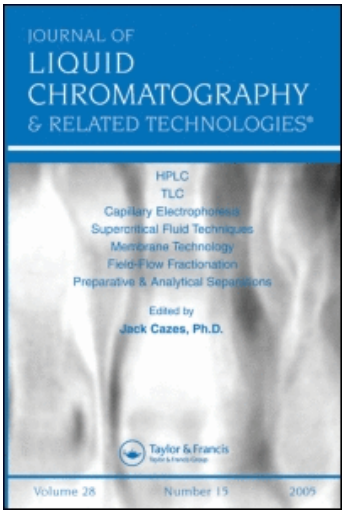

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Synthesis and Determination of N-Acetyloctopamine by HPLC with Electrochemical Detection. Bioassay in *Nippostrongylus Brasiliensis* (Nematoda)

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SYNTHESIS AND DETERMINATION OF N-ACETYLOCTOPAMINE BY HPLC WITH ELECTROCHEMICAL DETECTION. BIOASSAY IN *NIPPOSTRONGYLUS BRASILIENSIS* (NEMATODA)

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ABSTRACT

Determination of biogenic amines in invertebrates, by means of high-performance liquid chromatography (HPLC), is of considerable physiological significance. In insects, octopamine is released into the hemolymph in response to stress, and exhibits some myogenic properties in both insects and nematodes. The first part of the present paper deals with the synthesis of N-acetyloctopamine from octopamine and with its characterization. The second part deals, for the first time, with the detection of these biogenic amines in insects and nematodes using the chromatographic system with electrochemical detection. N-acetyloctopamine was detected in a chromatographic system enabling determination in biological

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samples. The interest of the used method is in the simultaneous detection of various biogenic amines. Improvements can certainly be made for detection of more specific to invertebrates compounds such as tyramine, octopamine and N-acetylated derivatives.

INTRODUCTION

Biogenic amines thought to act as neurotransmitters or neurohormones have been found in many invertebrates including insects (where noradrenaline is particularly abundant) and nematoda [1]. Two features of amine metabolism in invertebrates compared to the well known metabolic pathways in vertebrates are noteworthy: high levels of octopamine [2-4] and preponderance of the N-acetylation pathway in enzymatic degradation ([2], [5]).

In nematodes, the presence of N-acetyltransferase has been demonstrated recently [5], and consequently, the N-acetylated derivatives of dopamine and serotonin have been characterized and determined in *Nipppostrongyius brasiliensis* [4]. The assay of these derivatives is of considerable interest, at least in insects such as cockroaches [3], as their levels have been found to be influenced by sex, age, experimental housing and feeding conditions [6-10].

Although the N-acetylated derivatives of dopamine and serotonin can now be detected, N-acetyloctopamine, which is not yet commercially available, must be synthesized before being characterized in biological samples.

We currently employ a method of simultaneous detection of a variety of biogenic amines initially described in ref. [11] for vertebrate tissues, adapted for detection of additional compounds in cockroaches [3], and nematodes [1, 4, 12, 13]. Both octopamine and N-acetylated derivatives of dopamine and serotonin can thus be detected in the same assay.

The first part of the present paper deals with the synthesis of N-acetyloctopamine from octopamine, and its characterization. The chromatographic separation system was coupled with electrochemical detection employed in our laboratory for assay of biogenic amines in insects and nematodes. It enabled simultaneous detection of tryptophan (Trp), 5-hydroxytryptophan (5-HTP), 5-hydroxytryptamine (5-HT), 5-hydroxyindol acetic acid (5-HIAA), N-acetylserotonin (N-Ac-5-HT), tyramine (Tyr), dopamine (DA), dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT), homovanillic acid (HVA), noradrenalin (NAd), N-acetyldopamine (N-Ac.DA) and octopamine (OA).

EXPERIMENTAL

N-acetyloctopamine synthesis and characterisation :

Low yields and formation of several by-products were obtained by the classical method using direct N-acetylation of 1-(4-hydroxyphenyl)-2-aminoethanol (octopamine) with acetic anhydride and pyridine. In contrast, 1-(4-hydroxyphenyl)-2-(N-acetylamino)ethanol (N-acetyloctopamine) was readily available by selected N-acetylation in high yield (93 %) using octopamine and acetic anhydride in methanol (**Figure 1**).

Melting points were measured on a Mettler FP 52 and are uncorrected. The IR spectra were obtained using a Bomem MB-100. The frequency values are expressed in cm^{-1}. Nuclear magnetic resonance (^1H NMR) spectra were recorded

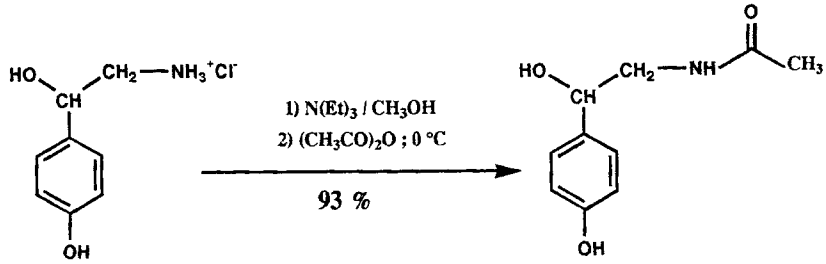

Figure 1 : synthesis of N-acetyloctopamine

on a Jeol 90 spectrometer. The chemical shift values are expressed in δ values (parts per million) relative to tetramethylsilane as an internal standard. High-resolution mass spectra were recorded on HP 5889A quadripolar. UV absorption spectra were recorded on a Uvikon 930 (Kontron) spectrometer in chloroform solution (wavelenghts are in nm). Analytical thin layer chromatography (TLC) was performed on Merck 60F-254 silica gel plates.

1-(4-hydroxyphenyl)-2-(N-acetylamino)ethanol: acetic anhydride (0.089 mL, 0.949 mmol) was added dropwise to a stirred solution of octopamine hydrochloride (0.15 g, 0.709 mmol) and triethylamine (0.11 mL, 0.709 mmol) in methanol (5 mL), at 0°C and under nitrogen atmosphere. After 1h, the mixture was evaporated and the product was crystallized from ethanol-petroleum ether, which gave 0.128 g (yield : 93 %) of pur product as colourless needles ; **Rf** 0.27 (dichloromethane-methanol, 9:1) ; **mp** = 204-206 °C ; **IR** (film) : 3400 (OH alcohol) ; 3400-3010 (NH amide) 1650 (CO amide) ; 1620 (C=C) ; **UV :** λ_{max} : 274 ; **^{1}H NMR** ($CDCl_3$) : δ = 2.02 (s ; 3H ; -CO-C<u>H</u>3) ; δ = 6,57 (m ; 1H ; -N<u>H</u>CO-). ; **MS** : 195 ($M^{\cdot +}$; 1%) ; 177 ($M^{\cdot +}$-18 ; 61%) ; 135 ($M^{\cdot +}$ - CH_2=C=O ; 100%) ; 107 (30%) ; 77 (24%).

Recovery of adult worms :

White rat parasited with the nematoda *Nippostrongylus brasiliensis* were maintained in the laboratory according to a previous protocol [14]. Coprocultures of faeces from infested rats were stored at 25°C and, after 6 to 9 days, third-stage larvae were collected from faecal cultures, rinsed and inoculated into rats by skin puncture (2500 larvae per 250 g female rat). Maturation needs migration to the lungs, then from the trachea to the oesophagus and onwards to the intestine, the larvae become adults. The first eggs appear in the faeces five to six days after infestation.

Rats were killed 7 days after infestation, internal mucus of the small intestine was taken off and placed in 0.15 M NaCl at 37°C for 1 hr. Nematodes were collected after sedimentation, then rinsed three times with physiological saline. Worms were then wiped dry, weighed and immediatly frozen at -20°C until assay.

Sample preparation :

Weighed nematodes were homogenized (1 mg/50 µl) in perchloric medium (0.4 N perchloric acid, containing 0.1 % EDTA-Na_2, 0.1 % sodium metabisulfite and 0.1 % L-cystein), using an Ultraturrax (PolyLabo, Paris, France). Proteins were precipitated with perchloric acid ; after centrifugation (Sorvall superspeed, 4°C, 20 min at 3000 *g*), 20 µl of supernatant were assayed for biogenic amine detection.

Separation and determination of biogenic amines :

Samples were analysed by means of chromatography with electrochemical detection according to [3, 13], with some minor adaptations. The

chromatographic system consisted of a Beckman 112 pump (constant flow rate of 0.9 ml/min); a Rheodyne injection valve with a 20 µl loop ; a reverse-phase column (Ultrasphere ODS, 5 µm, 150 x 4 mm, Beckman) and a Metrohm 641 VA electrochemical detector (sensitivity 1 nA) equipped with a glassy carbon electrode (set to 0.85 V potential versus a KCl/AgCl reference electrode) [1]. This potential was varied between 0.80 to 0.9 V to try and find the best conditions to detect N-acetyloctopamine in our chromatographic system.

The mobile phase was a mixture of phosphate buffer [0.1 M KH_2PO_4, heptane sulphonic acid (5 mM)] and methanol (92.5/7.5, v/v) ; pH was adjusted to 3.8 using 3 M KOH.

Total elution was obtained within 25 min. The system allowed the simultaneous detection of noradrenaline (NAd), 5-hydroxytryptophan (5-HTP), octopamine (OA), 3,4-dihydroxyphenylacetic acid (DOPAC), *N*-acetyldopamine (NADA), dopamine (DA), 5-hydroxyindoleacetic acid (5-HIAA), N-acetylserotonin (N-Ac-5-HT), tyramine (Tyr), homovanillic acid (HVA), tryptophane (Trp), 3-methoxytyramine (3-MT) and serotonin (5-HT), in the order of elution.

Chemicals

All reagents were of analytical grade ; methanol was provided from Merck, while tryptophan, 5-hydroxytryptophan, serotonin, 5-hydroxyindolacetic acid, N-acetylserotonin, noradrenaline, tyramine, octopamine, 3,4-dihydroxyphenylacetic acid, dopamine, 3-methoxytyramine, homovanillic acid and N-acetyldopamine were provided from Sigma Co.

RESULTS

Detection of N-acetyloctopamine :

20 µl of solutions containing 1 to 10 µg / ml of N-acetyloctopamine were injected and chromatographed at different potentials at a given sensitivity. A solution of 200 ng/20µl detected with an electrode potential of 0.85 V, produced a peak height of 18 cm, while no peak was observed at the retention time of octopamine (detection level of octopamine at 0.85 V : 18 ng/ml corresponding to 0.36 ng/20 µl) . This observation confirmed the purity and stability of the synthesized N-Ac.OA, which contains, even dissolved in buffer, less than 0.18% OA.

Retention times of the different amines in the chromatographic system are listed in **Table I**. Retention time of N-Ac.OA alone or after addition to the other substances was 3.80 ± 0.02 min (n = 15). This retention time is rather close to that of NAd (retention time 4.12 ± 0.04 min) which represents a difficulty for determination of this compound in biological samples from invertebrate tissues containing high levels of NAd.

A chromatogram of 20 µl of a standard solution containing tryptophan (8 ng), octopamine (4 ng), tyramine (2 ng), 5-hydroxytryptophan, 5-hydroxytryptamine, 5-hydroxyindol acetic acid, N-acetylserotonin, dopamine, 3-methoxytyramine, homovanillic acid, noradrenaline, N-acetyldopamine (0.4 ng of each) along with 40 ng of N-acetyloctopamine (N-Ac.OA) is shown in **Figure 2** (potential 0.9 V).

Table I : Retention time expressed in minutes for the different substances detected simultaneously in the analytical system.

Substances	Retention time (in min)
N-Ac-OA	3.80 ± 0.02
NAd	4.12 ± 0.04
5-HTP	5.33 ± 0.05
OA	5.53 ± 0.05
DOPAC	7.23 ± 0.06
NADA	7.28 ± 0.06
DA	9.48 ± 0.09
5-HIA	12.02 ± 0.12
N-Ac-5-HT	13.40 ± 0.12
Tyr	14.92 ± 0.13
HVA	16.07 ± 0.14
Trp	18.58 ± 0.19
3-MT	21.10 ± 0.15
5-HT	24.77 ± 0.24

In order to obtain a relationship between peak height and concentration of the solution, different amounts of N-Ac.OA were added to the standard solution . In **Table II**, peaks (expressed in cm) are given for OA and N-Ac.OA (various concentrations) at different potentials. The detector response for these two amines was dependent on the injected amount of the sample and of the elecrode potential (**Figure 3**). For N-Ac.OA as for OA and Tyr, peaks were well separated and a maximum response was observed at 0.9 V. The effect of the electrode potential was evaluated as the percentage fraction of the detector response at 0.9 V.

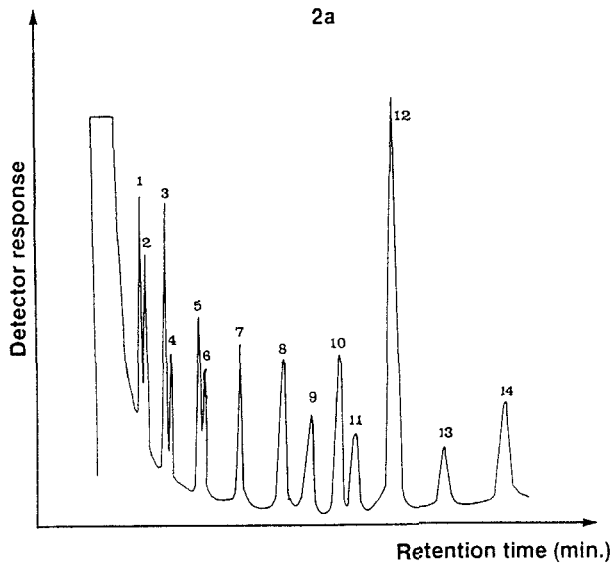

Figure 2 : Chromatogram obtained by injection of 20 µl of the standard solution containing, tryptophan (8 ng), N-acetyloctopamine (40 ng), octopamine (4 ng), tyramine (2 ng) and 0.4 ng of the other compounds (in the order of elution)

1. N-acetyloctopamine ; **2.** noradrenaline ; **3.** 5-hydroxytryptophan ; **4.** octopamine ; **5.** 3,4-dihydroxyphenylacetic acid ; **6.** *N*-acetyldopamine ; **7.** dopamine ; **8.** 5-hydroxyindolacetic acid ; **9.** N-acetylserotonin ; **10.** tyramine; **11.** homovanillic acid ; **12.** tryptophan ; **13.** 3-methoxytyramine ; **14.** serotonin.

2a : injection of 20 µl of a standard solution containing N-acetyloctopamine (40 ng).

2b : injection of 10 µl of standard solution containing N-acetyloctopamine (20 ng) and 10 µl of a solution containing N-acetyloctopamine (100 ng).

(continued)

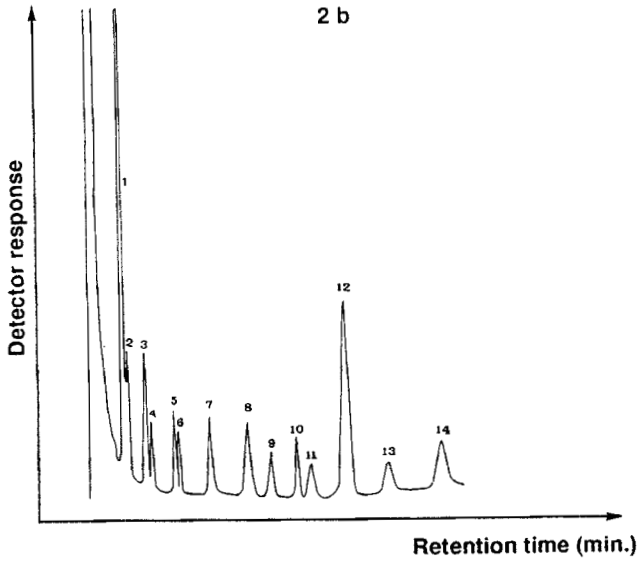

Figure 2 (Continued)

Table II : Peak heights expressed in centimeters after injection of a solution containing 2 µg/ml of octopamine or 1 to 10 µg/ml of N-Ac.OA at different electrode potentials.

	N-Ac.OA			OA
Potential	**1µg/ml**	**2 µg/ml**	**10 µg/ml**	**2 µg/ml**
0.80 V		1.1	6.3	0.1
0.83 V		3.7		0.5
0.85 V		6.4	18	1.3
0.87 V		11		2.8
0.90 V	11.6	28		4

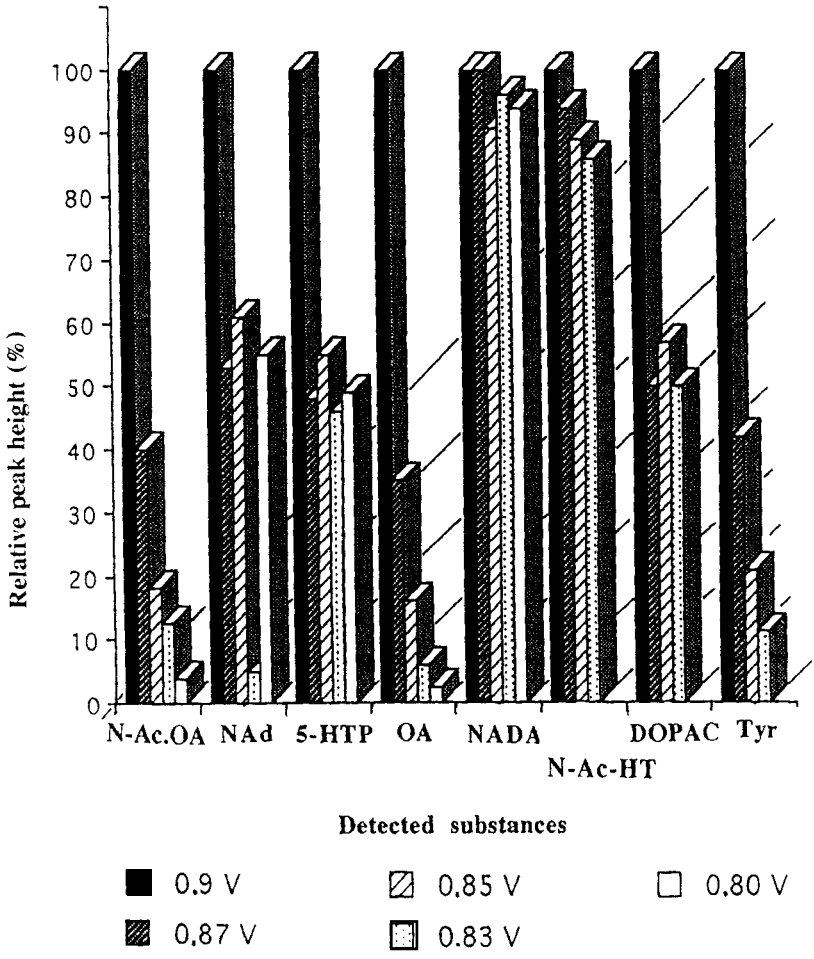

Figure 3 : Percentage response of different substances at different electrode potentials expressed as peak height relative to the peak height of the same substance at 0.9 volts.

Table III : Amine detection levels (ng/ml) of standard solution and correspondent concentrations in biological samples (1 mg of nematode / 50 µl buffer) at 0.90 and 0.83 V.

Reference substances	Standard solution (ng/ml)		Nematode samples (ng/g)	
	0.90 V	0.83 V	0.90 V	0.83 V
NAD	0.44	0.4	22	20
5-HTP	0.29	0.31	14.5	15.5
DOPAC	0.46	0.46	23	23
DA	0.53	0.49	26.5	24.5
5-HIA	0.53	0.58	26.5	29
Trp	4	44	200	2200
5-HT	0.83	0.85	41.5	42.5
N-Ac.OA	30.77	108.11	1538	5.405
OA	9.75	66.66	487.5	3333
NADA	5.88	3.071	294	153.5
N-Ac-5-HT	6.25	3.51	312.5	175.5
Tyr	3.85	16.66	192.5	833

Detection response expressed in ng of amines / ml of injected solution are listed for 0.90 V and 0.83 V in **Table III** which shows the detector responses to typical concentrations in biological samples on the basis of 1 mg of nematode / 50 µl buffer.

Assay in biological samples :

Chromatogram of a nematode sample is illustrated in **Figure 4a** (0.90 V), and detection levels are listed in **Table III.**

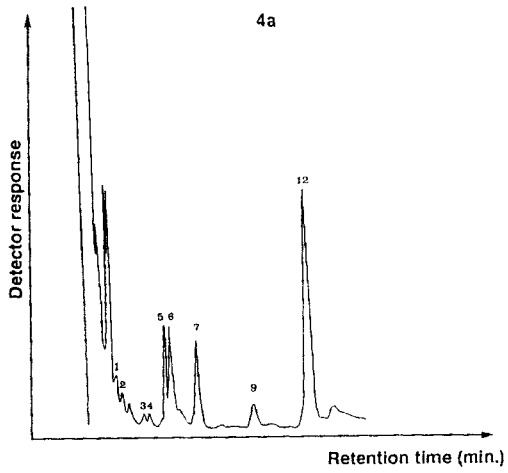

Figure 4 a : Chromatogram of the nematod sample

1. N-acetyloctopamine ; **2.** noradrenaline ; **3.** 5-hydroxytryptophan ; **4.** octopamine ; **5.** 3,4-dihydroxyphenylacetic acid ; **6.** N-acetyldopamine ; **7.** dopamine ; **9.** N-acetylserotonin ; **12.** tryptophan ; the other compounds were not detected in this sample.

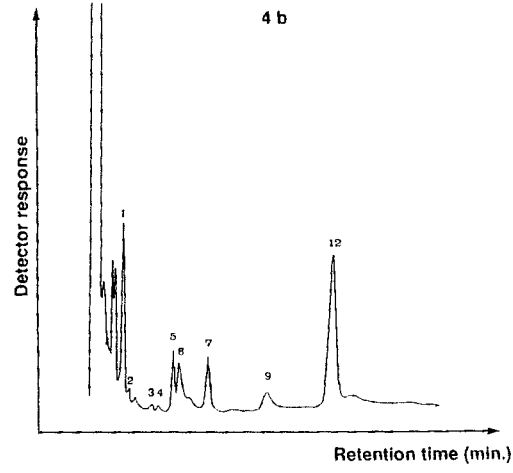

Figure 4b : Chromatogram of 10 µl of nematod sample mixed with the same volume of a solution of N-acetyloctopamine (2 µg/ml, 20 ng).

N-Ac.OA was detected in biological samples by *(i)* comparison of sample peaks with those from a mixture of reference substances with the same retention times as N-Ac.OA at different detection potentials; *(ii)* by spiking samples with a solution of N-Ac.OA (10 µl sample added to 10 µl of a 2 µg/ml solution of N-Ac.OA) (**Figure 4b**). The limit of sensitivity at 0.9 V was around 31 ng/ml of the solution.

DISCUSSION AND CONCLUSION

N-Ac.OA, a non-commercially available amine, was detected for the first time in a chromatographic system enabling quantitative analysis in biological samples. Determination of this amine in invertebrates is important since OA is of considerable physiological significance [2]. In insects, octopamine is released into the hemolymph in response to stress, and it has been shown to have myogenic properties in both insects and nematodes.

The interest of the used method is the simultaneous detection of various biogenic amines. One may remarks that the detection system is more sensitive to amines found in vertebrate nervous system and detection thresholds are lower for compounds such as Trp, 5-HTP, 5-HT, 5-HIAA, DA, DOPAC, 3-MT, HVA. Improvements can certainly be made for detection of compounds more specific to invertebrates such as tyramine, octopamine and N-acetylated derivatives. It should be noted that the levels of NAd and OA must be taken into account as they are often found at high concentration in invertebrates and may mask the N-Ac.OA peak. In such cases, samples will require appropriate dilution.

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