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Measurement of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in cultured rat mesencephalic neurons by high-performance liquid chromatography with electrochemical detection

Mayra Paolillo*, Stefania Preda, Gian Luigi Corona and Sergio Schinelli

Istituto di Farmacologia, Facoltà di Farmacia, Università di Pavia, Viale Taramelli 14, 27100 Pavia (Italy)

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ABSTRACT

An HPLC method with electrochemical detection for the simultaneous measurement of serotonin (5-hydroxytryptamine) and 5-hydroxyindoleacetic acid in primary mesencephalic cell culture is described. The serotonin and 5-hydroxyindoleacetic acid cell content was measured on different days of growth *in vitro*; after twelve days in culture the amounts of serotonin and 5-hydroxyindoleacetic acid detected were 916.0 ± 70.2 and 215.8 ± 15.5 pg per well, respectively. The heterogeneity of neurons in our cultures and their capacity to take up serotonin were assessed by measuring the amounts of exogenous serotonin taken up in the presence of different monoamine uptake inhibitors. This method, sensitive and reliable, can represent a valid alternative to the use of labelled compounds.

INTRODUCTION

The measurement of serotonin (5-HT) and its major metabolite 5-hydroxyindoleacetic acid (5-HIAA) could be a useful tool to investigate serotonin turnover, which plays an important role in several psychiatric disorders such as headache [1] and depression [2,3]. Many methods have been reported in the literature to quantify 5-HT and 5-HIAA in different *in vitro* and *in vivo* models using mainly fluorescence spectrometry and HPLC with electrochemical detection [5–8]. Low sensitivity and tedious clean-up procedures are the main disadvantages of these techniques [9,10]. Recently, the development of neural primary cultures containing serotoninergic cell bodies or post-synaptic 5-HT receptors has provided an interesting model to investigate the biochemical and pharmacological profile of serotoninergic transmission [4]. Primary cultures offer some evident advantages: (a) growth conditions can be easily controlled; (b) the type of cells under study can be characterized; (c) the intra- and extracellular levels of 5-HT and 5-HIAA can be assessed. Owing to the number and heterogeneity of cells present in the cultures, the amounts of neurotransmitter and its metabolites are very low and not easily detectable; for this reason, in the methods reported in literature radiolabelled precursors are employed to measure the synthesis of neurotransmitters [4].

In this study we have developed a method for the simultaneous determination of 5-HT and 5-HIAA in rat embryonic neurons grown *in vitro* using HPLC and coulometric detection. The short analysis time, the high sensitivity and the minimal prepurification steps make this method very suitable for cell culture studies.

* Corresponding author.

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EXPERIMENTAL

Reagents

All the analytes were purchased from Sigma (St. Louis, MO, USA). The stock standard solutions (1 m*M*) were prepared by dissolving the powders in double-distilled water and kept frozen at −80°C. Just before use, these solutions were diluted with mobile phase or water as indicated. The other reagents were of analytical grade and were obtained from common commercial sources.

Cell cultures

The ventral portion of the mesencephalic area of sixteen-day-old Wistar rat embryos (Morini, Italy) was dissected under microscopic control as previously reported [11] and cells were dissociated using a proteolytic enzyme method [12]. Briefly, the tissue fragments were incubated at 37°C for 45 min with 30 U/ml papain (Worthington Biochemical, USA) in Earle's balanced salt solution (EBSS) at pH 7.4 supplemented with 26 m*M* sodium carbonate, 1 m*M* cysteine, 0.5 m*M* EDTA and 0.01% (v/v) DNAse (Sigma). The fragments were centrifuged (70 *g* for 3 min) and then mechanically dissociated with a narrow fire-polished Pasteur pipette in EBSS containing 1 mg/ml bovine serum albumin (BSA), 1 mg/ml ovomucoid (Sigma) and 0.01% (v/v) DNAse. The cell suspension was layered over a cushion of EBSS containing 10 mg/ml BSA and 10 mg/ml ovomucoid, centrifuged at 70 *g* for 10 min and resuspended in the plating medium. Cell count was performed using a Neubauer modified chamber, and cell viability was >95% as determined by trypan blue exclusion. Cultures were grown in 24-multiwell plates (16-mm-diameter wells; Costar, Cambridge, MA, USA) previously coated with 10 μg of poly-L-lysine per well (M_r > 300 000, Sigma) in aqueous solution for 1 h at 37°C and rinsed twice with sterile water befor plating. The culture medium consisted of F12 nutrient mixture and Eagle's minimal essential medium (1:1) supplemented with 2 m*M* glutamine and 33.3 m*M* glucose containing 15% (v/v) heat-inactivated horse serum (Hyclone, USA). All media components were purchased from Gibco (USA). Cell cultures were plated at a density of 400 000 per well (200 000 per cm^2) unless otherwise specified and incubated at 37°C in a water-saturated atmosphere of 95% air–5% carbon dioxide for the time indicated. After three days *in vitro* (DIV) 1 μ*M* cytosine arabinoside (AraC) was added to the cells to inhibit astrocytic growth.

Sample preparation

The cellular contents of 5-HT and 5-HIAA were measured in mesencephalic cultures at different DIV and after loading the cells (12 DIV) with exogenous 5-HT in the presence of different uptake inhibitors.

In the latter experiments, after aspirating the incubation medium, the cells from each well were rinsed once with 1 ml of PBS (phosphate-buffered saline: 2.7 m*M* potassium chloride, 136.9 m*M* sodium chloride, 1.47 m*M* potassium dihydrogenphosphate, 8 m*M* disodium hydrogenphosphate) and preincubated for 15 min with 100 μ*M* pargyline to block 5-HT degradation. The uptake of exogenous 0.5 μ*M* 5-HT was measured in the presence of 1 μ*M* benztropine (BZT, dopamine uptake inhibitor), 1 μ*M* desipramine (DMI, noradrenaline uptake inhibitor) and 1 μ*M* fluoxetine (FLX, 5-HT uptake inhibitor) at 37°C for 15 min. The basal endogenous content of 5-HT was measured in untreated cultures. At the end of the incubation period the cells were washed twice with PBS and then scraped with a rubber policeman using a 0.1 *M* perchloric acid + 0.2% (v/v) Triton X-100 solution (500 μl) in mobile phase. The suspension was homogenized by ultrasonication for 15 s at 40% output (full scale, 50 W; Vibra Cell VC 50, Sonics, USA), centrifuged in Eppendorf tubes (8000 *g* for 5 min at 4°C, using a Sorvall RC-5B centrifuge equipped with an SH-MT rotor) and the supernatant was stored at −80°C until use. Aliquots of 20 μl were injected directly into the chromatographic system. The amount of 5-HT retained by the cells (total minus basal) in the presence of inhibitors was expressed as a percentage of the amount of 5-HT taken up in the absence of drugs.

HPLC analysis

The HPLC equipment consisted of a Waters 6000 A solvent delivery system pump, a Rheodyne Model 7125 manual injector, a Waters (Milford, MA, USA) Guard Pak precolumn packed with 10 μm particle size C_{18} material, a Beckman Ultrasphere 3 μm octadecylsilane (ODS) (75 mm × 4.6 mm I.D.) analytical column and an ESA 5100 A coulometric detector with a 5011 analytical cell. The first electrode potential was +0.05 V and the second was +0.35 V; the signal was monitored on the second electrode at the indicated full-scale sensitivities using an Omniscribe recorder (Houston Institute, Belgium). The mobile phase was 0.1 *M* potassium monophosphate buffered at pH 3.2 with concentrated orthophosphoric acid containing 0.1% (w/v) sodium octyl sulphate (SOS, Kodak, USA), 0.3 m*M* EDTA and 12% (v/v) methanol. The mobile phase was degassed under vacuum by filtration through a Millipore 0.2-μm-pore membrane; all analyses were carried out isocratically at room temperature (20–22°C) at a flow-rate of 1.0 ml/min. The concentrations of the analytes in the samples were calculated by measuring the peak heights, after correction for recovery, and by comparing them with the response obtained from known standard solutions.

The linearity of the response was calculated using the Pearson's correlation coefficient (r).

RESULTS

Chromatograms

A typical chromatogram of an aqueous standard solution containing 100 pg of 5-HT and 100 pg of 5-HIAA is shown in Fig. 1. The retention times were 4.1 min for 5-HT and 6.2 min for 5-HIAA. When an extract of the cultures was injected into the HPLC system, two peaks at the retention time of 5-HT and 5-HIAA were observed (Fig. 2). The identity of these compounds was checked with a co-elution experiment; a known amount of 5-HT and 5-HIAA (50 pg) was added to the sample before injection. The result is shown in Fig. 3; the area of the peaks increased but their shape was not affected, confirming that

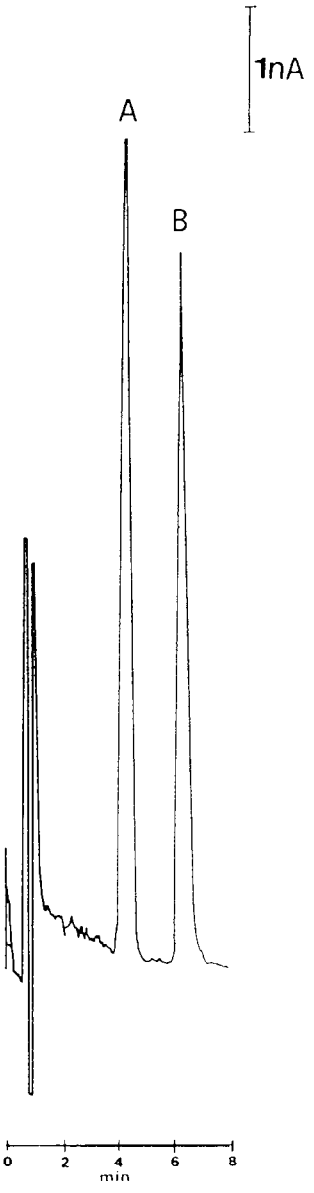

Fig. 1. Chromatogram of 100 pg of 5-HT (A) and 100 pg of 5-HIAA (B).

the compounds detected in the sample extract represent authentic 5-HT and 5-HIAA. The absolute recoveries, calculated for a spiked pool cell culture sample, were 92.5 ± 3.1% for 5-HT and 89.5 ± 4.2% for 5-HIAA ($n = 10$).

The detection limit, with a signal-to-noise ratio of 3, was 1 pg per injection for 5-HT and 5-HIAA; this value corresponds to about 30 pg per well.

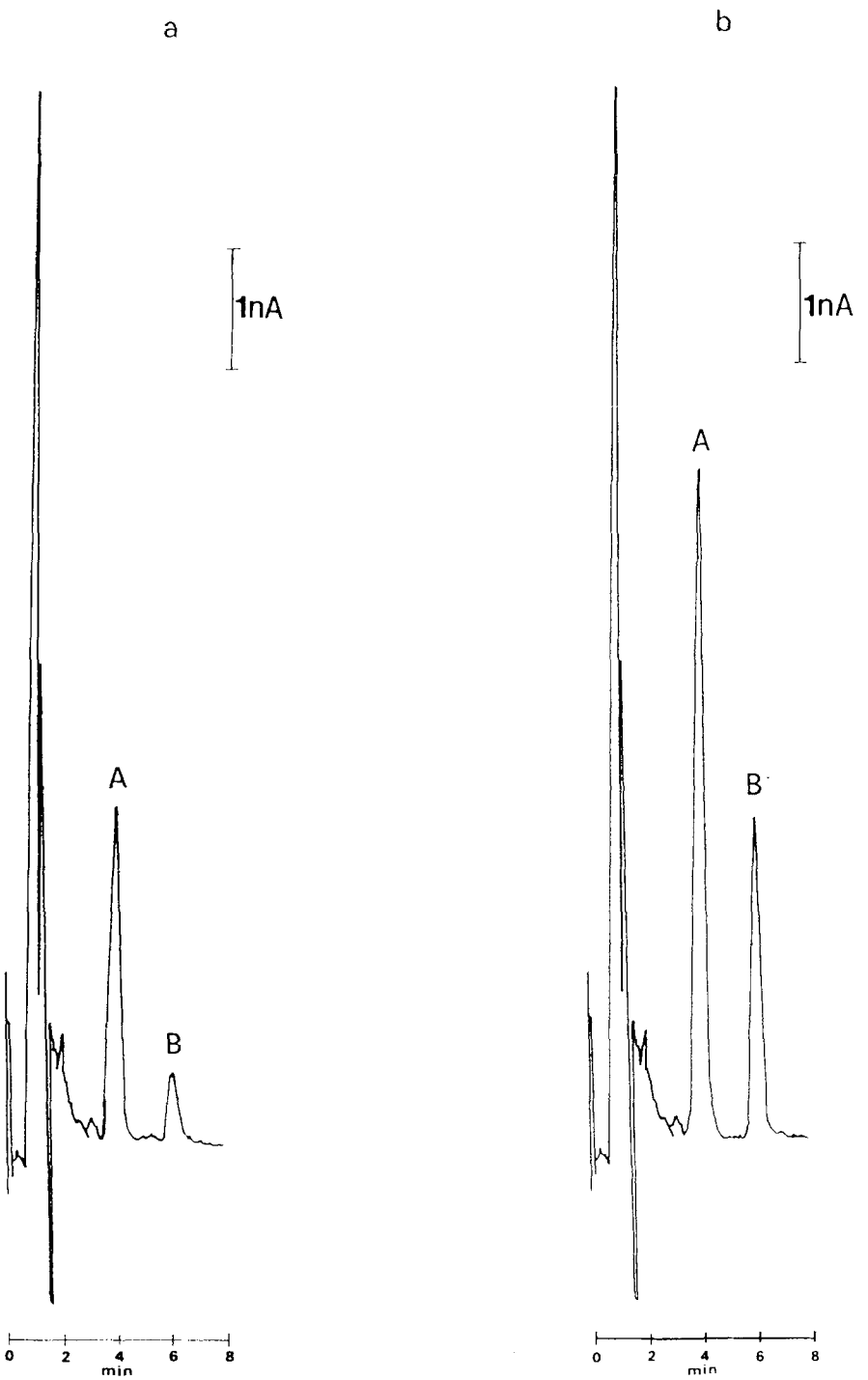

Fig. 2. (a) Chromatogram of mesencephalic cell extract at 12 DIV. (A) 5-HT; (B) 5-HIAA. (b) Chromatogram of mesencephalic cell extract at 12 DIV spiked with 1 ng of 5-HT (A) and 5-HIAA (B).

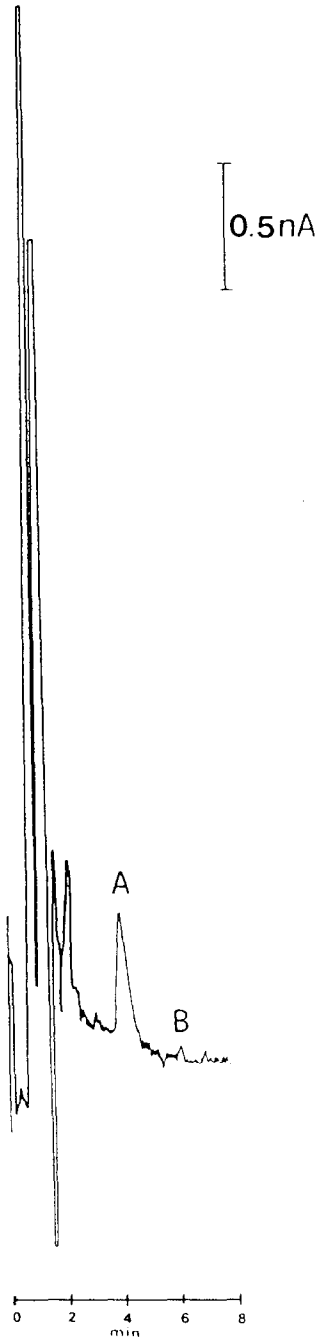

Fig. 3. Chromatogram of mesencephalic cell extract at 4 DIV. Peaks: A = 5-HT; B = 5-HIAA.

This sensitivity allows the measurement of 5-HT and 5-HIAA in cultures at 4 DIV (Fig. 3). Usually all the samples were injected after seven days of storage; stability experiments performed in frozen

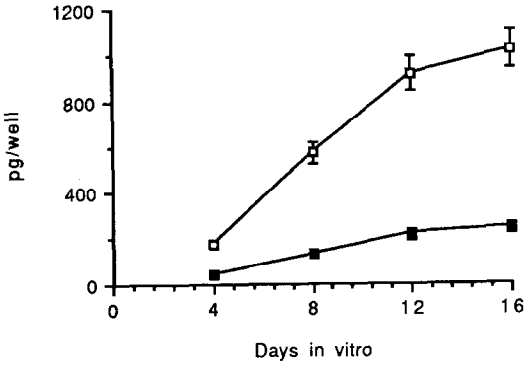

Fig. 4. 5-HT (□) and 5-HIAA (■) levels in mesencephalic cell extract at 4, 8, 12 and 16 DIV. Where not reported, S.D. is less than the area covered by the symbols.

supernatants stored up to fourteen days showed no statistically significant differences in the content of the two analytes. The within-run precision gave coefficient of variations of 1.7% for 5-HT and 2.1% for 5-HIAA, and the day-to-day precision was 2.6% for 5-HT and 3.0% for 5-HIAA ($n = 10$).

The detector response, measured by injecting increasing amounts of diluted standard, was linear over the range 10–1000 pg per injection for the two analytes; the equation of the line was $y = 6.0844 \cdot 10^{-2} + 0.13096x$ with $r = 0.9989$ for 5-HT and $y = -0.31214 + 0.14146x$ with $r = 0.9899$ for 5-HIAA. All the samples tested had levels of 5-HT and 5-HIAA within this range.

Cellular content of 5-HT and 5-HIAA

In rat embryonic mesencephalic cell cultures, grown *in vitro* for twelve days, the intracellular level of 5-HT is 916.0 ± 70.2 pg per well and of 5-HIAA is 215.8 ± 15.5 pg per well (mean ± S.D., $n = 9$). To test whether the synthesis of neurotransmitter in these neurons is time-dependent, the intracellular content of 5-HT was monitored at 4, 8, 12 and 16 DIV (Fig. 4). The amount of 5-HT increased linearly up to 12 DIV, with the maturation of the cultures reaching a plateau around 16 DIV. The pattern for 5-HIAA was similar, even though the amounts were lower; no substantial difference in the 5-HT/5-HIAA ratio

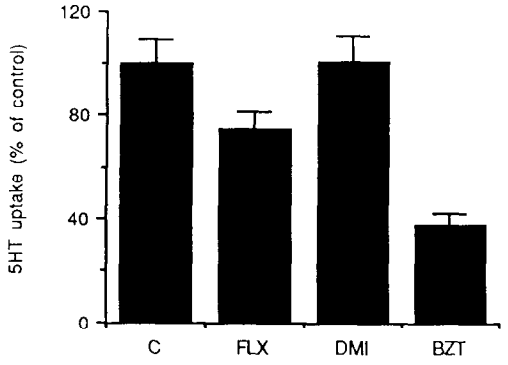

Fig. 5. Inhibition of exogenous 5-HT uptake by fluoxetine (FLX), desimipramine (DMI) and benztropine (BZT) in mesencephalic cells at 12 DIV; data are expressed as a percentage of uptake in controls (C, absence of inhibitors). For details see Experimental section.

was found during the time course of the experiment.

When the cultures at 12 DIV were incubated with exogenous 5-HT, the amount of 5-HT retained by the cells was differentially influenced by selective neurotransmitter uptake inhibitors (Fig. 5). DMI had no effect on 5-HT uptake, in contrast to the evident actions exerted by FLX (74% of controls) and BZT (38% of controls).

DISCUSSION

The most serious problems encountered in measuring neurotransmitters in neuronal primary cell cultures arise from the relative complexity of the matrix and from the trace amounts of analytes present. The high resolution of the chromatographic column used, coupled with the versatility and sensitivity of the dual coulometric detector, was the key factor that allowed us to set up this method. In order to obtain the best resolution for the two analytes, screening for the optimal chromatographic conditions was performed. The percentage of methanol added to the mobile phase was chosen to eliminate the interfering peaks co-eluting with the solvent front. The critical parameters were the pH and the ion-pair content of the mobile phase. The value of the former parameter influenced mainly the retention time of 5-HIAA (reverse correlation), while the amount of SOS had the effect of delaying the retention time of 5-HT. The first electrode (operating at 0.05 V) was used to oxidize the impurities present in the mobile phase; this potential did not interfere with the analytes, which were then detected at the second electrode. This configuration gave the best results in terms of signal-to-noise ratio at the highest sensitivities at the second electrode. The detection limit for 5-HT is well below the amount found in culture samples at 4 DIV, and hence the amount of serotonin synthesized can be measured in cell cultures even after a few days of plating.

Further chromatographic and electrochemical experiments were carried out to confirm the identity of compounds eluting at the retention time of 5-HT and 5-HIAA in our cell culture extract. When a sample was spiked with 5-HT and 5-HIAA standards and then injected, no change in the peak shape was noted. In addition, when some chromatographic parameters were varied, such as pH, percentage of methanol in the mobile phase or the amount of ion-pair and phosphate buffer, the 5-HT- and 5-HIAA-like material in the samples behaved in the same manner as authentic standards. Another electrochemical test was performed to check the identity of the peaks. The potential of the second electrode was set at different values and standard solutions plus a cell extract were then injected into the HPLC system. No difference in the profile between putative 5-HT and 5-HIAA and standard solution of the same substances was found. All these data confirm that the two peaks observed in our samples represent authentic 5-HT and 5-HIAA. As no peak appeared after 5-HIAA, up to seven samples can be processed in less than 1 h.

Our dissection procedure, resembling very closely the conditions used by other authors [13], probably includes a large portion of the cell bodies of serotonin-containing neurons located in the raphe nucleus at the level of brain stem. These cells, grown in culture, express the biochemical machinery necessary to synthesize, release and metabolize neurotransmitters [13]. In spite of this, the absolute levels of 5-HT and 5-HIAA are not comparable to the results reported in literature for

other tissue culture preparations [14] because of the great differences in the dissection protocol, brain areas considered and culture conditions.

The time-dependent increase in intracellular 5-HT levels in our cultures reflects the gradual development and maturation of the neurons with outgrowth of processes and branching of fibres from the soma, as reported in other brain areas in culture [15]. The plateau of the 5-HT content observed around 16 DIV is probably the resultant between the increasing amount of neurotransmitter synthesized per cell and the net number of surviving neurons, which slowly decreases over time in culture.

The heterogeneity of our cell preparation is well documented by the experiment involving exogenous 5-HT uptake. Other neurons present in our cultures, especially dopaminergic [16], can take up 5-HT from the culture medium, probably with less selectivity respect to the high-affinity re-uptake system present in serotoninergic neurons; dopaminergic neurons are present in large number in our cultures and this may account for the higher efficacy of BZT with respect to FLX in blocking the 5-HT uptake. The fact that DMI had no effect seems to suggest that cells with noradrenergic phenotype were absent or, if present, were not able to take up 5-HT. This approach to investigating the properties of neurotransmitter re-uptake systems in neuronal primary cultures could represent a valid alternative to protocols employing labelled compounds.

This method is simple, rapid and sensitive; these features make it very suitable for investigation of all the agents that might affect the synthesis, uptake and metabolism of 5-HT in neural primary cultures.

REFERENCES

1 M. Eison, *J. Clin. Psychopharm.*, 10 (1990) 26S.
2 G. R. Heninger, D. S. Charney and D. E. Sternberg, *Arch. Gen. Psychiatry*, 41 (1984) 398.
3 H. Y. Meltzer and M. T. Lowy, in H. Y. Meltzer (Editor), *Psychopharmacology, The Third Generation of Progress*, Raven Press, New York, 1987, p. 513.
4 J. B. Mitchel, K. Betito, W. Rowe, P. Boksa and M. J. Meaney, *Neuroscience*, 48 (1992) 631.
5 S. Sarre, Y. Michotte, P. Herregodts, D. Deleu, N. De Klippel and G. Ebinger, *J. Chromatogr.*, 575 (1992) 207.
6 M. De Vries and J. Odink, *J. Chromatogr.*, 564 (1991) 250.
7 J. R. Lee Chin, *J. Chromatogr.*, 528 (1990) 111.
8 M. Lema, J. Otero and J. Marcò, *J. Chromatogr.*, 547 (1991) 113.
9 P. Herregodts, Y. Michotte and G. Ebinger, *J. Chromatogr.*, 421 (1987) 51.
10 Y. Michotte, M. Moors, D. Deleu, P. Herregodts and G. Ebinger, *J. Pharm. Biomed. Anal.*, 5 (1987) 659.
11 M. L. Fitszman, A. Zuddas, M. Masana, J. L. Barker and U. di Porzio, *J. Neurochem.*, 56 (1991) 392.
12 J. E. Huettner and R. W. Baughman, *J. Neurosci.*, 6 (1986) 3044.
13 M. Yamamoto, H. W. M. Steinbusch and T. M. Jessel, *J. Cell Biol.*, 91 (1981) 142.
14 D. W. Y. Sah and S. G. Matsumoto, *J. Neurosci.*, 7 (1987) 391.
15 B. Berger, U. di Porzio, M. C. Daguet, M. Gay, A. Vigny, J. Glowinsky and A. Prochiantz, *Neuroscience*, 7 (1982) 193.
16 A. Prochiantz, U. di Porzio, A. Kato, B. Berger and J. Glowinsky, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 5387.