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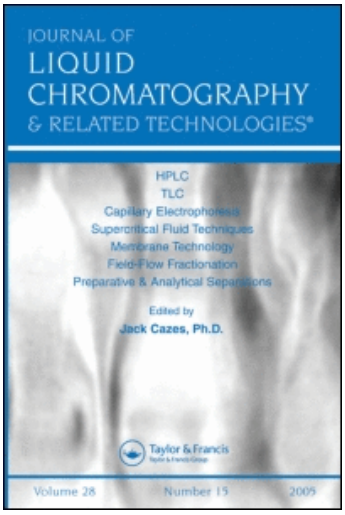

Journal of Liquid Chromatography & Related Technologies

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To cite this Article Girardie, J. , Allard, M. , Faddoul, A. and Neuzil, E.(1983) 'Separation of the Proteins of Locust Neurosecretory Endings with HPLC', Journal of Liquid Chromatography & Related Technologies, 6: 13, 2501 — 2512
To link to this Article: DOI: 10.1080/01483918308064917
URL: http://dx.doi.org/10.1080/01483918308064917

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(13), 2501-2512 (1983)

SEPARATION OF THE PROTEINS OF LOCUST NEUROSECRETORY ENDINGS WITH HPLC

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ABSTRACT

High performance liquid chromatography on gel permeation columns was use to analyse the protein and peptide content of the neurosecretory corpora cardiaca of the locust (*Locusta migratoria*). A variety of extraction conditions and mobile phase compositions were tested for their separation efficiency.

A good resolution, showing 12 peaks distributed within 5 distinct groups, could be achieved in 32 min with the fresh homogenate from only two corpora cardiaca (0.5 M Tris buffer, 0.3 NaCl, 0.1 % SDS, pH = 7.4 ; flow rate : 0.5 ml/min). Similar HPLC patterns were obtained with three fractions previously separated on a Sephadex G-100 column as well as with isolated neurosecretory vesicles.

HPLC then appears as a promising technique in the field of insect neurohormone isolation.

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INTRODUCTION

The minute quantity of available material is the main difficulty encountered in studying neurohormones. Contrasting with the numerous neurofactors isolated from 'higher' animals, the structures of which have been thoroughly determined, only two insect neuropeptides are well known (5) and their structure confirmed by synthesis. The proteins of the whole brain of locust (incorporating corpora cardiaca) have been studied using classical DEAE-cellulose and Sephadex chromatographic columns (1). In this paper, the HPLC technique performed on gel permeation columns has been used for analysing the proteins of dissected corpora cardiaca of the locust. HPLC appears to be a valuable technique in this new field, on account of its high sensitivity permiting a fast examination of very small quantities of biological material.

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MATERIAL AND METHODS

Preparation of Samples

Mature adult locusts (*Locusta migratoria*), reared in crowd, were sacrificed by decapitation and the neurosecretory lobes of corpora cardiaca rapidly removed. Three types of neurosecretory corpora material were used :

a) Crude extracts of corpora cardiaca were obtained by ultrasonification (CIT Alcatel apparatus ; 3 x 20 sec) followed by centrifugation. The precise conditions vary with the different experiments and will be detailed in the next section. The supernatants were injected directly into the chromatographic column.

b) Corpora cardiaca (2,000 dissected samples) stored in cold acetone (4°C) were collected by decantation, ultrasonicated in 0.01 M sodium acetate buffer, 0,15 M NaCl, pH = 7.4 and centrifuged (10.000 g ; 20 min). The extract was submitted to gel filtration on Sephadex G-100 (900 x 15 mn column) and eluted with the same medium.

c) The technique of Nordmann *et al.* (4) using a sucrose/metrizamide gradient of an osmolality value of 0.46 was used for the isolation of the neurosecretory vesicles from 500 corpora cardiaca.

Techniques

Two models of HPLC apparatus were used :

a) a Waters LCIV system, with a SF 770 low wavelength absorbance detector and a gel permeation column (Waters I 125, 7.8 mm x 30 cm).

b) a Beckman basic isocratic system with a 254 nanometers analyser optical unit, and a permeation column (Altex TSK, gel 2000 SW, 7.5 mm x 30 cm).

Samples (50 or 100 µl) were injected into the Waters column and 20 µl into the Altex column and monitored respectively at 215 and 254 nm.

In every case, HPLC columns were eluted at room temperature.

RESULTS

Influence of Different Chromatographic Parameters

We have first analysed by HPLC the crude extract from 2 fresh corpora cardiaca using different phases both as extraction and elution media :

EP_1 = distilled water ;

EP_2 = Tris 0.5 M ; NaCl 0.3 M ; pH = 7.4 ;

EP_3 = EP_2, with SDS 0.1 % ;

EP_4 = EP_2/MeOH, 2:1 vol/vol ;

EP_5 = H_2O/MeOH, 70:30 vol/vol.

EP_1 gave several peaks but this eluent phase was rapidly dismissed, as a high proportion of the biological material remains linked to the column. Increasing the ionic strength and buffering at pH = 7.4 (EP_2) dramatically improved the separation ; a further improvement was the addition of 0.1 % sodium dodecylsulfate (SDS), leading to the eluent phase EP_3 which gave a better resolution.

A typical chromatographic separation of the protein content of neurosecretory endings obtained with EP_3 is shown in fig. 1. Two high molecular weight fractions (Ia and Ib) were first eluted with a retention time (9-11 min) close to the retention time of serum albumin (MW : 67.000 ; t_r = 11 min). They were followed by a second apparently homogenous peak (II : t_r = 13 min) and by a heterogenous group III. The five constituent peaks (IIIa, IIIb, IIIc, IIId,

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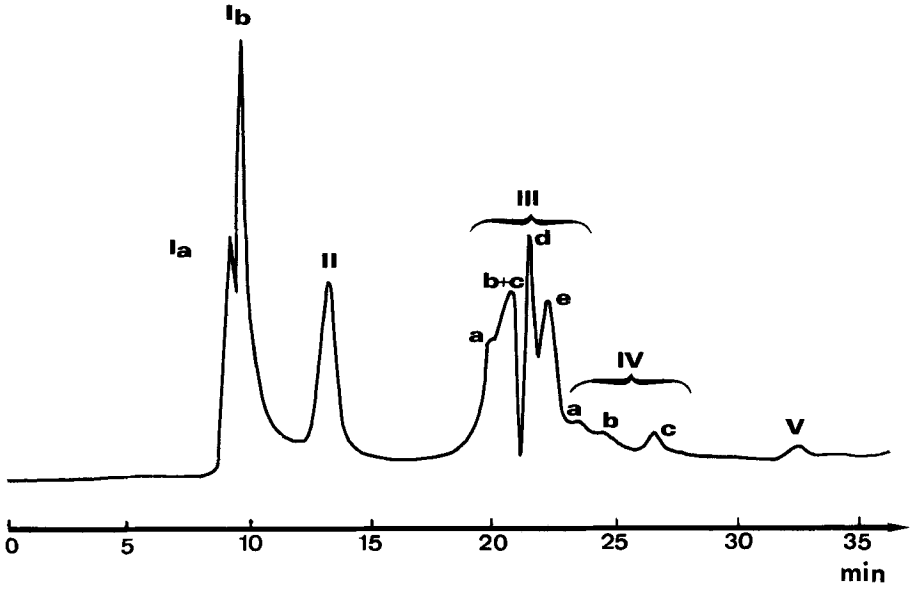

Figure 1.- HPLC separation of the proteins extracted from two fresh corpora cardiaca. The biological material was directly homogenized in the mobile phase EP_3 and centrifuged (10,000 g ; 20 min) ; the supernatant (20 µl) was loaded on an Altex column ; flow rate, 0.5 ml/min ; detection at 254 nm (0.01 AUFS) ; chart speed, 1 cm/min.

IIIe) with a retention time of 20-22 min correspond to fractions of molecular weight lower than ribonuclease (MW : 13.700 ; t_r = 17 min) and higher than insulin (MW : 6.000 ; t_r = 23 min). IIIa, IIIb and IIIc were poorly separated, especially IIIc which appeared more as a shoulder of IIIb than an individualized peak. The range of molecular weights of the III group peaks likely corresponds to the presence of neurosecretory products (1). The remaining elution peaks appeared on the diagram as a heterogenous group (IV : t_r = 23-27 min) with three minor peaks IVa, IVb and IVc. A last minor peak (V) was eluted with a retention time of 32-33 min (somatostatin, t_r = 32 min).

No major differences could be noted when modifying the concentration of Tris (0.01-0.5 M) or the final pH of the eluent

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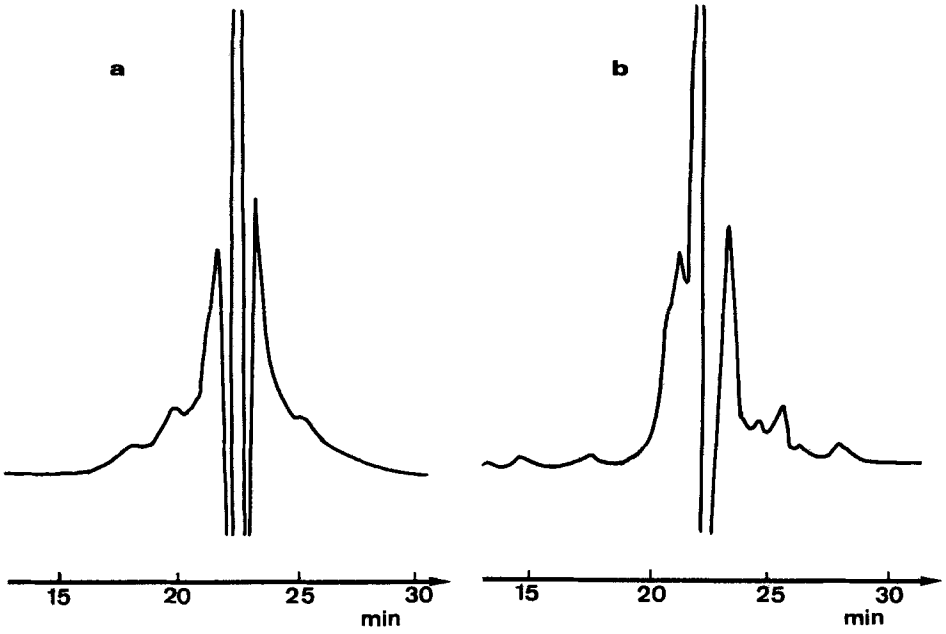

Figure 2.- Influence of methanol on the chromatographic profiles of group III peaks. The biological material (two fresh corpora cardiaca) was directly homogenized in either (a) EP_4 or (b) 30 % methanol ; in both cases, after a 20 min centrifugation at 10,000 g, the supernatant (20 µl) was loaded on an Altex column ; elution was performed with either EP_4 in (a) or EP_3 in (b) ; the chromatographic conditions were the same as in Fig. 1.

phase (pH = 3.0, 5.0 or 7.0) ; however, the higher salt concentration (0.5 M) and higher pH value (7.4) gave more reproducible results and were therefore finally selected. Addition of methanol (EP_4) considerably diminished the height of peak I ; peak II completely disappeared, and the different peaks of IV group were indistinct. The III group components were different, as only three peaks (instead of five) were noticeable ; the medium peak was the highest of the group, beginning and finishing in the negative part of the chart (fig. 2,a).

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The effect of methanol was also demonstrated with solvent phases deprived of electrolytes. With EP_5, peak I was almost suppressed and appeared as at least four small separated peaks. The different components of peak III appeared as a unique small peak. As with EP_4, peak II and IV were absent.

The separation was usually performed with a flow rate of 0.5 ml/min ; similar results were obtained with a flow rate of 0.8 ml/min, but a further increase (1 or 1,5 ml/min) led to a lesser resolution of the peaks.

The chromatographic columns were loaded with crude extracts obtained from a maximum number of 6 corpora cardiaca (80 µg of proteins) ; a higher quantity of extracts led to the saturation of the column ; this was proved by the emergence of a series of new peaks which ressembled the usual elution diagram, after a further injection of pure eluent.

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HPLC Analysis of Different Corpora Cardiaca Preparations

Crude methanolic extracts

Two fresh corpora cardiaca were extracted with 30 p. 100 methanol (sonification followed by centrifugation) and the chromatographic separation was performed with EP_3 as described. The modifications interested principally the two first peaks which almost disappear and group III, where two peaks were well resoluted (fig. 2,b). Increasing the methanol concentration to 70 p. 100 in the extraction medium with or without eliminating methanol under a stream of nitrogen, gave an extract very similar to the one obtained with 30 % methanol extraction.

Influence of storage

400 corpora cardiaca were sonificated in distilled water (4 ml) ; the homogenate was centrifuged (115.000 g ; 30 min) and the lyophilised supernatant stored at - 20°C. The lyophilised material was dissolved in EP_2 (4 ml) and analysed using EP_2. Peaks I and II almost disappeared ; the III group only showed four peaks. Contrary to the results obtained with fresh material, in which a very good reproductibility was always noticed, chromatograms from stored material showed a great variability in the four peaks

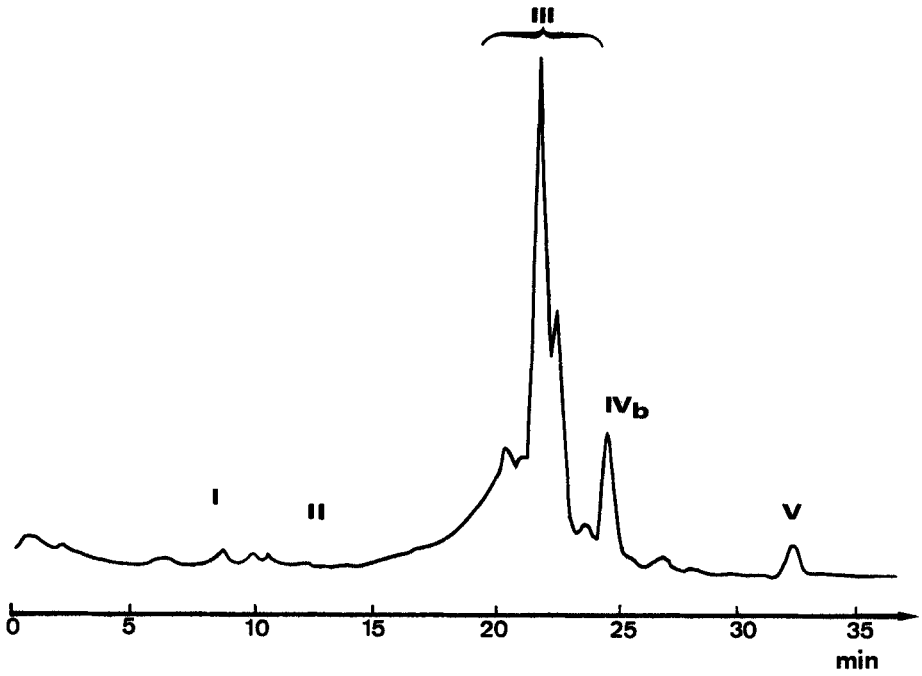

Figure 3.- Influence of storage on HPLC separation of the proteins. Corpora cardiaca (400 samples) were homogenized in distilled water (4 ml) : 30 min centrifugation at 115,000 g ; the supernatant was dissolved after lyophilisation in EP_2 (4 ml) ; 20 µl of the solution (corresponding to 2 corpora cardiaca) were loaded on an Altex column with the same chromatographic conditions as in Fig. 1.

of the III group, which differed in their heights from one batch to another and, in the same batch, from one injection to another. Storage of corpora cardiaca enhanced the importance of peak IVb and peak V (fig. 3).

Influence of a preliminary gel-filtration

Corpora cardiaca (2000 samples) were stored, extracted and centrifuged as previously described.

The extract was submitted to fractionation on Sephadex G-100. Three fractions a, b and c were collected at respectively 60, 67 and

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Figure 4.- HPLC separation of the proteins from fraction a obtained using Sephadex G-100 gel-filtration. Fraction a (50 µl collected at the top of the fraction) was loaded on a Waters column ; elution with EP_2 ; flow rate, 1.5 ml/min ; detection at 215 nm (0.01 AUFS) ; chart speed, 1 cm/min.

156 ml of elution volume. The a and b peaks were higher and sharper than c. Samples corresponding to the top of these peaks were submitted to HPLC with EP_2. The elution diagram of fraction a (fig. 4) is roughly similar to the one shown in fig. 1, with the exception of the different IV peaks which were all strongly enhanced. Peak I was absent in the elution diagrams of b and c fractions.

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Neurosecretory vesicles extracts

The technique of Nordmann *et al.*, applied to dissected neurosecretory corpora cardiaca previously submitted to Potter treatment, gives rise to two bands (vol : 1 ml ; d = 1.3812 and 1.3848) which were carefully collected. The two bands appeared as a homogenous suspension of intact neurosecretory vesicles, with only a few contaminating mitochondria, as confirmed by electron microscopy.

The vesicles were collected by centrifugation (40.000 g ; 20 min), resuspended in distilled water containing peptidase inhibitors and sonificated. After a series of freezing and defreezing processes, a last centrifugation (40.000 g ; 20 min) gave a supernatant and a pellet which were both analysed by HPLC with EP_2 as eluent. In both cases, the elution diagram was perturbed by a very large plateau region (ranging approximatively from t_r = 7 min to t_r = 13 min) due to sucrose and metrizamide. The plateau corresponded to the retention time of the last peaks of the III group and of all group IV peaks, thus masking the occurrence of these fractions. Sucrose and metrizamide could not be eliminated neither by ultrafiltration of the samples on a Millipore membrane (cutting off point 1000 mw) nor by monitoring the detector at 280 nm. However, the occurrence of peak I could be proved in the pellet and not in the supernatant (fig. 5).

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DISCUSSION

HPLC allowed a clear separation of the proteins of the neurosecretory endings of the locust. This could be achieved using a permeation column and a high osmotic strength elution medium, containing SDS. Our results agree with the conclusions of Jenik and Porter (2) and of Takagi et al. (6) : these two groups reported the beneficial influence of increasing the ionic strength and adding SDS in reducing the absorption of proteins and peptides to the matrix. The best separations were obtained with a low flow rate (0.5 ml/min). We have thus confirmed the previous results obtained by Meek and Rossetti (3), who emphazised the necessity of using a flow rate less than or equal to 1 ml/min.

The major advantage of HPLC was the possibility of exploring minute quantities of crude biological materials as the neurosecre-

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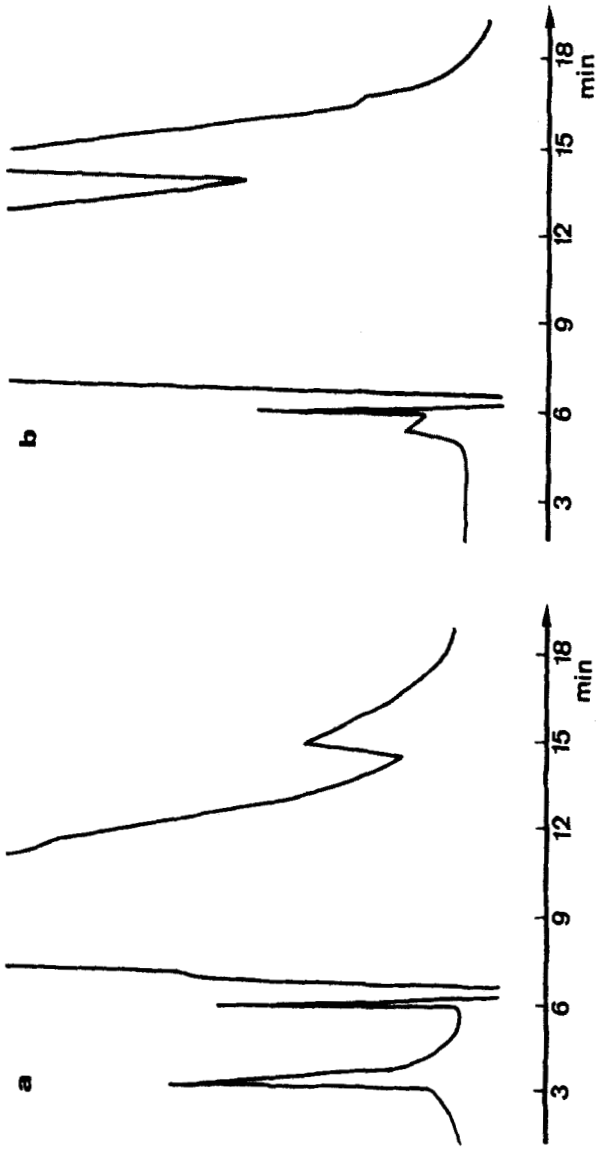

Figure 5.- HPLC analysis of the proteins from disrupted neurosecretory vesicles. (a) The pellet was homogenized in EP_2. (b) Supernatant. Chromatographic conditions as in Fig. 4.

tory endings of only two corpora cardiaca were needed, thus allowing analysis on fresh material in a wide variety of experimental conditions. A more heavier loading of the column diminished the quality of the separation, whereas the storage of the sample, an obligation when a larger weight of biological material is necessary, was followed by a low degree of reproductibility. Preliminary column gel-filtration did not improve the resolution.

Five groups of proteins of molecular weights ranging from 60,000 to 1,700 were distinctly separated. Peaks II and V always appeared homogenous ; Ia, Ib and II were present in all the samples analysed. Peaks belonging to groups III and IV showed the greatest variability in height. We generally observed that storage of the samples was associated with a decrease of group III peaks together with an enhancement of group IV peaks suggesting that the smaller compounds may be generated by an enzymatic attack of the larger protein molecules. Group III fractions include the range of molecular weights corresponding to the neurosecretory protein isolated from locust brain and corpora cardiaca by Freidel et al. (1). Using methanol in the extraction medium, an alcohol which allowed the extraction of two insect neuropeptides (5), was followed by a notable enhancement of peaks IIIb and IIIc.

Preparations of neurosecretory vesicles obtained by the technique of Nordmann et al. (4) appeared free from contaminating material and showed a good morphological quality. The constituants of the gradient unfortunately could not be eliminated and interfered with the subsequent spectrophotometric analysis. The exclusive occurence of the first compound in the pellet and its large molecular weight suggest that this compound can be a membrane protein, whereas group III compounds, entirely contained in the supernatant, appear to be neurosecretory products.

The results reported in this paper are currently applied to the isolation and biological characterization of the neurohormones of locust corpora cardiaca.

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