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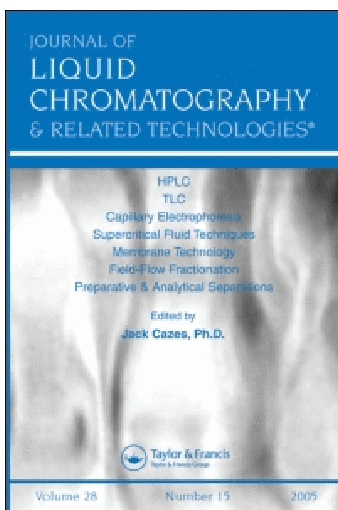

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Purification of Radioiodinated Peptides with PRP-1 Polystyrene Cartridges and HPLC: Application to Atrial Natriuretic Factor and Vasopressin

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PURIFICATION OF RADIOIODINATED PEPTIDES WITH PRP-1 POLYSTYRENE CARTRIDGES AND HPLC: APPLICATION TO ATRIAL NATRIURETIC FACTOR AND VASOPRESSIN

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ABSTRACT

A simple and rapid cleanup procedure is described for the purification of iodinated peptides using PRP-1 polystyrene cartridges following the radioiodination process. The method is validated using different volumes and solvent systems and compared to the standard Sep-Pak C_{18} procedure. In this study, the method is used to prepare ^{125}I-labeled atrial natriuretic factor and arginine-vasopressin which are further purified by reverse phase HPLC giving maximally obtainable specific activity required for the radioimmunoassays of these peptides.

INTRODUCTION

In recent years, reverse phase high performance liquid chromatography has been widely used to purify

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biologically active radioiodinated tracers free from unlabeled hormone (1-5), superseding the classical procedures using ion exchange chromatography (6,7) or gel filtration on Sephadex columns (8,9). The application of the iodination reaction mixture directly on the reverse phase column usually results in a very high background of radioactivity requiring tedious washing steps with a high risk of contamination. Initial cleanup procedure before the reverse phase chromatography has received little attention. We report here a simple radioiodination procedure by solid-phase method combined to a rapid pre-cleanup step to remove unincorporated iodine from radioiodinated peptides using a polystyrene cartridge, prior to chromatography on reverse phase columns. This method has been applied successfully to the preparation of high specific activity ^{125}I-labeled atrial natriuretic factor (ANF) and arginine-vasopressin (AVP).

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MATERIALS AND METHODS

Peptides

Rat [Ser^{99}-Tyr^{126}]-atrial natriuretic factor was obtained from Institut Armand Frappier, Laval, Canada. Synthetic [Arg^{8}]-vasopressin was purchased from Peninsula Laboratories (Belmont, CA, USA).

Chemicals

All reagents were analytical grade. Trifluoroacetic acid (TFA) and Iodo-beads were purchased from

Pierce (Rockford, IL, USA). Triethylamine (TEA) and acetonitrile were supplied by Baker (Phillipburg, NJ, USA) and Burdick Jackson (Muskegon, MI, USA), respectively. PRP-1 cartridges (Cat. No. 79508) and Sep-Pak C_{18} cartridges were from Hamilton (Reno, NV, USA) and Waters (Milford, MA, USA), respectively. Reverse phase HPLC columns used were octadecasilyl silica type (15 x 0.45 cm, 5 µm) and (25 x 0.4 cm, 5 µm) from Jones Chromatography (Columbus, Ohio, USA) and Bio-Rad (Richmond, CA, USA), respectively. The ANF antibody (Lot no. 6107) was from Peninsula Laboratories and the AVP antibody from Calbiochem (Lot no. 393079). Carrier-free $Na^{125}I$ was obtained from Amersham (Cat. no. IMS 300; Arlington Heights, IL, USA).

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Chromatographic Instrumentation

The chromatographic system consisted of dual pumps (Model 510), a gradient controller (Model 680), a fixed wavelenght UV detector (Model 441) set at 214 nm, a model 990 photodiode array detector, all from Waters Associates, and an injection valve (Rheodyne Model 7125; Berkeley, CA, USA) with a 2 ml loop. The eluted fractions (1 ml) were collected with a FRAC 100 fraction collector (Pharmacia, Uppsala, Sweden).

Radioiodination Process

A solid-phase method using Iodo-beads as the oxidizing agent (10) was used with some modifications for the radioiodination of both peptides. Briefly, the experimental conditions were as follows: 70 µl of 0.5 M potassium phosphate buffer (pH 7.0), 1 mCi of $Na^{125}I$

(2 µl), and 10 µg of the peptide (30 µl in 0.1 N acetic acid) were mixed in a polypropylene tube and the reaction was started by addition of two Iodo-beads. After a 20-minutes incubation period at 4°C, the iodination reaction mixture was diluted to 0.5 ml with cold 0.5 M potassium phosphate buffer and immediately passed through the PRP-1 cartridge.

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Pre Cleanup Procedure

The activation of PRP-1 cartridges was performed by flushing 1 ml of acetonitrile through the cartridge followed by 3 ml of TFA 0.1% for ANF or TEA-acetate buffer (0.02 M, pH 4) for AVP. The iodination reaction mixture diluted with phosphate buffer was applied on the PRP-1 cartridge using a 1 ml RNCP syringe (Chromatographic Specialties, Cat. no. H811331). The cartridge was then washed with 3 ml of TFA 0.1% or TEA buffer (for ANF and AVP, respectively) and the iodinated peptide was eluted with a mixture of TFA 0.1% or TEA buffer with acetonitrile as described in Table 1.

For comparison purposes, Sep-Pak C_{18} cartridges were used as follows: 10 ml of acetonitrile were flushed through the cartridge followed by 10 ml of TEA buffer (0.02 M, pH 4), and the iodinated peptides diluted in 0.5 M potassium phosphate buffer were applied on the cartridge. The cartridge was then washed with 10 ml of TEA buffer and the peptide eluted with a mixture of TEA buffer and acetonitrile as described in Table 2.

HPLC Purification of the Monoiodinated Peptides

For the separation of monoiodinated ANF from its unlabeled form and its diiodotyrosyl derivative, a gradient of acetonitrile from 15 to 55% in TFA 0.1% was performed in 60 minutes at a flow rate of 1 ml/min. For the purification of monoiodinated AVP, a gradient of acetonitrile from 0 to 30% in TEA buffer (0.02 M, pH 4) was performed in 60 minutes at a flow rate of 1 ml/min. The radioactivity of the eluent was monitored by counting small aliquots (5 μl) from each 1 ml fraction in a gamma counter (CliniGamma 1272; LKB, Broma, Sweden). The absorbance profile was monitored at 214 nm.

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Spectral Identification of the Peptides and Their Iodinated Derivatives

Spectra were taken with the on-line Waters 990 photodiode array detector combined to the high performance liquid chromatography system. The detection system consisted of the multichannel detector coupled to a microcomputer NEC AP III and a plotter, allowing rapid calculations of derivatives of the absorbance spectrum.

Determination of Immunoreactivity

For ANF, fractions corresponding to the major peak of radioactivity were collected and tested for immunoreactivity by a radioimmunoassay procedure reported previously (11). For AVP, immunoreactivity was evaluated on the fractions corresponding to the major peak of radioactivity by the radioimmunoassay procedure of

TABLE 1

Recovery of Iodinated Peptides from PRP-1 Cartridge after Using Different Solvent Systems and Eluting Solvent Volumes

Compound	Volume (ml)	Eluting Solvent	Recovery (%) (mean ± SEM, n=5)
ANF	0.3	ACN-TFA 0.1% (10:90)[a]	0
		ACN-TFA 0.1% (20:80)	0
		ACN-TFA 0.1% (30:70)	94.6 ± 4.3
		ACN-TFA 0.1% (40:60)	103.5 ± 2.1
	0.2	ACN-TFA 0.1% (40:60)	84.8 ± 3.0
	0.1	ACN-TFA 0.1% (40:60)	32.9 ± 3.8
AVP	0.3	ACN-TEA buffer (10:90)	0
		ACN-TEA buffer (20:80)	100.2 ± 1.6
		ACN-TEA buffer (30:70)	103.7 ± 3.2
	0.2	ACN-TEA buffer (30:70)	101.0 ± 2.2
	0.1	ACN-TEA buffer (30:70)	64.1 ± 6.2

[a] ACN, acetonitrile.

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Larose et al. (12). The immunoreactivities are expressed as % of bound radioactivity vs total radioactivity added (B/T).

RESULTS AND DISCUSSION

Pre Cleanup Procedure with PRP-1 Cartridge

The recovery of the iodinated peptides spiked in phosphate buffer using different solvent systems and eluting solvent volumes is reported in Table 1. The iodinated peptides could be recovered quantitatively

TABLE 2

Recovery of Iodinated Peptides from Sep-Pak C_{18} Cartridge after Using Different Solvent Systems and Eluting Solvent Volumes

Compound	Volume (ml)	Eluting Solvent	Recovery (%) (mean ± SEM, n=5)
ANF	4	ACN-TEA buffer (10:90)[a]	0
		ACN-TEA buffer (20:80)	1.2 ± 0.5
		ACN-TEA buffer (30:70)	55.6 ± 7.0
		ACN-TEA buffer (40:60)	72.5 ± 6.0
		ACN-TEA buffer (50:50)	75.1 ± 3.7
	3	ACN-TEA buffer (50:50)	73.2 ± 6.0
	2	ACN-TEA buffer (50:50)	64.9 ± 4.9
	1	ACN-TEA buffer (50:50)	39.4 ± 2.4
AVP	3	ACN-TEA buffer (10:90)	0
		ACN-TEA buffer (20:80)	85.4 ± 3.5
		ACN-TEA buffer (30:70)	94.9 ± 0.7
	2	ACN-TEA buffer (30:70)	92.1 ± 4.9
	1	ACN-TEA buffer (30:70)	21.9 ± 1.5

[a] ACN, acetonitrile.

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from the polymeric cartridge (PRP-1) with a minimum volume of eluting solvent (300 µl). The use of these cartridges offers some advantages over the Sep-Pak (Table 2): smaller volumes of elution solvent are required for a quantitative recovery of the tracers and the overall recoveries are better in particular for ANF. No leaking of the radioactive tracers from the PRP-1 cartridge could be observed during the washing steps with the buffers. The elution volume from the PRP-1 cartridge could be readily loaded on the HPLC

column, which reduces the risk of radioactive contamination. This pre cleanup system provides a very rapid and inexpensive method for the fast removal of excess unincorporated radioactivity as well as other possible reaction components used in the iodination process, such as chloramine T or metabisulfite (13), which could be harmful for labile peptides.

Purification of Peptides by Reverse Phase Chromatography

The radioactivity profile for ANF following the injection of the PRP-1 eluate onto the reverse phase HPLC column together with the absorbance profile following an injection of unlabeled ANF (5 μg) are shown in Figure 1. The unlabeled ANF and its monoiodinated derivative eluted at 45 and 52% acetonitrile, respectively. This separation was confirmed by the high specific activity of the tracer (1900 Ci/mmol) as determined by self-displacement according to Morris (14). The radioactivity peak eluting at 55% acetonitrile, which represents about 10% of the monoiodinated derivative, probably corresponds to the diiodotyrosyl derivative. The radioactivity and the absorbance profiles for AVP are reported in Figure 2. The high specific activity of the monoiodinated derivative (1430 Ci/mmol) was confirmed by the net separation of unlabeled AVP from its monoiodinated form which eluted at 18 and 25% acetonitrile, respectively. An insignificant amount of diiodotyrosyl derivative eluted at 29% acetonitrile.

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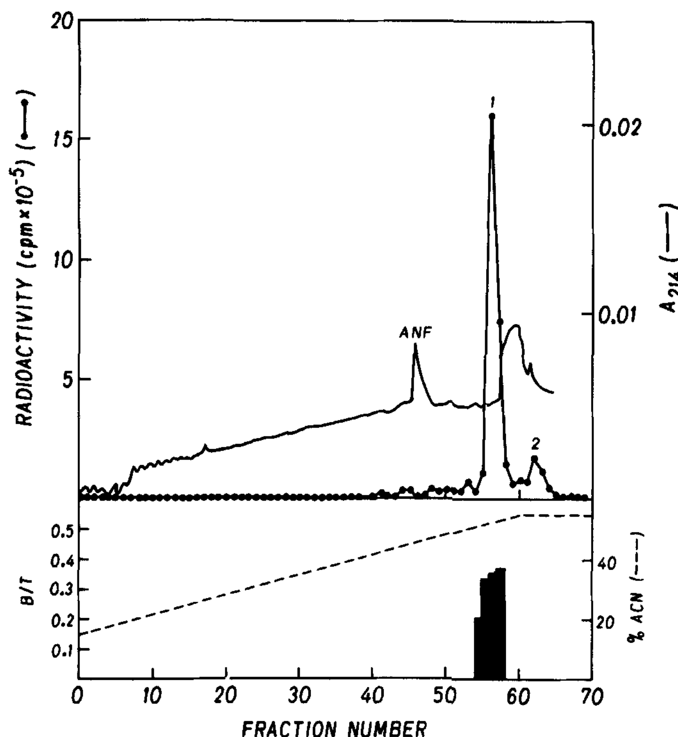

FIGURE 1: Reverse phase HPLC purification and immunoreactivity of the ANF monoiodinated derivative. Following the radioiodination procedure, the eluate from the PRP-1 cartridge was injected onto a reverse phase column (Bio-Sil ODS-5S) and eluted with a gradient of 15 to 55% acetonitrile in 0.1% TFA (dashed line). Fractions of 1 ml were collected and a 5-μl aliquot was counted for radioactivity (●—●). In parallel, the absorbance profile at 214 nm was monitored following a 5-μg injection of the unlabeled peptide (———). The immunoreactivity (B/T) of each fraction corresponding to the monoiodinated derivative peak was evaluated by a specific radioimmunoassay.

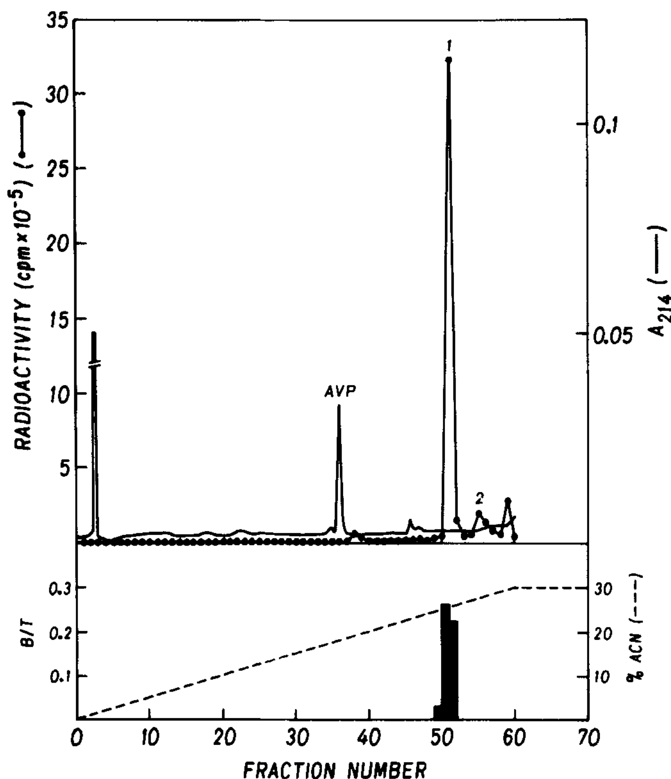

FIGURE 2: Reverse phase HPLC purification and immunoreactivity of the AVP monoiodinated derivative. Following the radioiodination procedure, the eluate from the PRP-1 cartridge was injected onto a reverse phase column (Spherisorb ODS-2) and eluted with a gradient of 0 to 30% acetonitrile in 0.02 M TEA-phosphate buffer (pH 4.0) (dashed line). Fractions of 1 ml were collected and a 5-µl aliquot was counted for radioactivity (•——•). In parallel, the absorbance profile at 214 nm was monitored following a 5-µg injection of the unlabeled peptide (——). The immunoreactivity (B/T) of each fraction corresponding to the monoiodinated derivative peak was evaluated by a specific radioimmunoassay.

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A very low background of radioactivity in both profiles could be noted, confirming the efficiency of the pre cleanup step for the removal of unincorporated radioactivity. The insignificant amount of diiodotyrosyl derivative in the profile of both peptides could be explained in part by the optimal iodination conditions with a molar ratio of peptide vs iodine higher than 2, or by the selective elution of the monoiodinated derivative from the PRP-1 cartridge. The incorporation yield of iodine into the monoiodinated derivative of both peptides was typically in the 35-40% range.

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Spectral Identification of Peptides and their Iodinated Derivatives

It is well established that spectra of tyrosine, monoiodotyrosine and diiodotyrosine show absorption at increasingly longer wavelengths respectively, when compared in either their ionized and unionized forms (15). Furthermore, multicomponent analysis of the second derivative spectrum provides a simple, rapid and accurate method for quantification of the chemical modification of aromatic residues in proteins (16). The second derivative spectra of the peaks corresponding to the elution volumes of native ANF, its monoiodinated and diiodinated derivatives were recorded in Figure 3. Effectively, a red shift for the monoiodinated and for the diiodinated ANF compared to the native form was observed, confirming the incorporation of iodine in the tyrosyl residue. A red shift for the monoiodinated and for the diiodinated AVP was also observed in Figure 4. However, the shift for the

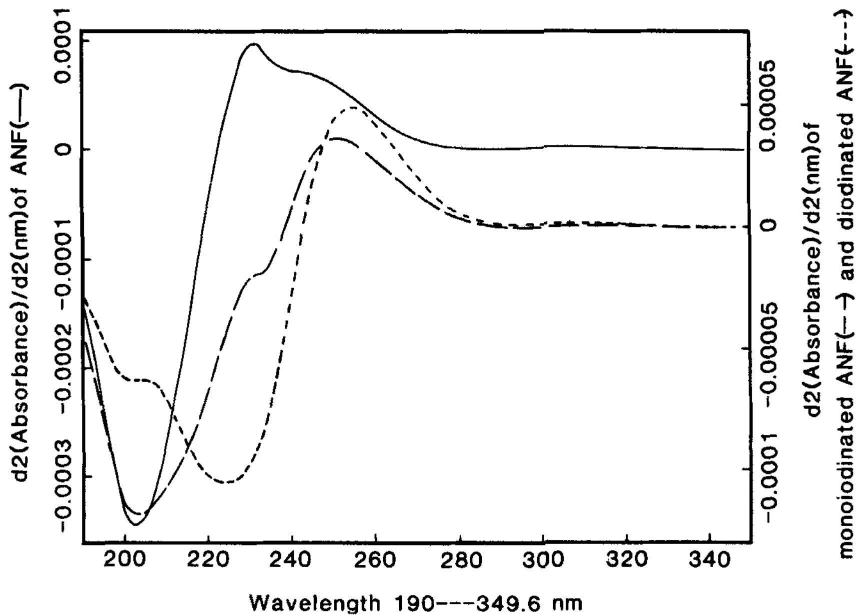

FIGURE 3: Second derivative spectra of native ANF (——), its monoiodinated (— —) and its diiodinated form (- - -).
Following the iodination procedure using non-radioactive iodine, the chromatographic separation of ANF from its iodinated derivatives was performed as described above. Spectra corresponding to the elution volume of ANF and its iodinated derivatives were recorded.

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iodinated AVP compared to its native form was less apparent.

Immunological Properties of the Tracers

The immunoreactivity of the purified tracers was confirmed by the high specific binding capacities (B/T) of the monoiodinated forms of ANF (40%) and AVP (30%), assessed by their respective radioimmunoassay procedure

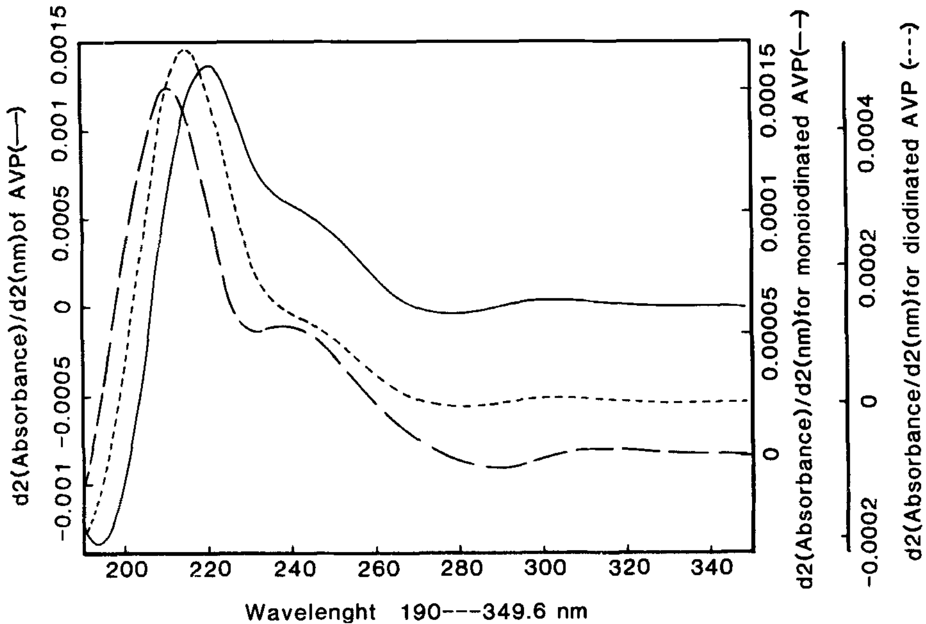

FIGURE 4: Second derivative spectra of native AVP (——), its monoiodinated (— —) and its diiodinated form (---).
Following the iodination procedure using non-radioactive iodine, the chromatographic separation of AVP from its iodinated derivatives was performed as described above. Spectra corresponding to the elution volume of AVP and its iodinated derivatives were recorded.

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(Figures 1 and 2). Nevertheless, very low specific binding to the antisera could be noted for the diiodinated derivatives of both peptides.

CONCLUSION

The major advantage of using the combination of PRP-1 cartridge and reverse phase HPLC for the purifi-

cation of radioiodinated peptides is that these cartridges provide a rapid and efficient way of removing unreacted free radioactive iodine while the resolving power of HPLC allows a rapid separation of the peptide derivatives. This methodology would be particularly useful for peptides with sensitive functional groups such as disulfide bonds present in the ANF and AVP structures. It could also be applied to peptides with methionine and tryptophane residues which are sensitive to oxidation in the iodination process. The tracers obtained following this procedure would have the high specific activity required for the sensitivity of radioimmunoassays or radioreceptor-assays.

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