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Note

High-performance liquid chromatography of tyrosine-related peptides with electrochemical detection

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A recent problem encountered in this laboratory involved the examination of a syringe for residues of an oxytocin solution containing 10 I.U./ml. The volume of liquid remaining in the syringe was small (*ca.* 10 μl). An extremely sensitive and specific method of analysis was therefore required. Bioassay techniques[1] are used to measure oxytocic activity of pharmaceutical preparations, and radioimmunoassays[2] have been developed for the determination of oxytocin in body fluids. However, these are inconvenient and oxytocin antisera are not readily available.

Several high-performance liquid chromatography (HPLC) systems have been reported for the separation of oxytocin[3,4], and since the equipment is already widely used in our laboratory it was the method of choice. Detection is usually by either ultraviolet (UV) absorption at wavelenghts between 200 and 220 nm or post-column derivatisation with fluorescence detection. Although sensitive, these detection methods are generally non-specific. Increased peptide specificity can be achieved at the expense of sensitivity by using UV absorption at 280 nm, which is characteristic of the amino acid residues tyrosine and tryptophan[5,6].

HPLC with electrochemical detection (ED) has been employed for the determination of a wide range of compounds[7]. Fleet and Little[8] have used the technique for the detection of amino acids, electroactivity reportedly being due to the NH_2 group. Thus peptides with a free terminal amino group should also be electroactive. Some amino acids however, have other more electroactive substituents. The most important of these are the phenol, indole and thiol substituents of tyrosine, tryptophan and cysteine, respectively. Peptides containing these amino acids have been shown to be electroactive[9,10], and therefore ought to be suitable for analysis by HPLC–ED. Such methods have been reported for the tripeptide glutathione[11] and for electroactive derivatives of other peptides[6]. The aim of this study therefore was to develop an HPLC–ED method for the determination of oxytocin that would be at least as sensitive as UV detection at 220 nm but more specific.

The discovery of the opioid peptides[12], and other biologically active peptides[5,13,14], will undoubtedly lead to the introduction of other peptide-related drugs, some of which may become subject to abuse. Since our involvement in the analysis of these pharmaceutically important substances is therefore likely to increase, it was of interest to apply the HPLC–ED method to a number of other tyrosine-related peptides.

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EXPERIMENTAL

Materials

Oxytocin solution (200 I.U./ml), lysine(Lys)-vasopressin, angiotensin II, leucine (Leu)-enkephalin, and [des-tyrosine[1] (Tyr)]-Leu-enkephalin were obtained from Sigma (London, Great Britain). The structures of these peptides are shown in Table I. The oxytocin solution also contained 0.9% sodium chloride and 0.5% chlorobutanol. Diluted solutions were prepared using distilled water and frozen when not in use.

Phosphate buffer solution (0.025 *M*) was prepared from K_2HPO_4 to give an unadjusted pH of 8.9. Acetonitrile (HPLC grade)–phosphate buffer (25:75) was employed for the analysis of oxytocin and Lys-vasopressin, and acetonitrile–phosphate buffer (15:85) was used for separations of Leu-enkephalin, (des-Tyr[1])-Leu-enkephalin and angiotensin II.

Apparatus

A reciprocating pump (Applied Chromatography services, Model 300) or metering pump (Metering Pumps, London, Great Britain) was used to deliver solvent at flow-rates of 2.1 ml/min and 1.2 ml/min, respectively. The column was a 15 cm × 4.6 mm I.D. stainless steel tube, slurry packed with octadecyl-trichlorosilane (ODS) modified silica (12% loading; particle size *ca.* 7 μm; prepared by a method previously described[15]).

The effluent was monitored at 220 or 280 nm by a variable-wavelength UV detector (Laboratory Data Control, Spectromonitor III) and by an electrochemical detector[16]. Working electrode potentials of the latter, relative to the silver–silver chloride electrode (SSCE), are given in the text. The electrochemical detector was coupled to the outlet of the UV detector and the chromatograms recorded simultaneously. Samples were introduced onto the column using a syringe-loading sample injection valve (Rheodyne, Model 7175) fitted with a 20-μl loop.

RESULTS AND DISCUSSION

Oxytocin

Suitable HPLC conditions for the separation of oxytocin were established using UV detection at 220 nm and are based on those reported by Krummen and Frei[17]. An ODS-modified silica column was used with a mobile phase consisting of a mixture of

TABLE I

PEPTIDE STRUCTURES

Peptide	*Structure*
Oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2
(Des-Tyr[1])-Leu-enkephalin	Gly-Gly-Phe-Leu
Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu
Angiotensin II	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
Lys-vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH_2

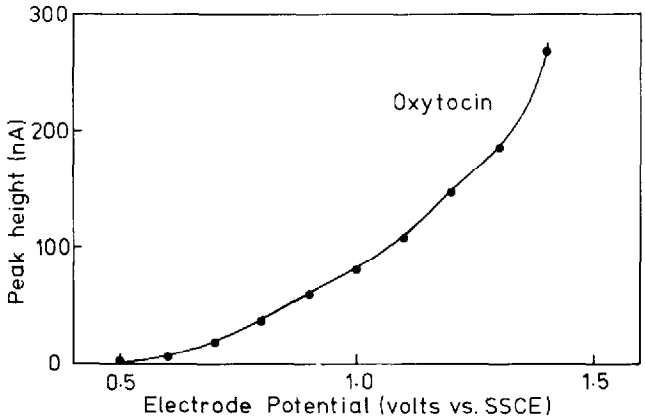

Fig. 1. Current–voltage curve for electrochemical detection of 20-μl injections of oxytocin solution (25 I.U./ml). Conditions: column (15 cm × 4.6 mm I.D.) of ODS-modified silica; mobile phase, acetonitrile–0.025 *M* K_2HPO_4 (25:75); flow-rate, 1.2 ml/min.

acetonitrile and phosphate buffer. The proportion of acetonitrile was adjusted so as to maximise sensitivity while maintaining adequate separation from the solvent. When acetonitrile–phosphate buffer (25:75) was used at a flow-rate of 2.1 ml/min, the retention time of oxytocin was 2.9 min (capacity factor, $k' = 4.1$). Under these conditions the preservative chlorobutanol was retained much longer ($k' = 11.1$), which increased the total analysis time to *ca.* 10 min.

In order to select a suitable operating potential for the electrochemical detection of oxytocin the relationship between detector response and working electrode potential was investigated. Aliquots of a diluted oxytocin solution (20 μl; 25 I.U./ml) were injected onto the column, and the effluent was monitored over a range of electrode po-

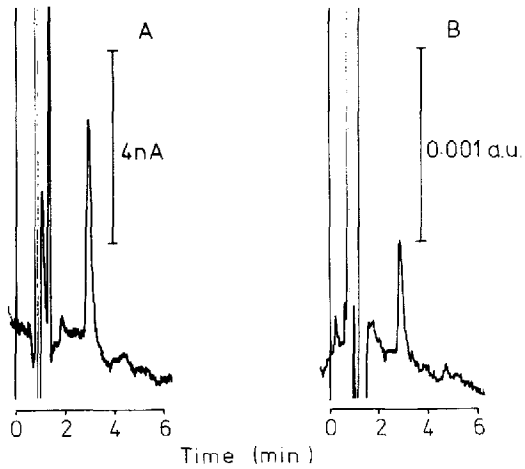

Fig. 2. Chromatograms of 20 μl of oxytocin solution (1.0 I.U./ml; 40 pmole injected). Conditions: column (15 cm × 4.6 mm I.D.) of ODS-modified silica; mobile phase, acetonitrile–0.025 *M* K_2HPO_4 (25:75); flow-rate, 2.1 ml/min. Detection: A, electrochemical, + 0.9 V *vs.* SSCE; B, UV, 220 nm.

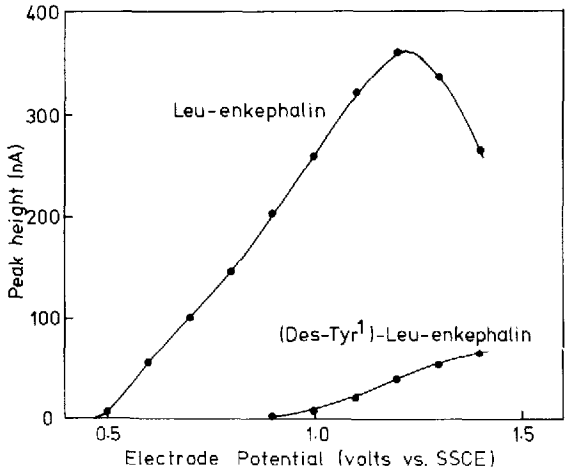

Fig. 3. Current–voltage curves for electrochemical detection of 20 μl of a mixture of (des-Tyr[1])-Leu-enkephalin and Leu-enkephalin (50 μg/ml each). Conditions: column (15 cm × 4.6 mm I.D.) of ODS-modified silica; mobile phase, acetonitrile–0.025 *M* K_2HPO_4 (15:85); flow-rate, 1.2 ml/min.

tentials starting at 1.4 V and decreasing by 100-mV steps until no significant detector response was observed. The resulting voltammogram shown in Fig. 1 was obtained by plotting peak height against operating potential.

An electrode potential of 0.9 V was found to give the optimum signal-to-noise ratio. The detection limit under these conditions is comparable to that obtained with UV detection at 220 nm. Fig. 2 demonstrates the signal-to-noise ratios of the two detectors for an injection of 40 pmole of oxytocin (20 μl; 1.0 I.U./ml). Although an enhanced electrochemical detector response is obtainable at higher electrode potentials the increase in background current is even greater, which leads to a reduction of the signal-to-noise ratio. In addition, a high electrode potential is undesirable as selectivity is lost.

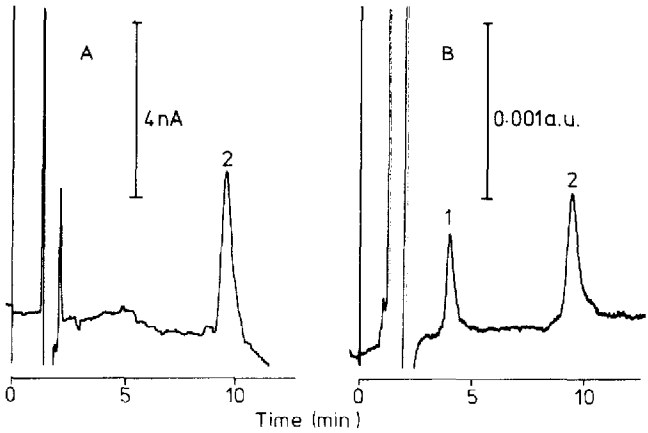

Fig. 4. Chromatograms of 20 μl of a mixture of (des-Tyr[1])-Leu-enkephalin and Leu-enkephalin (1 μg/ml each; 36 pmole injected). Detection : A, electrochemical, +0.9 V *vs.* SSCE; B, UV, 220 nm. Other conditions as in Fig. 3. Peaks: 1 = (des-Tyr[1])-Leu-enkephalin; 2 = Leu-enkephalin.

Peptide selectivity

One advantage of electrochemical over UV detection is the increased peptide selectivity which can be achieved without loss of sensitivity. Peptides containing tyrosyl or tryptophyl residues can be selectively detected using UV absorption at 280 nm, but electrochemical detection is more sensitive. Under the conditions described in Fig. 2, but monitoring at 280 nm, the response for oxytocin was only just distinguishable from the background noise.

Although most peptides are probably electroactive by virtue of the terminal amino group, it is possible, by selecting a suitable electrode potential, to eliminate completely any response due to this group. This can be demonstrated using Leu-enkephalin and (des-Tyr^1)-Leu-enkephalin. The current–voltage curves for these two peptides are shown in Fig. 3. The electroactivity of the latter presumably being due to the terminal amino group, whereas Leu-enkephalin contains an additional tyrosyl residue which is detected at a much lower electrode potential. The decrease in response for Leu-enkephalin above 1.2 V is probably due to deactivation of the electrode surface by electrochemical oxidation products of this peptide.

Fig. 4 shows the chromatograms obtained for a mixture of these two peptides (36 pmole each) which illustrates both the sensitivity and selectivity of the electrochemical detector. At an electrode potential of 0.9 V only the Leu-enkephalin is detected electrochemically, whereas both peptides are detected using UV absorption at 220 nm.

Other applications to peptide analyses

Two other peptides, angiotensin II and Lys-vasopressin were also examined although no attempt was made to optimise the HPLC conditions for maximum sensitivity. However, the relative response ratios of the two detectors for these peptides indicated

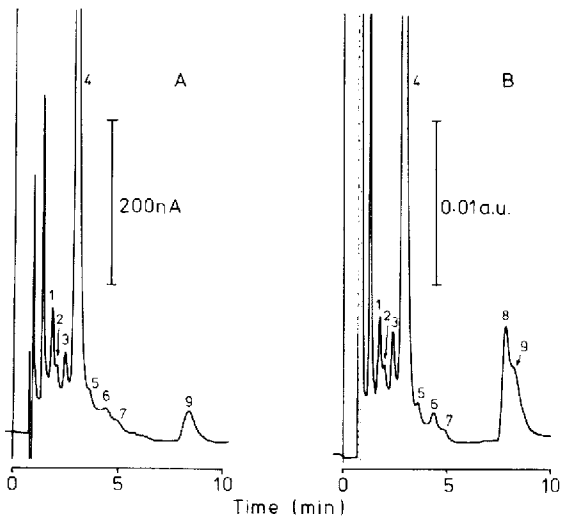

Fig. 5. Chromatograms of 20 μl of oxytocin solution (200 I.U./ml). Detection: A, electrochemical, +0.9 V *vs.* SSCE; B, UV, 220 nm. Other conditions as in Fig. 2. Peaks: 4 = oxytocin; 8 = chlorobutanol; other peaks are by-products.

that the sensitivity of electrochemical detection at 0.9 V would be comparable to that obtained using UV detection at 220 nm.

HPLC with UV detection has also been used for the determination of by-products and additives present in synthetic oxytocin formulations[18]. Some of these compounds are electroactive and additional information can be obtained from the relative response of the two detectors. A comparison of the detectors for the analysis of an oxytocin solution (20 μl; 200 I.U./ml) is shown in Fig. 5. Peptide by-products of oxytocin detected by UV absorption at 220 nm are also detected by electrochemical oxidation at 0.9 V. The different response ratios for the various by-products are indicative of the relative number of electroactive animo acids present in each peptide. On the chromatogram showing the UV detector response one of the by-products is partially masked by the chlorobutanol peak. However, by using electrochemical detection this interference is eliminated since chlorobutanol is not electroactive at this electrode potential.

For the selective detection of tyrosine-related peptides, electrochemical detection provides a more sensitive alternative to UV absorption at 280 nm. Detection limits comparable to or better than those obtained using non-specific UV absorption at 220 nm are possible, although actual sensitivities will depend on the number and type of amino acid residues present.

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