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Microanalysis of serotonin by high pressure liquid chromatography with electrochemical detection

Microanalyse de la sérotonine par chromatographie liquide haute pression équipée d'un détecteur électrochimique

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RÉSUMÉ

L'analyse de la sérotonine (5-HT) par chromatographie liquide haute pression avec un détecteur électrochimique a été adaptée pour quantifier des quantités inférieures au picogramme. Nous démontrons que le potentiel d'oxydation de la sérotonine est étroitement lié au pH de la solution tampon utilisée comme phase éluente. Le comportement électrochimique de la 5-HT dans un tampon acétate à pH 4,00 ou dans un tampon phosphate à pH 6,80 permet une quantification de cette molécule jusqu'à des quantités de l'ordre de 10^{-18} g. La quantité de courant d'oxydation est proportionnelle à la quantité de sérotonine analysée. La réponse électrochimique de la 5-HT est différente pour les très faibles quantités dosées dans la gamme des attogrammes et pour celles des picogrammes. Deux courbes sont enregistrées en fonction de la réponse électrochimique de la 5-HT : l'une pour des dosages dans la gamme des 10^{-18} g et l'autre pour des dosages dans la gamme des picogrammes et au-delà. Dans la gamme de concentrations qui encadre le point de jonction des 2 courbes de calibration, le dosage de la 5-HT n'est pas fiable. La méthode a été appliquée pour doser la 5-HT sur des coupes à congélation de moelle épinière de rats. Cette méthode qui utilise une extraction organique en une étape pourrait ouvrir de nouvelles perspectives pour la quantification de la 5-HT dans des localisations précises du système nerveux central sans avoir recours à l'utilisation de molécules radiomarquées. ▲

Mots clés : *sérotonine, chromatographie liquide haute pression, détection électrochimique, microdosage.*

ABSTRACT

Serotonin (5-HT) analysis by high pressure liquid chromatography with electrochemical detection (HPLC-ED) has been designed to perform micro assays below the picogram range. We demonstrated that the oxidation potential of 5-HT was tightly linked to the pH of the buffer used as eluent phase. The electrochemistry of 5-HT in pH 4.00 acetate buffer and in pH 6.80 phosphate buffer allowed quantification down to 2 × 10^{-18} g. The oxidization current was linked to the 5-HT amount added. The electrochemical response of 5-HT was different for low amounts in the 10^{-18} g range and for amounts above the picogram range. Two curves were obtained according to the electrochemical response of 5-HT: the first one was for the 10^{-18} g range and the second one was for the picogram range and above. It thus appeared between concentration ranges that assays are not be feasible. This method has been used to analyse 20 µm frozen slices of rat spinal cord. These data open new insights into scarce quantification of 5-HT without the use of radio-elements. ▲

Key words: serotonin, high pressure liquid chromatography, electrochemical detection, micro assay.

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VERSION ABRÉGÉE

La chromatographie liquide haute pression (HPLC) est très largement utilisée dans le domaine des neurosciences pour l'étude des amines biogènes. Les méthodes proposées, bien qu'elles permettent le dosage simultané de plusieurs constituants, ne permettent pas de procéder à des dosages en deçà du picogramme.

Nous présentons une méthode qui permet l'extraction organique de la sérotonine (5-HT) en une seule étape, et d'effectuer des dosages dans une gamme de concentration allant jusqu'à 10^{-18} g. Appliquée à l'étude d'échantillons biologiques, il a été possible de quantifier la 5-HT sur des coupes à congélation de moelle épinière de rats.

Une étude préalable de la réponse électrochimique de la sérotonine (courbes intensité-potentiel) effectuée avec le détecteur électrochimique de la chaîne HPLC permet de choisir le meilleur pH et le meilleur potentiel d'oxydation de la 5-HT dans un tampon donné. L'une des conséquences des profils des courbes d'oxydation obtenues avec la sérotonine réside dans la précision avec laquelle le potentiel d'oxydation doit être choisi pour obtenir la meilleure réponse électrochimique du détecteur. Les meilleures oxydations en tampon acétate ont été obtenues à pH 4,00 pour E_{ox} = 574 ± 10 mV et pour un tampon phosphate, à pH 6,80 pour E_{ox} = 521 ± 21 mV. Avec l'un ou l'autre de ces systèmes, le profil de la réponse électrochimique de la 5-HT peut se décomposer en 2 droites en fonction de la quantité analysée : l'une pour la gamme des 10^{-18} g et l'autre pour la gamme de concentration supérieure ou égale au picogramme. Les courbes de calibration sont proportionnelles à la quantité de 5-HT injectée. La droite de régression obtenue avec la courbe de calibration pour les standards dans la gamme des 10^{-18} g est de même qualité (r^2 = 0,994) que celle obtenue pour les standards dans la gamme supérieure ou égale au picogramme (r^2 = 0,997). Pour les faibles concentrations, dans la gamme des 10^{-18} g, nous vérifions que la destruction thermique de la 5-HT s'accompagne d'une disparition du signal correspondant sur le chromatogramme. Le point de jonction des droites exprimant la réponse électrochimique de la 5-HT délimite une gamme de concentration dans laquelle la quantification de cette molécule est difficilement réalisable (instabilité du temps de rétention, faible rapport signal/bruit). Appliquée aux échantillons biologiques, cette méthode permet en une seule étape l'extraction organique et l'analyse quantitative de la 5-HT issue de coupes à congélation de moelle épinière de rats.

Ces résultats ouvrent de nouvelles perspectives dans les microdosages des amines biogènes et cette technique pourrait être un précieux outil d'analyse pour suivre la production de 5-HT, par exemple, par des cellules sérotonergiques. ▲

The chemical identification of biological compounds (*i.e.* carbohydrates) is usually performed by the combined use of techniques such as gas chromatography and mass spectrometry which are usually considered as the most specific. Unfortunately, this method is not applicable on scarce material recovered in very low amounts below the picogram range. In the neurochemical field, the detection of indolamines within biological fluids or within brain tissues in both research and routine laboratories is performed by high pressure liquid chromatography (HPLC) analysis with fast cyclic voltametry, electrochemical or fluorometric detection [1-6]. The sensitivity of data reported is usually over the range of some picograms. Attempts have been made to investigate discrete areas of the central nervous system using the HPLC for neuromediator assays [7, 8]. Thus basic research studies on neurotransmitters require more sensitive detection techniques for investigation within more precise areas of the nervous system or within cell cultures. The presence of such molecules in amounts below the picogram range in these biological samples cannot be quantified or characterized. We describe here a quantification method for serotonin in the magnitude of attogram (10^{-18} g) without the use of any radionuclides. Moreover, we have found the existence of a singular region within the femtogram range in which the behaviour of these molecules in the eluent phases was very peculiar and the assay was not accurate. On both sides of this range, the calibration curves exhibited a linear dose-response fitting together with stable retention time of the molecule. We have also focused our attention on the detector response but not on parameters which are involved in the retention time of the molecule (*i.e.* flow rate, temperature, addition of organic solvent or sodium octyl sulphate within the mobile phases [9, 10]. In addition, the present report has studied a one step extraction procedure for 5-HT from any biological sample (*i.e.* the central nervous system tissues or media) without the use of both perchloric acid and alumina.

Materials and methods

Materials

The standard serotonin (5-hydroxytryptamine creatinine sulphate complex, M_w 405.40); di-sodium hydrogen phosphate, $2H_2O$ and sodium dihydrogen phosphate, $2H_2O$ were purchased from Sigma (St Louis, USA). Acetic acid was purchased from Prolabo (France) and chloroform ($CHCl_3$, HPLC grade) purchased from Carlo Erba.

HPLC

The analyses were performed on a high pressure liquid chromatography system using an electrochemical detector (HPLC-ED, Waters M464 and M460, Milford, MA, USA). This M464 detector was equipped with an Ag/AgCl *versus* glassy carbon electrode with surface area of 7.06 mm^2. The width of the cell was 50 µm for the M464 model. A third electrode, the auxiliary electrode, which allowed the setting of the potential between the mobile phase and the working glassy carbon electrode, was also present. For the voltamogram analysis, the scan rate was 2 $mV.sec^{-1}$ and the potential started from 0 to 1,800 mV with a 100 µA full range intensity. The 5-HT standard solutions used for scanning were from 0.4 to 1 $mg.ml^{-1}$. In isocratic conditions the time constant was set to 1 s and the current was 10 nA full scale. The HPLC-ED system was also composed of an U6K injector and a M510 pump (both from Waters) equipped with a pulse free solvent delivery. The flow rate was 1 $ml.min^{-1}$ and 2 different mobile phases were used:

(1) system 1: 0.1 M sodium acetate (M_w 82.03), 1 mM EDTA (M_w 292.2) with the pH adjusted to 4.00 with acetic acid and; (2) system 2: 0.1 M sodium dihydrogen phosphate, $2H_2O$ (M_w 156.01), 0.1 M di-sodium hydrogen phosphate, $2H_2O$ (M_w 177.99), 1 mM EDTA, and the pH adjusted to 6.80 with NaOH. Each mobile phase was degassed under vacuum by filtration through a 0.45 µm membrane (Millipore, HA type). It should be emphasised that no counter ion such as sodium octane sulphonate nor organic solvent was added to the mobile phase. In addition, before each analysis for both the voltamograms and the isocratic chromatography, a blank injection with the eluent phase alone was performed in order to ensure that no more molecules were present into the HPLC-ED system (*i.e.* seringue, injector and column). The seringue was washed 10 times with the successive solvents: 6 N nitric acid, water, 90% methanol, water, eluent phase. In contrast to usual indolamines analysis, the mobile phases were never recycled, except during rest periods. Serotonin vials older than 6 weeks were discarded. Before each calibration curve, a first HPLC analysis was performed with a run time of 60 min with an injection of 1ng of the standard to control both the retention time of the standard and the purity of the molecule. The analytical column was a microbondapack phenyl 10 µm particle size, 300 mm length from Waters (minimum plates 4,500). The column was isolated from the detector using teflon screw devices. Chromatograms were processed on a compatible PC/AT computer terminal with an expert chromatography software (Waters, Baseline 810, version 3.1).

Extracts

The spinal cords from male Wistar rats were dissected and placed on ice. Lumbar segment was placed onto cryostat chuck with some drops of embedding medium (Tissue tek, Miles laboratories) and allowed to freeze at -30° C. A new extraction procedure was used based on the insolubility of 5-HT into $CHCl_3$. Thirty-four serial transversal sections of the lumbar segments were cut at 20 µm and placed into an eppendorf tube. Samples were homogenized by sonication on ice at 20 kHz, 7 W for 4 x 15 s in 20 µl of methanol/1mM EDTA in water (2/1; v/v) and 20 µl $CHCl_3$. The aqueous phase was separated from the organic phase by centrifugation for 10 min at 17,600 g (Sigma 2K15, FRG) at 6° C. Percentage recovery was 96.2% for controls performed in the same conditions using a 1 $mg.ml^{-1}$ 5-HT solution as a reference. The protein layer which was at the interface between the aqueous and the organic phase was assayed for the protein content [11], and the supernatant assayed by HPLC. These biological extracts can be stored at 4° C for at least 1 week without any significant lost of HPLC reactivity.

Results

The pH influence of the mobile phase on the serotonin response intensity through the detector had been investigated. As shown in *Figure 1*, 2 independent mobile phases were studied. In acetate buffer (system 1), the best oxidization current was obtained at pH 4.00 with a detector response of 3,162 $\mu A.M^{-1}$. Around pH 4.00, the oxidation response of serotonin decreased when both pH increased or decreased. The *Figure 1* also showed that a pH variation of 0.25 units induced a mean decrease for the detector response of 20%. In phosphate buffer (system 2), *Figure 1* showed that the maximum oxidation of the 5-HT occurred at the pH value of 6.80 and with a detector response of 2,675 $\mu A.M^{-1}$. In addition, as for the acetate buffer, a 0.20 pH unit increase induced a mean decrease for the detector response of 20%. The pK values for 5-HT, 4.90 and 9.80, did not correspond to either the maximum detector response (pH 4.00 and pH 6.80) or to the minimum detector response observed (pH 5.50 and pH 7.80). The pH value of 4.00 in system 1 and a pH value of 6.80 in system 2 was thus adopted for subsequent HPLC-ED analysis in isocratic conditions.

The relationship between the applied potential and the oxidation current can be visualised by curves. As shown in *Figure 2*, the voltamogram curve in acetate buffer alone at pH 4.00 *(Fig. 2A, curve b)* consists of an exponential curve starting around 810 mV. The oxidization of 5-HT within this system *(Fig. 2A, curve a)* exhibited a polyphasic sigmoid-like curve. The first sigmoid-like curve ranged from 325 mV to a maximum of 574 mV, while the second one ranged from 720 mV to a maximum of 980 mV. Over this last value, 2 minor additional waves could be observed. Thus, the oxidization of the mobile phase alone started after the first oxidation of 5-HT had occurred but before the second oxidation of the molecule had been totally achieved. The experimental shape of the dynamic voltamogram curve was a successive merging of waves in which each apex was separated by valley. Therefore, the oxidation potential for each wave has a narrow range of validation and a minor shift in this potential value significantly decreased the detector response. In subsequent analysis, when an acetate buffer was used, a pH 4.00 buffer and a 574 mV oxidation potential were applied. In a pH 6.80

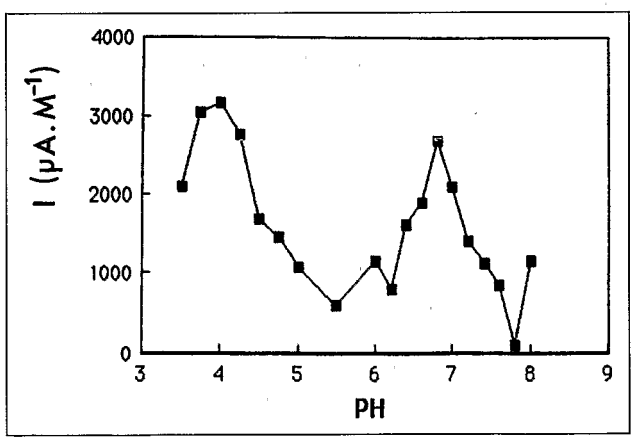

Figure 1. ***Oxidation response of serotonin from pH 3.50 to 8.00.*** *Values were obtained from voltamograms performed as in* Materials and methods. *From pH 3.50 to 5.50, the oxidation responses were given for an acetate buffer whereas from pH 6.00 to 8.00 they corresponded to a phosphate buffer. Values correspond to the first wave potential of the anodic current of 5-HT in the buffer minus the potential of the buffer alone taken at the same abscissa. The intensity of the detector response on the Y axis is expressed as $\mu A.M^{-1}$ of standard 5-HT creatinine sulphate complex.*

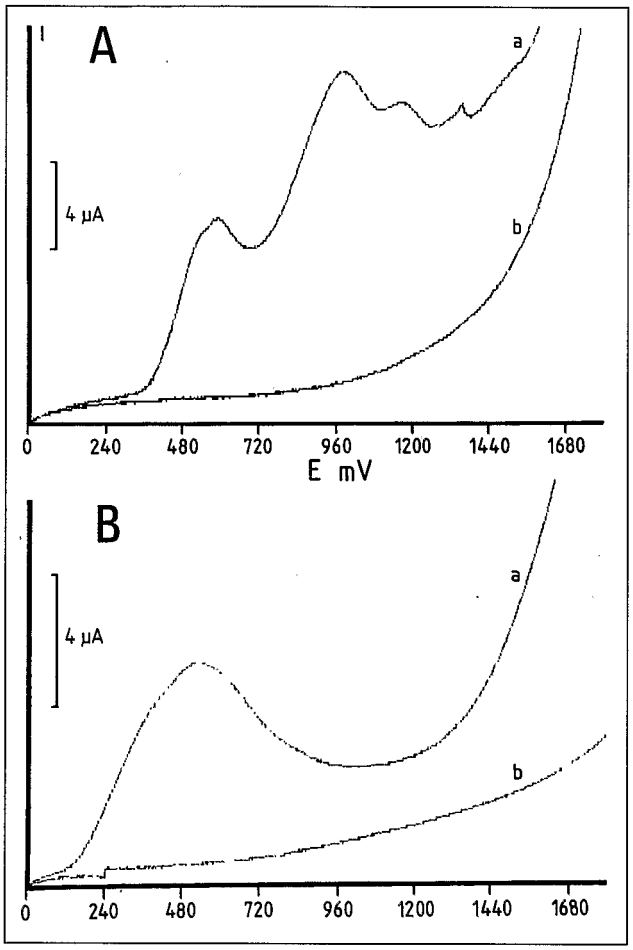

Figure 2. ***Voltamograms of a 2.5 10^{-3} M solution of 5-HT in supporting electrolyte of 0.1 M acetate buffer, pH 4.00 (A) and 0.1 M phosphate buffer, pH 6.80 (B).*** *The reference electrode was Ag/3M-NaCl saturated AgCl. Line (a) represents the data for 5-HT and line (b) represents the buffer.*

Table I
Oxidation potentials of 5-HT in acetate buffer (from pH 3.5 to 5.50) and phosphate buffer (from pH 6.00 to 8.00)

pH	E_0	E_{ox}	pH	E_0	E_{ox}
3.50	341	596 ± 5	6.20	129	273 ± 17
3.75	350	600 ± 5	6.40	103	324 ± 10
4.00	374	574 ± 10	6.60	111	374 ± 30
4.25	340	613 ± 22	6.80	126	521 ± 21
4.50	284	424 ± 23	7.00	71	323 ± 11
4.75	231	494 ± 10	7.20	124	400 ± 24
5.00	284	515 ± 33	7.40	71	238 ± 6
5.50	189	389 ± 6	7.60	60	331 ± 63
6.00	200	405 ± 5	7.80	154	321 ± 39
			8.00	81	306 ± 16

Data obtained from the voltamograms of 5-HT creatinine sulphate complex. E_0 was the potential oxidation starts and correspond to the potential at which the voltamogram of the 5-HT had diverged from that of the buffer alone. E_{ox} was the potential values of this standard molecule given for the apex of the first wave. Potentials were expressed as mV.

phosphate buffer *(Fig. 2B)* the oxidation curve of 5-HT exhibited a single sigmoid-like curve *(Fig. 2B, curve a).* Oxidation started at 126 mV and was totally achieved at 521 mV whereas the buffer alone began to oxidize around 600 mV *(Fig. 2B, curve b).* The voltamograms from pH 3.50 to pH 7.80 are summarised in *Table I.* The oxidization potential starts and the oxidization potential ends of the experimental voltamograms are designated as E_0 and E_{ox}, respectively. As shown for each pH value of the eluent phase, there is a corresponding unique value for both the oxidization start and oxidization end of the molecule. The lower oxidation potential starts were obtained for pH values above 6.2 and not below. From pH 3.75 to pH 4.25, the oxidization started between 325 mV and 350 mV, whereas from pH 6.20 onwards the oxidization potentials started between 70 mV and 280 mV. These observed differences were also true for the oxidization potential ends. Indeed, for pH less than 6.20, the oxidation potential for 5-HT were between 400 and 610 mV whereas it was between 200 and 400 mV above pH 6.20. However for pH 6.80 the oxidation potential was 521 mV. According to the shape of the experimental intensity-potential curves where the apex of the wave was followed by a valley in the both eluent phases, the potential divergence for the given oxidation ranged from 5 to 63 mV.

As shown in *Figure 3,* 10 attograms of 5-HT were sufficient to allow an accurate detection of this molecule in this system. The signal-to-noise ratio within the chromatogram was 16.5. An overlay of chromatograms up to 200 attograms (ag) was illustrated in *Figure 3A.* It is shown that the elution time of the standard 5-HT was 11.04 min (± 0.10) in all cases. In addition, the area of each standard peak was used as the base for calculating concentrations. The calibration curve *(Fig. 3B)* exhibited a linear dose-response with the mathematical expression: 5-HT amount = $-7.02\ 10^{-3}$ + 186.7*R (where R is the response factor for 5-HT). The correlation coefficient was r = 0.997. With an approximation of 7.10^{-3}, this curve could be extrapolated through the origin. To be sure that the detected peaks in the attogram range were the standard 5-HT, the molecule (300 attograms) was destroyed by heating (70° C, 60 min) and analysed by HPLC in the same conditions. *Figure 4* exhibited the overlay chromatograms of the 5-HT standard *(Fig. 4, curve a)* and the heated standard *(Fig. 4, curve b).* The crude 5-HT standard molecule (RT 11.14) has disappeared and at least 2 unknown products were formed.

In contrast, overlayed chromatograms from the picogram range *(Fig. 5)* showed that they were co-axial over 1 picogram. The retention times for 5-HT were shortened as compared to the attogram range and its values were 10.90 ± 0.06 min. As for the attogram range, the corresponding calibration curve has the mathematical expression: 5-HT amount = $-6.37\ 10^5$ + $2.18\ 10^9$R. The correlation coefficient r was 0.95. This curve did not extrapolate to the origin. The apparent detection limit according to this experimental equation was $6.37\ 10^5$ fg or 637 pg obtained for R = 0. If a 50 pA intensity current allowed a peak detection onto the chromatogram, with respect to the detector response for 5-HT (*i.e.* 3,162 $\mu A.M^{-1}$) this value would correspond to an amount of 5-HT of about 64.1 pg into 10 µl. This value has been considered of the same magnitude as that found for the detection limit given by the calibration curve. However, the extrapolations from these data did not match well with the obtained sensitivity. Indeed, users

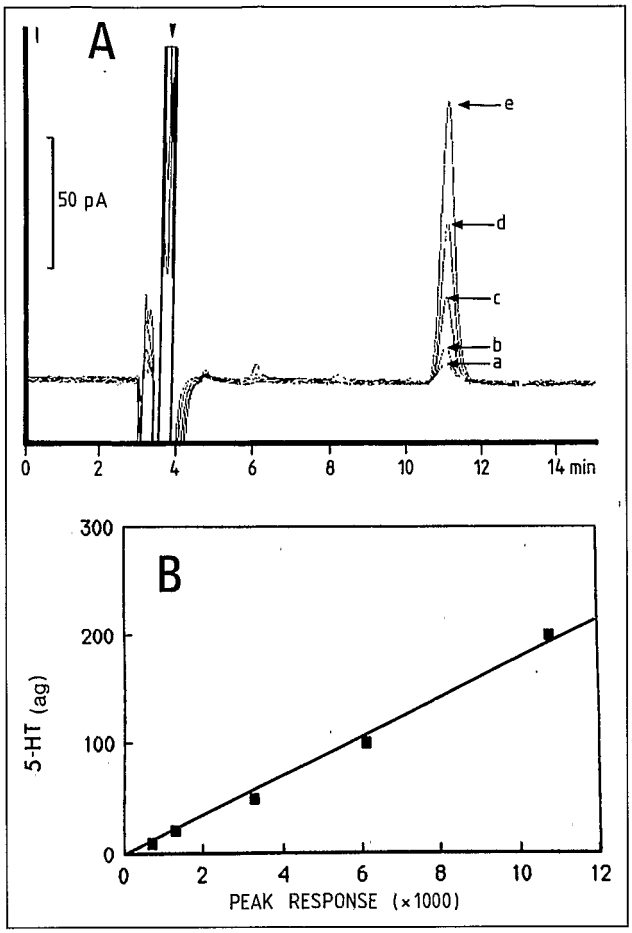

Figure 3. ***Chromatograms of 5-HT within the attogram range and the corresponding calibration curve.*** *Overlay chromatograms of 5-HT up to 200 attograms* ***(A).*** *Dilutions were performed from a 1 mg/ml standard solution by successive dilutions carried out with calibrated Gilson pipetman: a = 10 ag; b = 20 ag; c = 50 ag; d = 100 ag; e = 200 ag. Calibration curve* ***(B)*** *for the attogram range. Chromatograms performed in 0.1 M acetate buffer with an electrode potential fixed to 574 mV. The background current baseline was set to zero just before the injection of the standard.*

usually reached a detection limit of some picograms, as was our case initially. Surprisingly however, between 1 femtogram and 1 picogram, the retention time of 5-HT shifted between 10.90 and 11.04 min. No calibration curve could be fitted with a correlation coefficient better than 0.20. The major fact was that some chromatograms were difficult to resolve on account of a low signal-to-noise ratio of the standard peak. The detection limit obtained for a biological extract analysis was displayed in *Figure 6.* Serotonin extracted from lumbar spinal-cord slices was analyzed (chromatogram 1) and compared to the 12.5 10^{-18} g of the standard 5-HT processed in the same conditions (chromatogram 2). The signal-to-noise ratio was higher than 4, and the biological sample contained a 5-HT amount estimated to 2 x 10^{-18} g (923 ± 134 ng.g^{-1} of wet tissue).

Discussion

We have demonstrated that it is possible to assay molecules down to the attogram range with HPLC-ED without the radionuclides. It was also the case for capillary electrophoresis [12] or in the femtogram range for HPLC with fluorescence detection [13]. However, for concentrations ranging in the femtograms, 5-HT displayed a peculiar behaviour in its retention time as well as in its oxidation response which are not compatible with an accurate determination of this molecule. Below this femtogram range, we

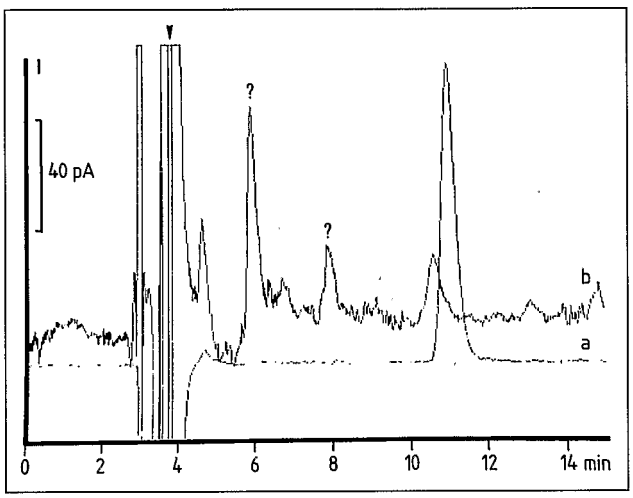

Figure 4. ***Chromatograms of the standard 5-HT (curve a) and a 5-HT solution heated at 70° C for 1 h (curve b).*** *HPLC analysis was performed in the same conditions as stated in materials and methods. Arrow represents the void volume. The eluent phase was 0.1 M acetate buffer and the potential was set at 574 mV.*

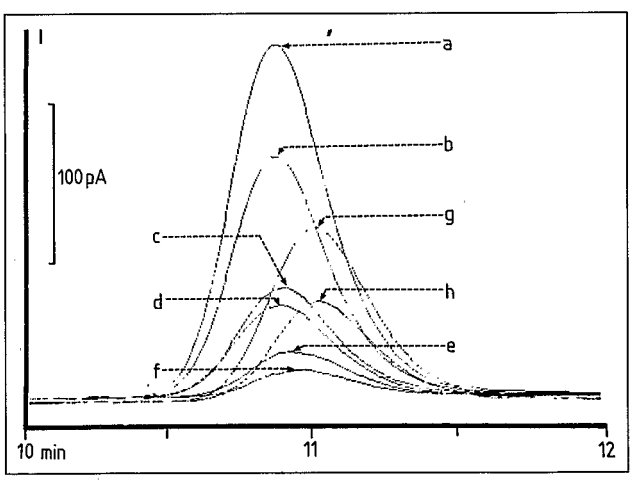

Figure 5. ***Overlay chromatograms from 500 picograms to 100 attograms at the same scale.*** *a: 500 pg; b: 250 pg; c: 100 pg; d: 1 pg; e: 500 fg; f: 250 fg; g: 200 ag; h: 100 ag. RT = 11.04 for g, h (and for the attogram range as shown in* Fig. 3B*). RT = 10.90 for a, b, c, d. The femtogram range had no stable RT. Note the peak height of g as compared to d (1 pg), e (500 fg) and f (250 fg). Chromatograms of standard 5-HT run in 0.1 M acetate buffer. The potential was set at 574 mV.*

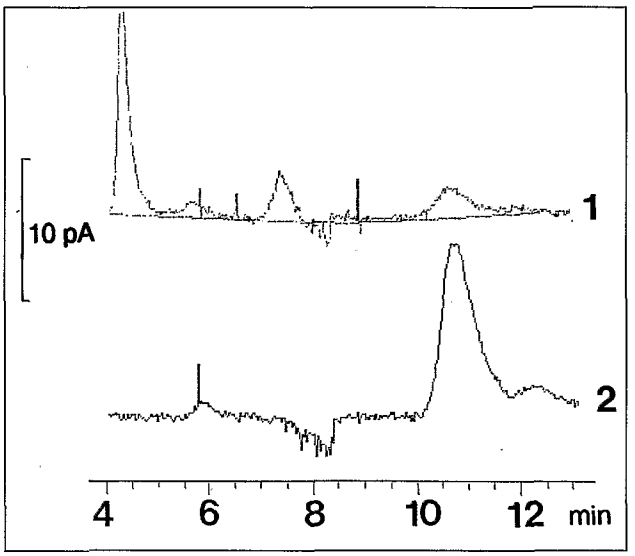

Figure 6. ***Electrochemical detection of serotonin in the attogram range from rat lumbar-spinal-cord slices (chromatogram 1) and 12.5 ag standard 5-HT (chromatogram 2) run in the same conditions with pH 4.00 acetate buffer as eluent phase.***

have demonstrated that the calibration curve type recover a linear dose-response, and that the curve passes through the origin. We presently demonstrate that outside this femtogram range, assays can be performed. As with other amperometric detectors, high background current was first recorded but it then decayed while electrodes equilibrated. To perform assays within the 10^{-18} g range, at least 12 h were necessary to obtain background levels from peak-to-peak below 0.1 pA. If this background level cannot be reached within a day, a new eluent phase must be prepared. The best background levels were obtained with the pH 4.00 acetate buffer. The practical recommendations already given by previous authors [1, 14, 15] must be noted when working with sub picograms amount of 5-HT.

Detection within the attogram range has raised several questions. Could there have been a detection artifact? After the injection of heated 5-HT, the loss of the 5-HT peak together with the appearance of metabolites on the chromatogram indicates that the 5-HT molecule must be present within the injection sample to produce an electrical signal with a retention time identical to that of standard 5-HT. Thus, peaks on the chromatogram within the attogram range were due to genuine organic molecules.

Both the eluent phases alone exhibited a typical exponential curve which characterised the slow system whereas the voltamogram curves of 5-HT exhibited a bi-phasic sigmoid-like curve in acetate buffer and only a single sigmoid-like curve in phosphate buffer. The intensity of the oxidation limit current involved several parameters [16, 17] one of which is the concentration of a given molecule in solution. This parameter is linked to the activity of the molecule (in the physico-chemical meaning) and is dependent on the pH of the solution. During the oxidation process, as the diffusion layer increases, the current falls and thus peak voltamograms were observed. In addition, several events might explain the polyphasic sigmoïd-like curve of 5-HT in acetate buffer: (1) the chemical structure of the molecule exhibits at least 2 sites for an oxidation process, each of which could oxidise at its own potentials; (2) with regard to the pK of 5-HT (pK_1 = 4.90, pK_2 = 9.80) a hypothesis would be that in acetate buffer the low divergence between the pH of the buffer and the pK_1 might allow oxidation of the more acidic function of the molecule. These could explain the presence of the merging waves; (3) the composition of the acetate buffer and not the phosphate buffer, might allow the successive oxidations of all the oxidable sites of 5-HT. In phosphate buffer, the phenomena could not occur or would be attenuated. Indeed, the 5HT voltamograms performed at pH 6.00 with both system 1 and system 2 displayed a bi-phasic sigmoid like curve within system 1 and a single like sigmoid curve within system 2 (data not shown). So, the electrochemistry behaviour of 5-HT might be attributed to the nature of the buffer itself which appears as a key parameter for the electrodetection of 5-HT. In order to totally avoid the eluent oxidization response, it must be emphasised that the detector response for 5-HT in the pH 4.00 acetate buffer has been given for the first maximum of the sigmoid curve and not at the apex of the second sigmoid one. Therefore, care must be taken when working to high potential values where a lot of molecules would have been oxidised, and thus a number of pitfalls could obscure the results. In general, above 750 mV, the mobile phase oxidization interferes with that of 5-HT and thus could obscure low amounts of amines. This fact is particularly true for phosphate buffer where the oxidization of the buffer itself starts at 420 mV. Users must be vigilant to the applied potential, which in general is set to high potential for the analysis of a wide range of amines in the same chromatogram [18]. In addition, taking into account the experimental shape of the dynamic voltamograms, large divergences in applied potentials around our published data are not advisable. For a known molecule, the best detector response was linked closely to both the applied potential and to the pH used, together with the composition of the buffer itself. Indeed, it was clearly demonstrated that there was no linear relationships between the pHs and the oxidation potentials.

The random region is characterised by a shift in the retention time of 5-HT between the two stable retention times of the linear standard curve (*i.e.* the attogram and over the picograms range). The observed abnormalities in the detector response within this femtogram region might be linked to physico-chemical phenomena on the surface of the cell detector such as the 5-HT monolayer formation. This fact deserves additional investigations.

The amount of 5-HT found in the spinal cord was in agreement with that reported in previous studies [19-22]. Therefore, HPLC analysis with an electrochemical detector has allowed the detection as little as 10^{-18} g of 5-HT. This means that it is now possible to assay biological samples which contain at least 15,000 genuine molecules. Together with the recent advance in the use of functional microdialysis probe during long term transplantation [23], this technique may be a precious tool for 5-HT trace analysis by grafted serotonergic neurons. ▼

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