and 500 mV.

TABLE III

BASAL EXTRACELLULAR CONCENTRATIONS OF 5-HT AND 5-HIAA IN RAT HIPPOCAMPAL DIALYSATES

| Compound | Concentration (mean ± S.D., $n=6$) |
|---|---|
| 5-HT | 17.6 ± 2.9 fmol per 20 min |
| 5-HIAA | 7.4 ± 1.5 pmol per 20 min |

tem in at least a 100-fold higher concentration than 5-HT. In this case the sensitivity needed for low concentrations of neurotransmitters is not required. Moreover, at the 0.2 nA/V scale, the currents measured are too high (> 1 V) making integration of these peaks impossible. Therefore, setting the dual potentiostat at two ranges (0.2 nA/V and 10 n/AV) allows for accurate estimation of both the 5-HT and the 5-HIAA concentrations in the dialysate.

In our experiments basal release of 5-HT is expressed as fmol per 20 $\mu$l, and extracellular 5-HIAA as pmol per 20 $\mu$l. Results are given in Table III.

## CONCLUSION

A microbore LC assay is described for the determination of 5-HT and 5-HIAA in brain dialysates without the need for a re-uptake inhibitor added to the perfusion fluid. The detection limit is less than 1 fmol injected for all the compounds studied. The use of dual ED enhances confidence in the analysis of the dialysates. The sub-femtomole detection limits allow several different assays to be carried out on the same 20-min dialysate sample.

With the use of microdialysis growing in importance for pharmacokinetic studies, microbore LC systems might become essential in overcoming analytical limitations for the determination of low concentrations of drugs in plasma.

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