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PREPARATIVE PURIFICATION OF *PLASMODIUM FALCIPARUM* CIRCUMSPOROZOITE PROTEIN SYNTHETIC POLYPEPTIDES BY DISPLACEMENT CHROMATOGRAPHY

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SUMMARY

Displacement chromatography was used for the preparative purification of a synthetic polypeptide that is a promising malaria vaccine. It was prepared by solid-phase synthesis and contains two important epitopes of circumsporozoite (CS) protein of *Plasmodium falciparum* sporozoite. With apparatus typically employed in analytical high-performance liquid chromatography (HPLC) and on a 250 × 4.6 mm I.D. reversed-phase column, up to 50 mg of crude polypeptide were purified in a single run and with a yield higher than 95%. The results demonstrate that displacement chromatography is suitable for the isolation of several milligrams of a pure polypeptide from a complex mixture that is difficult to separate even by analytical HPLC. In such a preparative application, displacement appears to be superior to elution chromatography as used traditionally.

INTRODUCTION

Numerous studies have demonstrated that reversed-phase high-performance liquid chromatography (RP-HPLC) is a powerful technique for polypeptide analysis[1]. Concomitantly, reversed-phase chromatography has been the main method used for the preparative separation of synthetic and natural peptides[2].

In approaching preparative chromatography, if the amount of injected sample is kept low, it may be possible to maintain the separation obtained on the analytical scale by using similar chromatographic conditions[3]. Neverthless, as the loading is increased, the retention times become strongly dependent on the sample concentration and the peak shapes become so distorted that the recovery may be significantly reduced[4]. Displacement chromatography has been demonstrated to be able to overcome such high loading problems, because the separation of feed components in the displacement mode takes place when the concentrations of the components are sufficiently high to be in the non-linear range of their absorption isotherms[5].

In practice displacement chromatography is carried out in the following steps. First, the column is conditioned with the carrier solution, which permits a subsequent complete absorption of the feed on the stationary phase. Second, a relatively large sample is loaded on the column. Third, a solution containing the displacer, which is

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absorbed more strongly than any of the feed components on the stationary phase, is passed through the column. During the third stage, the feed components begin to be displaced by the displacer, moving down the column, and because of their competitive absorptions they become separated in adjacent bands of pure components[5]. After each displacement run, the displacer is washed out with a suitable regenerant to permit the reuse of the columns[6].

Recently, applications of displacement chromatography in the isolation of peptide hormones obtained from extractions procedures have been reported[7–9]. The successful results prompted us to apply this technique to the purification of biologically active synthetic peptides.

RI-$(NANP)_3NA$ is a 29-amino acid residue polypeptide, whose sequence is shown in Fig. 1; it elicits a high-titre antibody response in mice and the IgGs produced react specifically with *Plasmodium falciparum* sporozoites of infected mosquitoes, which are responsible for malaria infection. This synthetic peptide, which combines two epitopes of *P. falciparum* CS protein, was designed to overcome the genetically restricted response of $(NANP)_n$ sequences[10].

H-Lys-Pro-Lys-His-Lys-Lys-Leu-Lys-Gln-Pro-Gly-Asp-Gly
1 5 10

-Asn-Pro-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro
15 20 25

-Asn-Ala-OH
29

Fig. 1. RI-$(NANP)_3NA$ sequence.

EXPERIMENTAL

Material

The RI-$(NANP)_3NA$ polypeptide was synthesized by solid-phase synthesis on a polyamide matrix[11], as reported elsewhere[12]. Benzyldimethyldodecylammonium bromide (BDMDA), trifluoroacetic acid (TFA) and triethylamine were supplied by Fluka (Buchs, Switzerland); the last two compounds were distilled prior to use. Methanol and acetonitrile (HPLC grade) were purchased from Merck (Darmstadt, F.R.G.). Water was purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.). CM-52 cellulose ion-exchange resin was obtained from Whatman (Clifton, NJ, U.S.A.).

All the chromatographic eluents were filtered on a 0.45-μm Millipore filter and degassed by purging with helium.

Apparatus and procedures

Displacement chromatography was carried out on an Aquapore RP-18 (7 μm, 300 Å) column (250 × 4.6 mm I.D.) (Brownlee Labs., Santa Clara, CA, U.S.A.), installed in an HPLC system composed of two Model 303 pumps for the carrier and displacer solution, a Model 802C manometric module, a Model 811 dynamic mixer and an Apple II-Plus gradient controller (Gilson, Villiers-le-Bel, France), a Model

7125 injector (Rheodyne, Cotati, CA, U.S.A.) with a 3-ml loop, a PU 4025 UV detector (Pye Unicam, Cambridge, U.K.) and a Model 2210 recorder (LKB, Bromma, Sweden). The column effluent was collected in 0.1-ml fractions by a Model 2070 Ultrarack II (LKB).

The apparatus for preparative elution chromatography consisted of a Model 590 programmable HPLC pump (Waters Assoc., Milford, MA, U.S.A.), an axial compression column (250 × 20 mm I.D.) (ISA Jobin Yvon, Longjumeau, France), packed with 40 g of LiChroprep RP-18 (25–40 μm; Merck), a PU 4025 UV detector (Pye Unicam) and a Model 2210 recorder (LKB). The flow-rate was 8 ml/min and the eluent, collected in 12-ml fractions by a Model 2070 Ultrarack II (LKB), was monitored at 225 nm.

HPLC of the crude material and chromatographic fractions was performed on a Vydac 201TP54 C_{18} (5 μm, 300 Å) column (250 × 4.0 mm I.D.) (Separation Group, Hesperia, CA, U.S.A.) and using an HPLC system assembled from two Model 114M pumps, a System Organizer, a Model 165 UV detector and a Model 450 Data/System Controller (Beckman, Fullerton, CA, U.S.A.) and a Model BD-41 recorder (Kipp & Zonen, Delft, The Netherlands).

The presence of the displacer in the collected fractions of the displacement runs was checked by HPLC on a LiChrosorb-DIOL column (250 × 4 mm I.D.; Merck) under isocratic conditions with 25 m*M* triethylammonium trifluoroacetate solution as eluent.

RESULTS AND DISCUSSION

The peptide mixture from the synthesis was passed through a CM-52 cellulose column and in Fig. 2 the results of ion-exchange chromatography are depicted. The

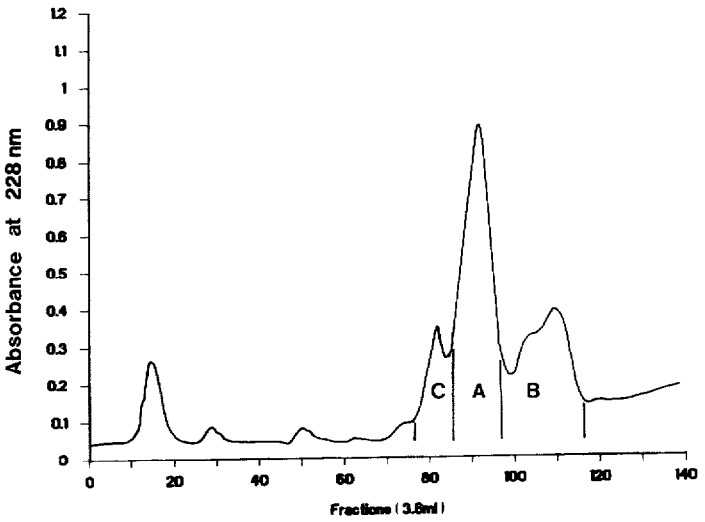

Fig. 2. Ion-exchange chromatography of RI-$(NANP)_3$NA mixture. Column, 450 × 25 mm I.D. CM-52 cellulose; eluent, linear gradient from 0.1 to 0.5 *M* ammonium acetate; pH, 6.1; flow-rate, 36 ml/h; amount of sample loaded, 400 mg of crude mixture.

three main peaks (A, B and C) were collected separately. The amino acid analyses of these fractions were very similar as well as the HPLC profiles shown in Fig. 3A–C. The fused peaks around t_R = 7.1 min (Fig. 3) represented peptide material, while the large peak at t_R = 5.5 min was a chromatographic artefact also present in blank runs.

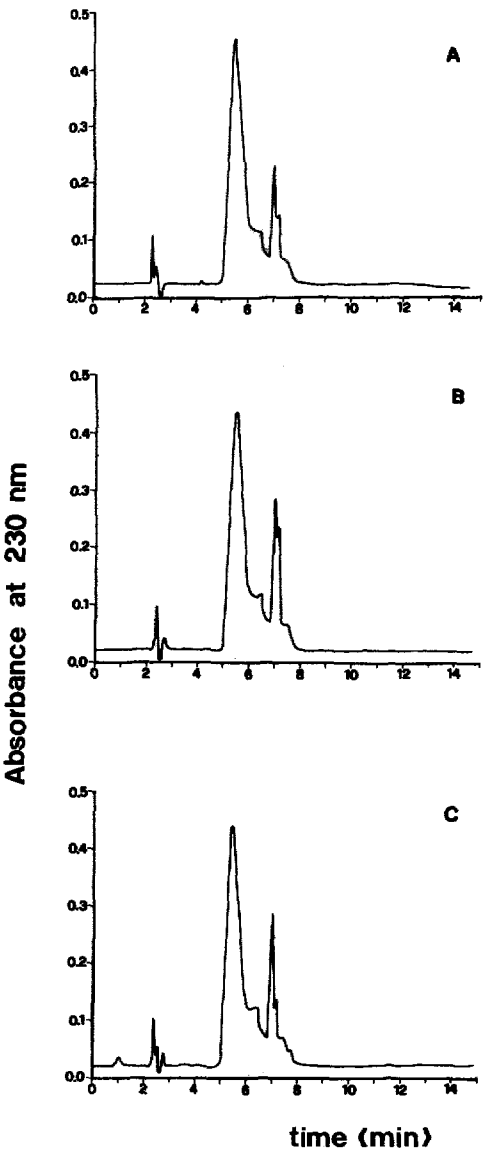

Fig. 3. Analytical chromatograms of Fractions A, B and C. Column, 5-μm Vydac 201 TP54 C_{18} (250 × 4.0
Fig. 4. Displacement chromatography of A. Column, Aquapore RP-18 (7 μm, 300 Å) (250 × 4.6 mm I.D.); carrier, 0.1% TFA; displacer, 50 m*M* BDMDA in 0.1% TFA; flow-rate, 0.1 ml/min; temperature, 23°C.

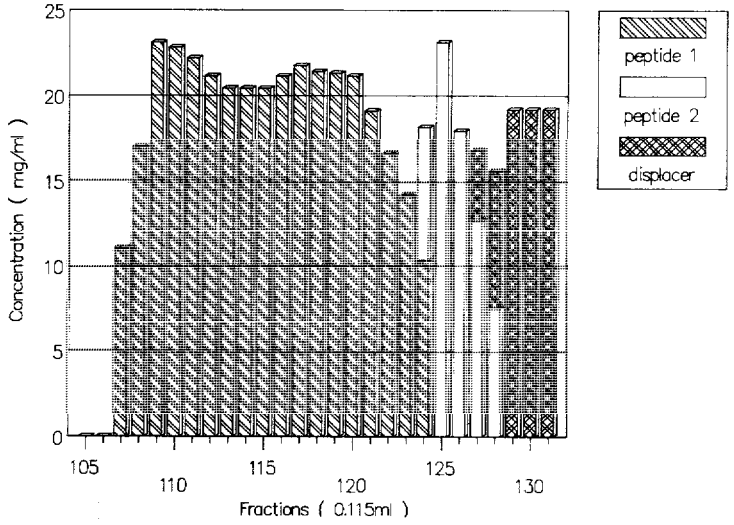

Fig. 4. Displacement chromatography of A. Column, Aquapore RP-18 (7 μm, 300 Å) (250 × 4.6 mm I.D.); carrier, 0.1% TFA; displacer, 50 m*M* BDMDA in 0.1% TFA; flow-rate, 0.1 ml/min; temperature, 23°C.

Attempts to find isocratic eluents suitable for preparative elution chromatography were unsuccessful. When 20 mg of A were loaded on a 250 × 20 mm I.D. LiChroprep RP-18 (25–40 μm) column with 0.1% TFA as the eluent containing 0.1% TFA and 2% acetonitrile they were eluted unresolved at the void volume of the column.

The purification of 45 mg of A by displacement chromatography, using BDMDA as the displacer and 0.1% TFA solution as the carrier, is shown in Fig. 4. The histogram depicted represents the quantitative RP-HPLC analysis of the collected fractions, carried out in the following manner. A 5-μl volume of each fraction was diluted with 100 μl of water and the solution was analysed under the same conditions as

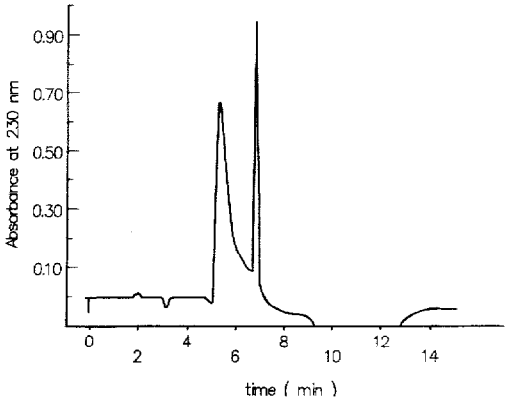

Fig. 5. Typical analytical chromatogram of a fraction containing pure peptide 1. Column, 5-μm Vydac 201 TP^{54} C_{18} (250 × 4.0 mm I.D.); eluent, 0.1% TFA with a linear gradient of acetonitrile from 0 to 20% in 15 min; flow-rate, 1.5 ml/min; sample size, 20 μl; detection, UV absorbance at 230 nm.

TABLE I

AMINO ACID ANALYSES OF PEPTIDES 1 AND 2 ISOLATED BY DISPLACEMENT CHROMATOGRAPHY

Amino acid	*Residues per mol*		
	From RI-(NANP)$_3$NA sequence	*Peptide 1*	*Peptide 2*
Aspartic acid	9	8.80	7.96
Glutamic acid	1	1.09	1.20
Proline	6	6.46	6.21
Glycine	2	1.89	2.00
Alanine	4	3.80	4.04
Leucine	1	1.09	1.00
Histidine	1	0.99	0.98
Lysine	5	4.56	4.50

in Fig. 3. A typical RP-HPLC profile of a fraction containing pure peptide 1 is shown in Fig. 5.

Fractions 107–123 were pooled and lyophilized; 34.5 mg of pure product (peptide 1) were recovered. Its retention time in RP-HPLC was 7.1 min and the amino acid analysis, reported in Table I, gave the corrected amino acid ratios of the desired peptide. A 5.4-mg amount of a product with a retention time of 7.3 min (peptide 2), was recovered from fractions 125–126. Its amino acid analysis (Table I) showed that it was a peptide missing an Asx residue relative to peptide 1. In this purification, 97% of the total amount of RI-(NANP)$_3$NA present in the crude mixture (calculated as the sum of pure collected material plus the peptide present in the zones overlapping with the impurities) was recovered in pure form.

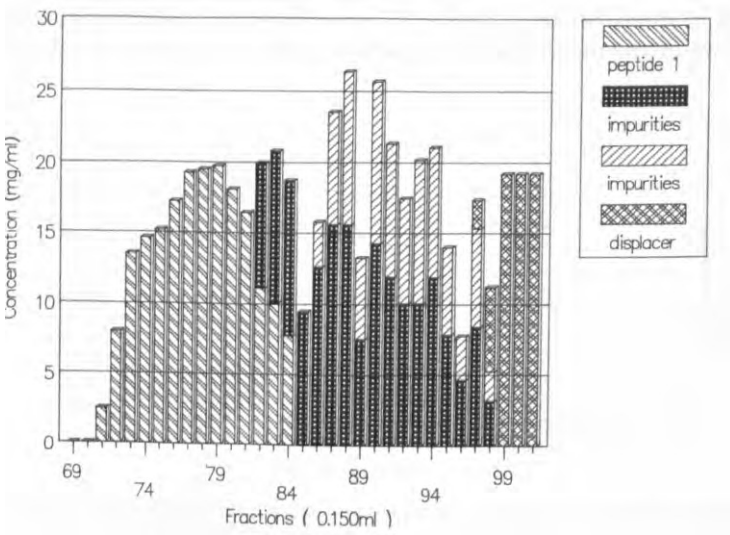

Fig. 6. Displacement chromatography of B. Conditions as in Fig. 4.

Under the same chromatographic conditions as in Fig. 4, 40 mg of B were purified and the relative histogram is shown in Fig. 6. The pooled fractions 71–81 gave 24.5 mg of peptide 1, with a yield of 85%; the impurities, which were collected as unresolved products from fractions 85–88, had retention times close to 7.3 min. Coelution analysis, carried out by injecting under the same conditions as in Fig. 3 a mixture of this fraction pool with pure peptide 2 (1:1), indicated that none of the major peaks of the pool coincided with that of peptide 2.

An increase in the amount of sample loaded to 100 mg of B caused complete saturation of all the stationary phase and part of peptide 1 started to elute at the void volume of the column as a gaussian peak, purified through a frontal chromatographic mechanism, and part as a square band typical of a displacement mechanism, during the displacer run.

In a first attempt to purify C, 60 mg of the material were loaded under the same displacement chromatographic conditions as applied to both A and B but, in contrast with the previous results the products were eluted close to the void volume rather than displaced. The low recovery of peptide material (20 mg) indicated that C was rich in salts. When this peptide material, made free from major amounts of salts in the first attempt to purify, was rerun under the previous conditions, separation of the products by displacement chromatography occurred (Fig. 7) and 2.52 mg of pure peptide 1 were obtained from fractions 92–93. The unresolved peptide impurities collected from fractions 94–98 were different from those of B, even though the retention times were very close.

After each displacement run, the column was washed with 25 ml of pure methanol at a flow-rate of 1.5 ml/min and reconditioned with the 0.1% TFA carrier solution; no reduction of the column efficiency was detected after the regeneration procedures.

The purification of synthetic peptides is a challenge, because in the course of synthesis they are frequently contaminated by very closely related by-products. An

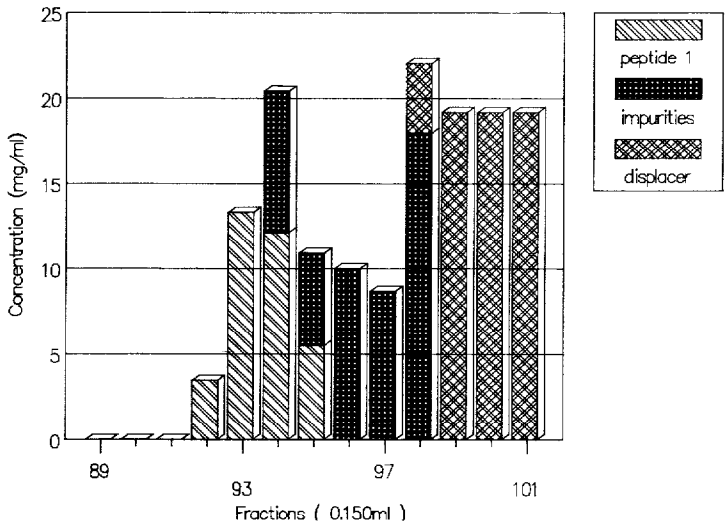

Fig. 7. Displacement chromatography of C. Conditions as in Fig. 4.

indication of this aspect can be deduced from Fig. 2, where fractions A, B and C of the RI-$(NANP)_3$NA mixture, isolated by ion-exchange chromatography, show similar amino acid analyses and HPLC profiles, and from Fig. 3A–C, where the retention time of the desired peptide is so close to those of the impurities that it is impossible to separate them efficiently by reversed-phase preparative elution chromatography. In spite of this, the reported purifications (Figs. 4, 6 and 7) by displacement chromatography clearly demonstrate the potential of this technique in preparative liquid chromatography.

The choice of BDMDA as the displacer was based on the consideration that, as the peptide products were separated in gradient runs always starting from 0% of organic solvent, a mixed hydrophobic–hydrophilic interaction is probably responsible for the retention of these products. Under such conditions, for a compound to be a proper displacer it should be absorbed on the stationary phase more strongly than any feed components in both hydrophobic and hydrophillic interactions, reacting with the C_{18} chains and with the free silanol groups still present in the reversed stationary phase. Tetraalkyl halides with long alkyl chains have these properties. Indeed, it has been reported that they work as hydrophobic displacers of peptides in the reversed-phase mode[7–9] and as hydrophilic displacers of polar compounds in the normal-phase mode[13].

CONCLUSION

The high level of purity required for biopharmaceutical products has substantial ly increased the importace of preparative liquid chromatography in purification and isolation processes. In this respect, the purification of synthetic peptides, which are important therapeutically, has received particular emphasis. The remarkable properties of displacement chromatography compared with the elution mode, namely a high ratio of the amount of sample loaded to the amount of stationary phase (20 mg per gram of stationary phase), the reduced volume of eluent per milligram of purified material (1 ml/mg), the collection of concentrated pure peptide (20 mg/ml) and the use of the same equipment and columns employed in HPLC, make this technique a powerful tool for the purification of peptides at levels of several milligrams, which are usually obtained in solid-phase synthesis.

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