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DETERMINATION OF NEUROPEPTIDES IN DISCRETE REGIONS OF THE RAT BRAIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

High-performance liquid chromatography (HPLC) in combination with electrochemical detection (ED) is suitable for measuring accurately the levels of oxidizable neuropeptides in small samples of brain tissue. We have determined by HPLC–ED and a two-column-switching technique the levels of leucine- and methione-enkephalin in rat brain. By a sequential two-column HPLC–ED method we have also determined the levels of cholecystokinin tetrapeptide and octapeptide sulphate in various regions of the rat brain. The values found are in good agreement with those reported previously based on radioimmunoassay. We conclude that, due to its simplicity, HPLC–ED is an attractive alternative to existing methods for the determination of some neuropeptides in biological material.

INTRODUCTION

In the last decade more than twenty peptides have been described in the mammalian central nervous system (CNS), localized in specific neurons, and their number is still growing[1,2]. However, the rôle of these neuropeptides in the physiology and pathology of the CNS is at present poorly understood, although there is evidence that they might be involved in several neurological diseases[3–6]. Progress in neuropeptide research will rely on specific and sensitive, yet simple methods for their quantification in biological material.

The levels of neuropeptides found in brain tissue and cerebrospinal fluid are generally low, usually in the pmol/g range. The classical method for the determination of neuropeptides is the radioimmunoassay (RIA), which it is highly sensitive and selective enough to measure peptides even in crude extracts of biological material. However, appropriate antibodies have first to be raised for each peptide and, in addition, long incubation times (up to 4 days) can make RIA quite tedious. The most serious drawback of currently used RIA is, however, the lack of specificity for a given neuropeptide molecule: antibodies suitable for RIA are raised with specificity towards a certain sequence in the molecule of interest. Since in many cases the biologically active neuropeptide molecule, *e.g.*, cholecystokinin-8 sulphate (CCK-8S), is derived from larger precursor molecules having different biological activities, *e.g.*, CCK-33,

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a "specific" antibody will cross-react with these precursors and occasionally also with smaller (metabolite?) molecules, *e.g.*, CCK-4[7,8]. Therefore, data obtained with RIA are correctly expressed as "neuropeptide-like immunoreactivity".

The problem of specificity which is seriously limiting the scope of "classical" RIA can be eliminated by separating the neuropeptide of interest from cross-reacting peptides using high-performance liquid chromatography (HPLC)[9]. Usually, a gradient/reversed-phase HPLC system is used and the neuropeptide is collected for subsequent RIA, according to the retention times of calibration chromatograms obtained with large, UV-detectable amounts of peptide standards. Such a method based on HPLC–RIA has recently been published for the determination of CCK-8s and CCK-4 in the rat CNS[10]. While HPLC clearly improves the specificity of RIA it unfortunately also makes it more expendable. Continuous "on-line" detection of neuropeptides at the end of the HPLC column would eliminate some of the remaining disadvantages associated with RIA. Conventional HPLC detectors, such as UV and fluorescence, are generally not sensitive enough to quantify the low levels of neuropeptides in biological material[11]. Electrochemical detection (ED) has recently evolved as a versatile, highly sensitive detection method in HPLC[12,13]. Since many neuropeptides contain oxidizable amino acids, theoretically they should be good candidates for ED. We show that HPLC in combination with ED (HPLC–ED) is a simple, yet sensitive method, allowing the accurate determination of neuropeptides with oxidizable amino acids in brain tissue without prior derivatization.

MATERIALS AND METHODS

Apparatus

The chromatograph used consisted of two M 6000 pumps (Waters, Milford, MA, U.S.A.), a 7125 injection valve (Rheodyne, Berkley, CA, U.S.A. with a 20-μl loop, three 7000 switching valves (Rheodyne) and a LC-4A electrochemical detector (BAS, W. Lafayette, IN, U.S.A.). The applied potential was +1.0 *V vs.* a Ag/AgCl reference electrode. A glassy carbon detector cell (Type TL-5, BAS) with the following PTFE gaskets was used: for low flow-rates (0.4 and 0.6 ml/min), TG-2M; for high flow-rates (1 ml/min), TG-5M. As backpressure regulator, a worn-out HPLC column, immersed in a temperature-controlled water-bath, was used. Over a temperature range of 20–70°C the backpressure varies between approximately 1000 and 2000 p.s.i. The exact, matching backpressure was obtained by adjusting the temperature of the water-bath.

Reagents

Leucine–enkephalin, methionine–enkephalin, cholecystokinin tetrapeptide and *tert.*-butyl-oxycarbonyl (BOC)-cholecystokinin octapeptide were obtained from Bachem (Bubendorf, Switzerland). Sulphated cholecystokinin octapeptide was prepared by R. Huguenin (Sandoz, Basle) from BOC-cholecystokinin octapeptide according to published procedures[14]. The enkephalin analogue FK 33-824 [Tyr-D-Ala-Gly-MePhe-Met(O)-ol] was synthesized at Sandoz[15]. All other chemicals used were of the highest commercially available analytical grade (Merck, Darmstadt, F.R.G.) and used without further purification.

RESULTS AND DISCUSSION

Quantification of neuropeptides by HPLC–ED

Although it was recognized some time ago[16] that certain amino acids such as tyrosine, tryptophan and cysteine are oxidizable at potentials used in electrochemistry, few practical attempts have been made to detect electrochemically peptides containing such amino acids. Fig. 1 shows the separation and electrochemical detection of a standard mixture (100 ng each) of three naturally occurring neuropeptides and one synthetic analogue, FK 33-824. It is evident that ED might be sufficiently sensitive to detect directly physiological amounts of neuropeptides in biological material.

The main problem encountered in neuropeptide analysis from biological material by HPLC–ED arises from the coextraction of huge amounts of electrochemically active compounds, resulting in a big, interfering void peak. Since the sensitivity in HPLC does not only depend on the detector used, but to a great extent also on the peak shapes, the long retention times, necessary to separate the neuropeptide of interest from the large void, will lead to broad peaks and hence reduce the overall sensitivity of the system. In addition, when electrochemical detectors are set at high gain, solvent gradients, often used in peptide HPLC, are unsuitable because of unstable, shifting baselines. For successful determination of neuropeptides in biological material by HPLC–ED, the chromatographic conditions (column length, internal

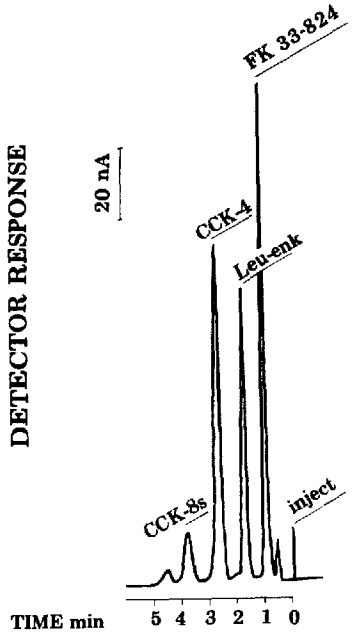


Fig. 1. Separation and electrochemical detection of neuropeptide standards. Three naturally occurring neuropeptides (cholecystokinin tetrapeptide = CCK-4, 100 ng; cholecystokinin octapeptide sulphate = CCK-8s, 100 ng; leucine enkephalin = leu-enk, 100 ng) and one synthetic enkephalin analogue (FK 33-824[15], 100 ng) were applied to a RP-18 column (2.1 × 150 mm) and detected electrochemically. Mobile phase: 150 m*M* phosphate buffer, pH 5.5, containing 11.5 vol.% 1-propanol; flow-rate 0.6 ml/min. Temperature: 45°C. Electrochemical detector: glassy carbon; potential, + 1.0 V *vs.* Ag/AgCl reference electrode; sensitivity, 200 nA per 1 V full scale.

diameter, packing material, buffer composition, temperature, etc.) have to be selected carefully.

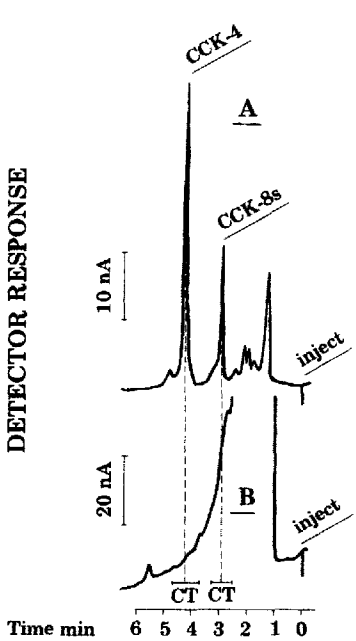
In our experience[17] it is rather difficult to separate the neuropeptide of interest from a large void peak using a single, isocratic system when the retention time should not exceed 10 min. Direct determination is possible, *e.g.*, for CCK-8s, which occurs in relatively high concentration in the cerebral cortex of the rat. In most cases, however, it will be necessary to use a two-column system: on the first column the neuropeptides are separated from the large, interfering void peak, whereas the second column separates the neuropeptide(s) from other compounds having the same or a similar retention time on the first column. The neuropeptides are quantified by ED after elution from the second column, by comparison of their peak heights with those of known amounts of standard peptides. The two columns can be related in two ways: (1) sequential two-step HPLC, where the neuropeptides eluted from the first column are collected according to the retention times determined with peptide standards and, after evaporation, reinjected onto the second column; or (2) column-switching HPLC, where the effluent of the first column is diverted to the second column during a short time period, determined by the retention time of the neuropeptide(s) on the first column. The successful application of the two methods and a critical evaluation will be discussed below.

Determination of CCK-4 and CCK-8s in the rat brain by sequential two-step HPLC–ED

CCK-4 and CCK-8s can be determined by HPLC–ED in biological material by a sequential two-column procedure. Fig. 2A shows a calibration chromatogram obtained on the first column in the two-step assay. A mixture of CCK-4 and CCK-8s standards (100 ng each) was injected and the exact collection time (CT) for each peptide was determined. Fig. 2B shows a typical chromatogram of a crude rat cerebral cortex extract on the first column. Due to a massive, broad void peak, the small amount of CCKs present in the rat CNS (see Table I) cannot be detected. Therefore, CCK-4 and CCK-8s are collected separately according to the CTs determined in the calibration run (Fig. 2A). After evaporation under reduced pressure, the peptide fractions are separately applied to a second column, operated under different chromatographic conditions. The separation and electrochemical detection of CCK-4 and CCK-8s from rat cerebral cortex on the second column is represented in Figs. 3 and 4, respectively.

It is noteworthy that on the second HPLC column the retention times of CCK-4 and CCK-8s are completely different, both relatively and absolutely, when compared to those on the first column. On the first column CCK-4 has a longer retention time than CCK-8s, whereas on the second column it is eluted before CCK-8s. This results in an overall better separation of the neuropeptide peaks from accompanying impurities that would have similar retention times on a single column. In addition, it also makes more reliable the identification of the peaks, based on two completely different and independent retention times.

The sensitivity of this HPLC–ED procedure, evaluated by injection of a pure CCK-4 standard, is better than 0.1 pmol, giving a signal-to-noise ratio of 3. However, in our opinion, a more significant measure is the detection limit when the extraction procedure from biological material and, hence, also the interference from impurities



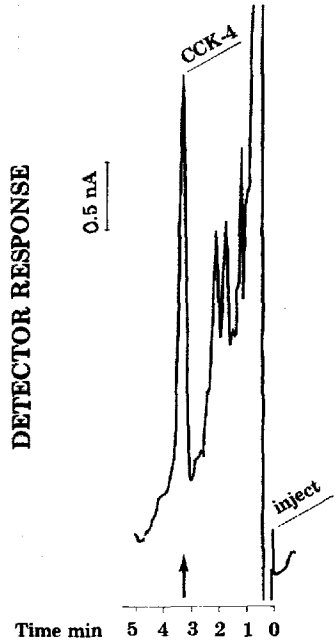


Fig. 2. Chromatograms of the first ("preparative") step in the two-step assay of CCK-4 and CCK-8s. Column: RP-18, 4.6 × 125 mm. Mobile phase: 40 m*M* phosphate buffer, pH 5.5, containing 15.5 vol. % 1-propanol; flow-rate 1.0 ml/min. Temperature: 47°C. ED as in Fig. 1. A, Calibration of the column with CCK-4 and CCK-8s standards, 100 ng each. Collection times (CT): CCK-8s, 2.75–3.6 min; CCK-4, 3.85–4.85 min. B, Chromatogram of a crude extract of rat cerebral cortex.

Fig. 3. Chromatogram of the second ("analytical") step in the two-step-assay of CCK-4 and CCK-8s. Electrochemical detection and quantification of CCK-4. Column: RP-18, 2.1 × 50 mm. Mobile phase: 200 m*M* phosphate buffer, pH 5.5, containing 11.5 vol % 1-propanol; flow-rate 0.4 ml/min. Temperature: 30°C. ED as in Fig. 1, except sensitivity: 5 nA per 1 V full scale. Retention times: CCK-4, 3.35 min, indicated by an arrow; CCK-8s, 8.4 min.

in the extract is included. In Fig. 3 the peak of CCK-4 is about 30 times higher than the baseline noise. This peak corresponds to 5 pmol CCK-4 from approximately 150 mg brain tissue.

The levels of CCK-4 and CCK-8s were measured in different regions of the rat CNS using this sequential two-step HPLC–ED technique. The data obtained

TABLE I

CCK-4 AND CCK-8s LEVELSIN THREE REGIONS OF THE RAT BRAIN, AS DETERMINED BY HPLC–ED AND HPLC–RIA[10]

Brain region	*CCK-4*		*CCK-8s*	
	HPLC–ED, $\bar{X} \pm$ S.D., $n = 6$ (pmol per g tissue)	*HPLC–RIA, $\bar{X} \pm$ S.D., $n = 36$ (pmol per g tissue)*	*HPLC–ED $\bar{X} \pm$ S.D., $n = 6$ (pmol per g tissue)*	*HPLC–RIA $\bar{X} \pm$ S.D., $n = 36$ (pmol per g tissue)*
Cortex	33 ± 5	29 ± 5	417 ± 28	436 ± 15
Hippocampus	20 ± 8	23 ± 7	53 ± 18	116 ± 15
Striatum	51 ± 12	20 ± 4	141 ± 31	131 ± 12

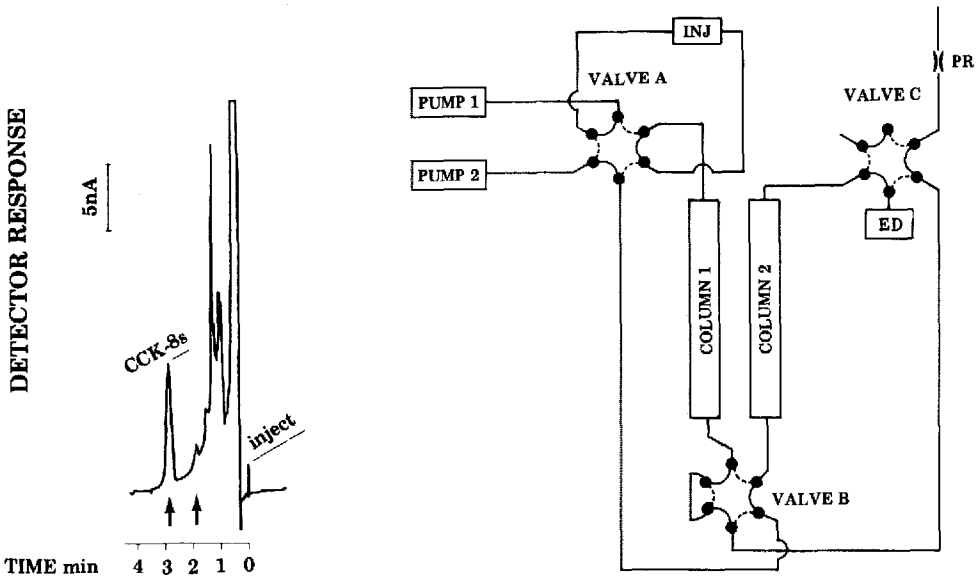


Fig. 4. Chromatogram of the second ("analytical") step in the two-step assay of CCK-4 and CCK-8s. Electrochemical detection and quantification of CCK-8s. Conditions as in Fig. 3 except: temperature, 47°C; ED sensitivity, 50 nA per 1 V full scale. Retention times: CCK-8s, 2.85 min; CCK-4, 1.8 min, indicated by arrows.

Fig. 5. Column-switching HPLC system used for the assay of leu-enk and met-enk. Under standby conditions (———) the flow from pump 1 goes to valve A, to the inector (INJ), back to valve A, through column 1 to valve B, to valve C and finally, over a backpressure regulator (PR), to waste. The flow from pump 2 goes to valve A, to valve B, through column 2 to valve C and finally, through the electrochemical detector (EC), to waste. By switching valve C (---------), the detector is connected to the end of column 1. This allows the calibration of column 1 with enkephalin standards in order to determine the exact switching times of valve B. During an analysis, valve B is switched (---------) during the time the enkephalins are eluted from column 1 (previously determined by injection of standards), in order to divert the flow from column 1 to column 2. The backpressure regulator is set at a pressure equal to that of column 2. This results in constant pressures, independent of the position of valve B, and hence precise flows and retention times. In order to test the performance of column 2, enkephalin standards can be directly injected onto it by switching valve A (---------).

(mean values from six animals) are summarized in Table I. They are in good agreement with recently published values using HPLC–RIA[10].

Determination of leucine–enkephalin and methionine–enkephalin in the rat brain by column-switching HPLC–ED

Leucine–enkephalin (leu-enk) and methionine–enkephalin (met-enk) can be determined simultaneously in biological material by HPLC–ED, using a two-column switching technique. Fig. 5 is a schematic representation of the equipment used, showing how column 1 is connected through a switching valve B to column 2. Appropriate chromatographic conditions are chosen to separate leu- and met-enk from the large void peak in column 1. Both neuropeptides have the same retention time (2.15 min) on column 1 and are diverted together to column 2 by switching valve B between 1.75 and 2.6 min. Column 2 separates leu-enk from met-enk and other com-

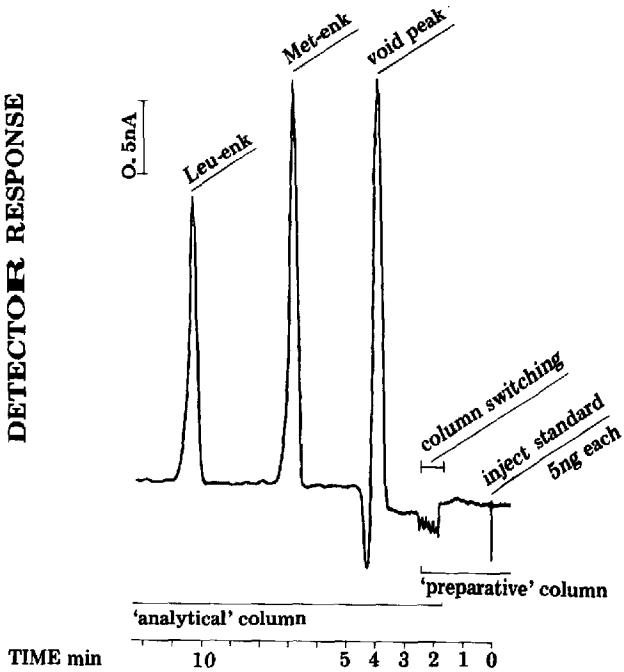


Fig. 6. Chromatogram of leu- and met-enk standards obtained with the column-switching HPLC system. Column 1 ("preparative column"): anion exchange, 3.0 × 70 mm. Temperature: 50°C. Column 2 ("analytical column"): RP-18, 3.0 × 150 mm. Temperature: 30°C. The mobile phase and flow are the same for both columns: 300 m*M* phosphate buffer, pH 5.5, containing 14.5 vol. % 1-propanol; 0.4 ml/min. Electrochemical detector: glassy carbon; potential +1.0 V *vs.* Ag/AgCl reference electrode; sensitivity, 5 nA per 1V full scale. Retention times: column 1, leu-enk and met-enk, 2.15 min; column 1 + column 2, met-enk, 6.75 min; leu-enk, 10.15 min. Switching times: 1.75–2.5 min.

pounds having the same retention time on column 1 and, hence, being also diverted to column 2.

The chromatogram of a standard (5 ng leu-enk + 5 ng met-enk), passed through both columns and detected electrochemically, is shown in Fig. 6. The linearity of the method was tested by repeated injections of various amounts of standards (1–50 ng of leu-enk and met-enk). A proportional detector response (peak heights) was obtained in the range tested. The coefficient of variation for the lowest amount injected (1 ng) was less than 5% ($n=4$) for both, leu-enk and met-enk. Fig. 7 shows a chromatogram obtained from whole rat brain (minus brain stem and cerebellum): a crude extract, equivalent to 19 mg brain tissue, was injected and passed through the two columns. The levels of leu-enk and met-enk, calculated from such chromatograms by comparing the peak heights to those of known amounts of standards, are: leu-enk, 0.09 ng per mg tissue; met-enk, 0.24 ng per mg tissue. They are similar to values reported in the literature[18] obtained by using RIA: leu-enk, 0.03–0.05 ng per mg tissue; met-enk, 0.1–0.2 ng per mg tissue. From Fig. 7 it can be concluded that the signal-to-noise ratio would allow operating the ED at considerably higher sensitivity, which can be used either to reduce the amount of tissue per assay or enlarge the peak heights.

The advantage of the column switching technique is evident: since it can easily be fully automated by microprocessor-controlled switching of valve B, this method is very rapid and has a high capacity. However, the overall sensitivity of the sequential

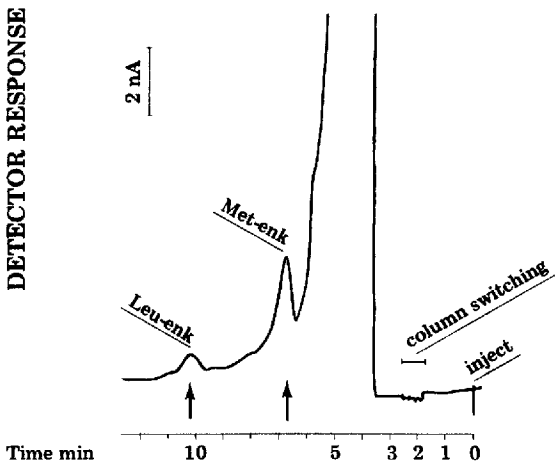


Fig. 7. Chromatogram of crude rat brain extract, obtained with the column-switching HPLC system. Electrochemical detection and quantification of leu- and met-enk. Conditions in Fig. 6 except: detector sensitivity, 20 nA per 1 V full scale. The arrows indicate the retention times of pure standards. The amount of tissue extract injected (20 μl) corresponds to 19 mg fresh brain tissue.

two-column technique is better, because after collecting the fractions from the first column, the volumes are reduced and hence more sample in a smaller volume is injected onto the second column. This results in bigger and sharper peaks. In addition, two completely independent column–mobile phase systems can be chosen with the sequential method, whereas with the switching technique it is preferable to use the same mobile phase and flow-rate for both columns in order to have a stable detector baseline and well equilibrated columns during the entire analysis.

CONCLUSIONS

We have shown that HPLC–ED is sensitive enough to determine peptides with oxidizable amino acids in biological material without derivatization. The comparatively simple and rapid assay procedure and the unequivocal sample identification should complement existing methods. It will be helpful in gaining insights into post-translational processing mechanisms of neuropeptides and will be used to investigate pharmacological effects on neuropeptide levels and turnover.

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