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Large-scale purification of the synthetic peptide fragment 163–171 of human interleukin-β by multi-dimensional displacement chromatogaphy[a]

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ABSTRACT

Multi-dimensional chromatography has been used successfully in the displacement mode for the purification of the synthetic peptide H–Val–Gln–Gly–Glu–Glu–Ser–Asn–Asp–Lys–OH, the fragment 163–171 of human interleukin-β. This peptide can mimic several of the *in vivo* and *in vitro* immunostimulatory activities of the entire protein, except for the inflammatory effect. A large-scale procedure has been developed to purify the synthetic peptide by reversed-phase (RP) and ion-exchange (IE) displacement chromatography (DC) in a single run without any pretreatment. Masses from 100 mg to about 35 g of the unpurified compounds synthesized by a solid-phase technique on a Merrifield-type resin and obtained by acidolytic cleavage from the solid support, can be purified in this way. In the RP-DC mode the carrier and the displacer were aqueous solutions of 0.1% trifluoroacetic acid and 50 m*M* benzyltributylammonium chloride, respectively, whereas in the IE-DC mode the carrier was water and the displacer 50 m*M* ammonium citrate solution. RP-DC and IE-DC were also performed in series by directing the effluent of the RP column onto the IE column. Peptide purities and recoveries greater than 96 and 90%, respectively, were obtained.

INTRODUCTION

Of the various biologically active substances used in drug treatments, peptides are acquiring increasing therapeutic relevance. As peptides usually show a strong effect at low doses, they need to be synthesized in the gram to kilogram range. The mild conditions required for their purification make preparative liquid chromatography the method of choice for the development of efficient process technologies [1].

Synthetic peptides obtained through homogeneous or solid-phase procedures are frequently contaminated with closely related impurities resulting from unavoidable side-reactions, such as sequence deletions, racemization, the incomplete removal of the side-chain protecting groups and the multiple addition of amino acid residues. The target peptide and most of the peptide contaminants are difficult to separate as a

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result of their chemical and structural similarities and purification to chemical homogeneity is often a very demanding task.

Generally, in preparative liquid chromatography, once the purity levels are fixed, most effort is addressed to increase the volume of production by increasing the loading capacity of the stationary phases. It has recently been shown that to maximize the throughput, the stationary phases should be considerably overloaded [2]. As column overloading favours displacement effects, displacement chromatography (DC) can be considered as a suitable purification process in the large-scale production of synthetic peptides [3].

The reported applications of DC in the peptide field have been restricted to purifications in the range of hundreds of milligrams [4–7]. This paper reports a study of the potential of this technique for the purification of the crude synthetic peptide interleukin-1-β (IL-1β)-(163–171) on the multi-gram scale. The peptide samples were used in the DC runs without prior manipulation so that they contained, in addition to the peptide impurities, salts and derivatives produced during the deblocking and the final acidolytic removal of the peptide from the polystyrene resin.

In addition to maintaining the ability of the natural protein to activate T-cell functions without inducing an inflammatory response [8], the peptide IL-1β(163–171) has been shown to act as an adjuvant of IL-1β for both the T-helper-dependent and T-helper-independent immune response [9]. Therefore, the suggested use of this peptide as an adjuvant in vaccine formulations adds further significance to the preparative purification, as large amounts of this peptide will be required for future clinical trials.

EXPERIMENTAL

Materials

Benzyltributylammonium chloride (BTBA) and trifluoroactic acid (TFA) were supplied by Fluka (Buchs, Switzerland). Methanol and acetonitrile [high-performance liquid chromatography (HPLC) grade] were purchased from Merck (Darmstadt, Germany). Diammonium hydrogencitrate, ammonium chloride and aqueous ammonium hydroxide were provided by Carlo Erba (Milan, Italy). Triammonium citrate was obtained by neutralizing the diammonium hydrogencitrate to pH 7 with ammonium hydroxide.

Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA). The aqueous eluents were filtered through a 0.45-μm cellulose acetate filter and the organic eluents through a 0.5-μm PTFE filter; all the eluents were degassed with helium prior to use.

Apparatus

The analytical chromatograph was assembled from two Model 114 pumps, a System Organizer, a Model 165 UV detector and a Model 450 data/system controller (Beckman, CA, USA) and a Model BD-41 recorder (Kipp & Zonen, Delft, Netherlands). A flow diagram of a typical displacement chromatograph has been reported previously [3].

The DC runs in reversed-phase (RP) mode were carried out on a 250 × 4 mm I.D. LiChrosorb RP-18 column (10 μm, Merck) in the milligram range. The HPLC

equipment consisted of a Model M-45 pump (Waters Assoc., Milford, MA, USA) with the reservoirs of carrier, displacer and regenerant solutions connected via a four-way valve, a Model 7010 injector (Rheodyne, CA, USA) with a 10-ml loop, a PU-4025 UV detector (Pye Unicam, Cambridge, UK) to monitor the displacement chromatogram at 260 nm and a Model 2210 recorder (LKB, Bromma, Sweden). The column effluent was collected with a Model 2070 Ultrarack II fraction collector (LKB).

The RP-DC separations were carried out in the gram range on axial compression columns of 20, 40 and 80 mm I.D. (Jobin Yvon, Longjiumeau, France), packed with LiChroprep RP-18 (25–40 μm) (Merck). The pump was a Model 590 programmable HPLC pump equipped with a four-solvent selecting valve (Waters Assoc.). The effluents, monitored by a Model PU 4025 UV detector at 260 nm, were collected with a Model 2211 Superrac fraction collector (LKB).

The 4, 20, 40 and 80 mm I.D. columns were equilibrated with the carrier for 20 min at flow-rates of 0.4, 8, 32 and 128 ml/min, respectively. The same flow-rates were used to regenerate the stationary phase with methanol after the displacement runs. Taking into account the equilibration, elution and regeneration steps, the total chromatographic time for the displacement runs was about 260 min. The presence of the displacer agent in the fractions was monitored by thin-layer chromatography or spectrophotometry [10]. The displacement chromatographic run in the ion exchange (IE) mode was carried out in the milligram range on a Mono-Q 50 × 5 mm I.D. column (10 μm) with a fast protein liquid chromatography (FPLC) system equipped with three P-500 pumps and a Model Frac 100 fraction collector (Pharmacia, Uppsala, Sweden). The IE-DC on the gram scale was performed on a 350 × 10 mm I.D. column packed with Q-Sepharose Fast Flow (45–164 μm, Pharmacia). The chromatographic apparatus consisted of Model 590 pump with the four-solvent selecting valve (Waters Assoc.), a Model UV-M detector at 214 nm and a Model Rec 481 recorder (Pharmacia).

Analytical IE chromatography was performed to analyse some collected fractions and to detect the presence of the IE-DC displacer using a FPLC system with a Mono Q column.

The multi-dimensional displacement chromatographic run was monitored online by dedicated equipment as described previously [11]; a 33 × 4.6 mm I.D. Supelcosil LC-18-DB column (3 μm, Supelchem, Milan, Italy) was used under isocratic conditions with 0.1% TFA in acetonitrile–water (4:96) as the eluent.

Fast atom bombardament mass spectrometry (FAB-MS) was carried out in the Analytical Department of ENIRICERCHE (Monterotondo, Rome, Italy).

RESULTS

The analytical chromatogram of the crude mixture after the acidic cleavage of the peptide from the resin is shown in Fig. 1. Compounds of peptidic nature consitute about 50% (w/w) of the total material.

The peak at the retention time (t_R) 3.29 min corresponds to the target peptide IL-1β(163–171), whereas that at t_R 4.14 min is the peptide impurity with the glutamine residue replaced by the glutamic acid residue, (Gln)IL-1β(163–171), most probably arising from the acid-catalysed hydrolysis of the Gln side-chain carboxyamide

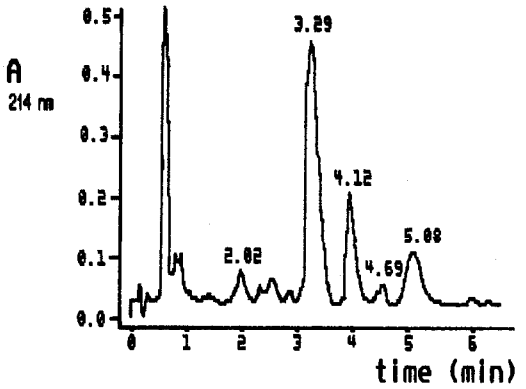
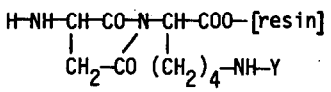


Fig. 1. RP-HPLC of crude synthetic mixture of IL-1β(163–171). Sample: 40 μg of mixture in 20 μl. Eluents: A, 0.1% TFA; B, 0.1% TFA in acetonitrile–water (60:40). Gradient: 3–6% B in 3 min, 6–3% B in 2 min, 3–3% B in 2 min. Flow-rate, 1.5 ml/min; column, 33 × 4.6 mm I.D. LC-18 DB Supelcosil.

during the peptide assembly on the solid support. The peak at t_R 4.96 min is an aminosuccinimide derivative originated from the ring closure of the aspartyl residue during the acylation step of the C-terminal lysine α-NH_2:



The identities of the target peptide and both peptide impurities have been assessed by FAB-MS.

The peak at t_R 2.02 min has been tentatively attributed to the Asp–Lys-β isopeptide: H–Val–Gln–Gly–Glu–Glu–Ser–Asn–NH–CH(CH_2–CO–Lys–OH)–COOH, as its area increased on treatment with 1 *M* sodium hydroxide solution with the concomitant reduction of the peak at t_R 4.96 min.

The peak at t_R 5.08 min was associated with an impurity, the identity of which has not been determined and which co-eluted with the target peptide in an analytical IE-FPLC separation carried out on a 50 × 5.0 mm I.D. Mono-Q column with a gradient from 5 to 100 m*M* of disodium hydrogenphosphate at pH 7.0 in 20 min.

The optimum conditions for a convenient purification procedure for IL-1β (163–171) in RP-DC wer first tested on a small scale using a 250 × 4.0 mm I.D. LiChrosorb RP-18 column.

The concentration of the mixture to be fed onto the column was determined in a preliminary study and it was found that the complete adsorption of the material onto the stationary phase, an essential step for the displacement mechanism, did not occur at concentrations greater than 10 mg/ml. The RP-DC was carried out by loading 100 mg of the untreated crude mixture onto the column using 0.1% TFA as the carrier and 50 m*M* BTBA as the displacer. The results obtained are shown in Fig. 2, where the histogram is constructed on the basis of the RP-HPLC analysis of the collected fractions.

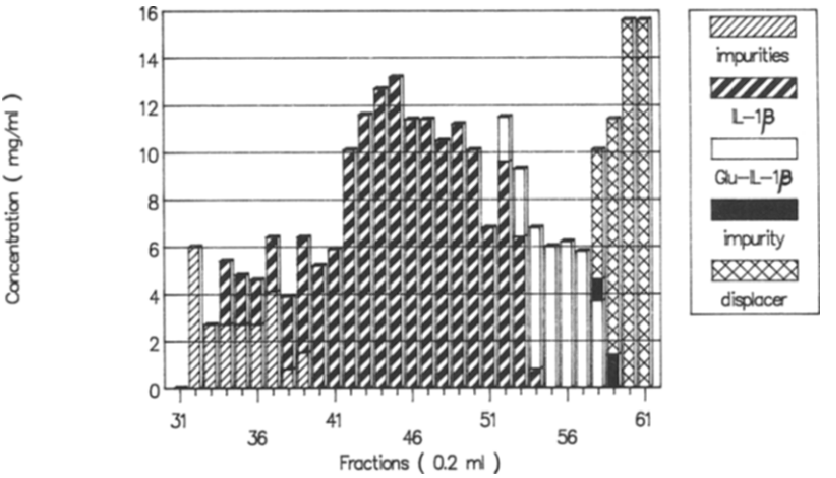


Fig. 2. Displacement histogram of RP-DC. Flow-rate, 0.1 ml/min; column 250 × 4 mm I.D. LiChrosorb RP-18; 98 mg of the mixture; pooled fractions, 40–51.

Multi-gram purification was obtained by adopting a scaling-up procedure similar to that described for the large-scale purification of the cysteine derivative Fmoc–Cys(Trt)–OH [11]. The 4.0 mm I.D. LiChrosorb RP-18 column used in the preliminary experiments was replaced in turn by 20, 40, and 80 mm I.D. LiChroprep columns, while the linear velocity of the sample and displacer solution (0.017 cm/min), the sample concentration (10 mg/ml) and the ratio of the loaded sample per gram of stationary phase (45 mg/g) were kept constant.

The results are summarized in Table I and the relative histograms are shown in Figs. 3–5. The purity levels of the desired peptide were greater than 95% in the pooled fractions collected.

The regeneration of the stationary phase after each displacement run was achieved with 2 ml of methanol per gram of stationary phase. As the relatively low yield was a result of the overlap between the bands of the target peptide and the impurity (Glu)IL-1β(163–171), which has a different net charge with respect to IL-1β (163–171), an attempt was made to improve the resolution of the bands by operating in the IE-DC mode. The run was carried out on a small scale by loading 50 mg of the

TABLE I

EFFECT OF SCALING-UP PROCEDURE ON PURIFICATION OF THE PEPTIDE

Column size (mm)	Stationary phase (g)	Loaded sample (g)	Yield[a] (%)	Peptide recovery (g)
250 × 4	1.8	0.098	78.2	0.024
260 × 20	45	2.0	78.8	0.55
260 × 40	200	9.0	81.8	2.4
260 × 80	800	35.5	78.2	8.1

[a] Calculated as collected peptide in the pure form over the total amount present in the crude mixture.

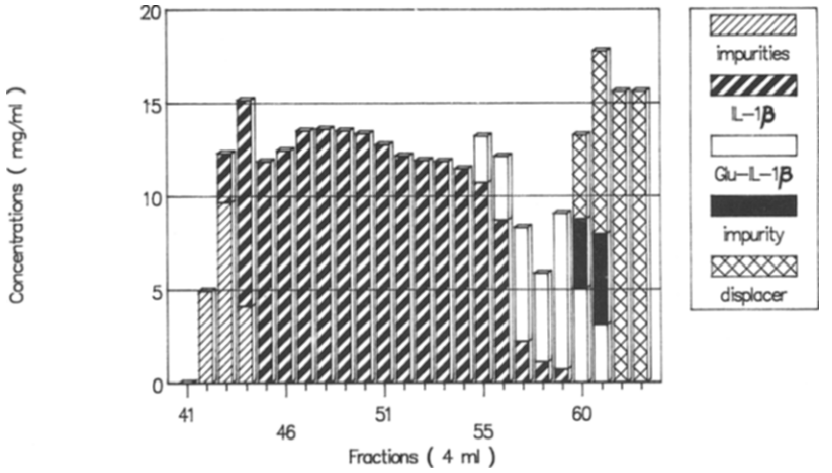


Fig. 3. Displacement histogram of RP-DC. Flow-rate, 2 ml/min; column, 260 × 20 mm I.D. LiChroprep RP-18; 2.0 g of the mixture; pooled fractions, 90–110.

material onto a 50 × 5.0 mm I.D. Mono Q column, using water (pH 7.0) as the carrier and 50 m*M* ammoniun citrate as the displacer.

The displacement separation gave a reduced overlap zone, less than 5% of the IL-1β(163–171) band, between the two eluted bands. However, as expected from the results of the analytical IE-FPLC experiments, the impurity appearing at t_R 5.08 min in Fig. 1 was present in all the fractions containing IL-1β(163–171).

However, as this impurity can easily be removed in RP-DC, it was decided to combine the advantages of both the DC modes by carrying out the purification with two columns connected in series, the first operating in the RP-DC mode and the

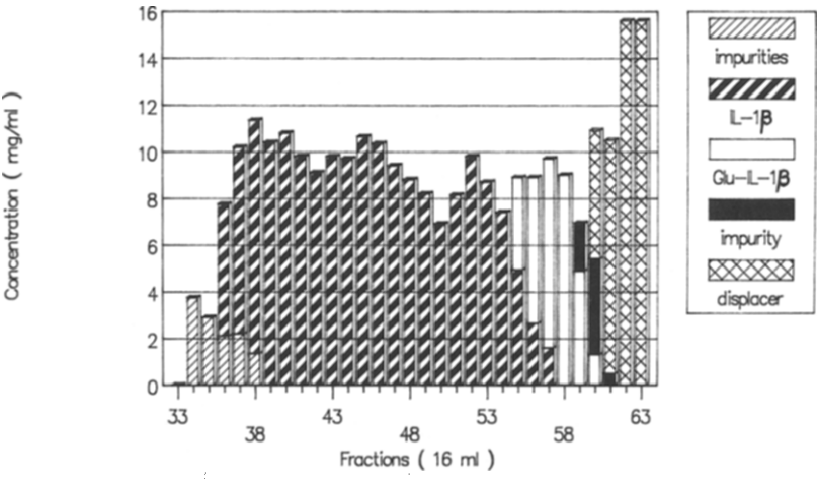


Fig. 4. Displacement histogram of RP-DC. Flow-rate, 8 ml/min; column, 260 × 40 mm I.D. LiChroprep RP-18; 9 g of the mixture; pooled fractions, 74–108.

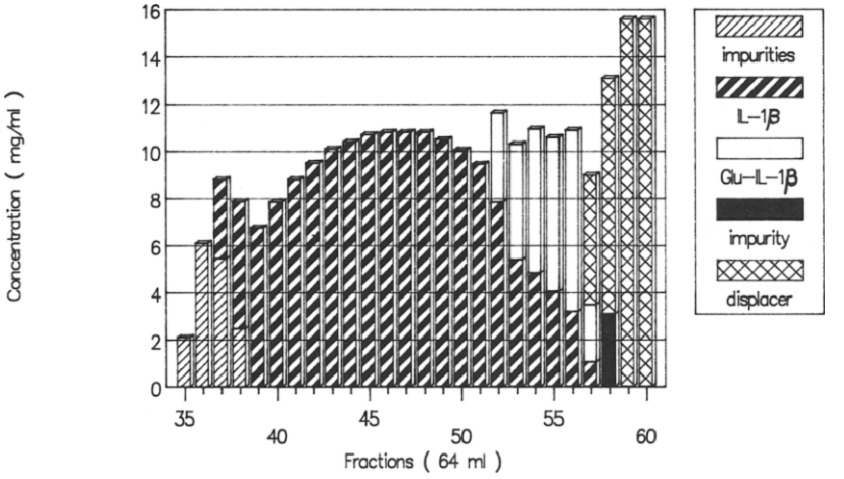


Fig. 5. Displacement histogram of RP-DC. Flow-rate, 32 ml/min; column, 260 × 80 mm I.D. LiChroprep RP-18; 35.5 g of the mixture; pooled fractions, 77–103.

second in the IE-DC mode (Fig. 6). The effluent of the 260 × 20 mm I.D. LiChroprep RP-18 column, monitored on-line, was directed, through valve 1 onto the 350 × 10 mm I.D. Q Sepharose FF column just as IL-1β(163–171) left the column. Immediately before reaching the top of the IE column, the pH of the effluent was changed from

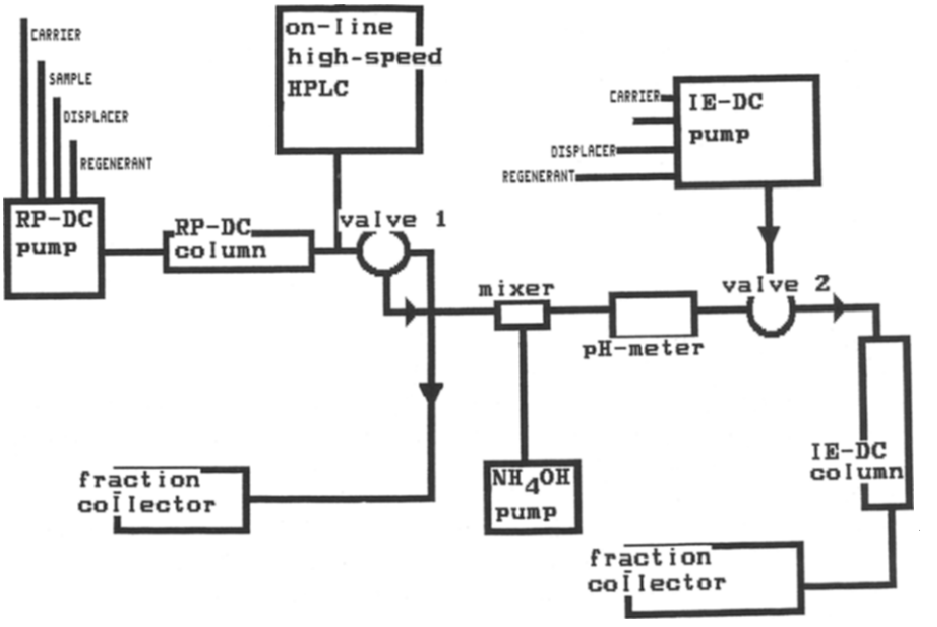


Fig. 6. Schematic diagram of chromatographic apparatus used for RP-DC and IE-DC in series.

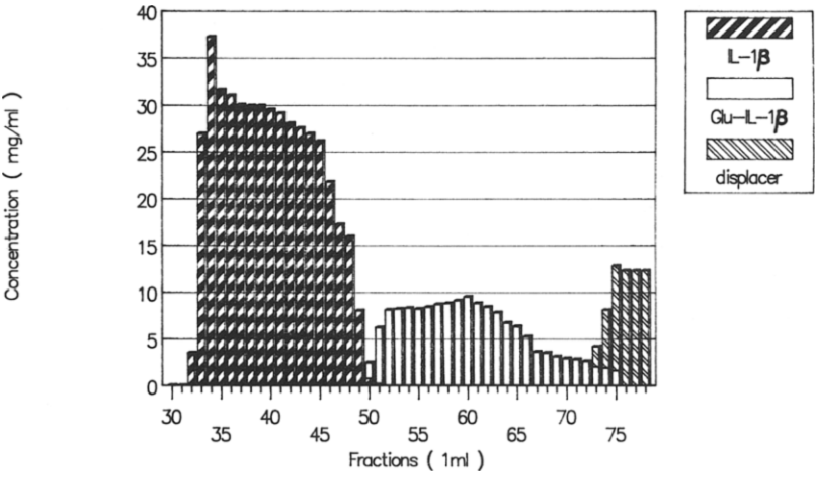


Fig. 7. Displacement histogram of IE-DC in the technique using RP- and IE-DC in series. Conditions of RP-DC as in Fig. 3. IE-DC conditions: flow-rate, 0.5 ml/min; column, 350 × 10 mm I.D. Q Sepharose Fast Flow; pooled fractions, 32–49.

3.0 to 7.0 to ensure that a complete adsorption of all the compounds onto the ion-exchange stationary phase occurred.

After the disappearance of the target peptide in the effluent and before the appearance of the unknown impurity at t_R 5.08 min, loading to the IE column was stopped and the stationary phase was conditioned with the carrier. The displacer solution was then pumped through the IE column at a linear velocity of 0.01 cm/s.

The resulting displacement histogram is given in Fig. 7. The chromatographic yield increased to over 90% and the purity of the IL-1β(163–171) recovered by lio-phylization was greater than 98%.

DISCUSSION

A straightforward scaling-up procedure has been developed for the purification of 100 mg to 35.5 g of the peptide IL-1β(163–171) using DC by simply widening the column diameters using the same conditions as in the small-scale separation of the target peptide. This result suggests that the purification of even larger amounts of crude peptide preparations could be easily achieved using DC.

Compared to the previously reported RP-HPLC purification of the same peptide in the elution mode [12], twenty times more crude material can be loaded per gram of stationary phase and a purer peptide obtained (>95% *versus* >90%) with fourteen times less solvent.

In the RP-DC experiments, 1.3 ml of eluents, included the regenerant solvent, were consumed per milligram of purified peptide. Using RP-DC and IE-DC in series, higher purity levels (>98%) and recovery (>90%) have been achieved. As reported previously stationary phases with a larger particle diameter and lower column costs do not reduce the separation efficiency of the experiments at the gram scale [11].

It is often difficult to find a suitable displacer for the DC runs; however, these

data confirm that the tetraalkylammonium halides represent a class of modulated hydrophobicity compounds that can be used advantageously for the purification of a large number of peptides in RP-DC [4–7, 13]. It is less difficult to find an IE-DC displacer as, theoretically, any compound with a net charge higher than that of the compound to be purified could be a potential displacer [14].

As far as the oligopeptides are concerned, the search for a displacer is facilitated further as conformational effects are less important or even negligible with respect to proteins. Therefore, a compound with a net charge one unit higher than that of the target compound can be used. This concept, previously applied with success to the purification of the retro-inverso tetrapeptide analogue of tuftsin, was effective for the purification of IL-1β(163–171) [15]. Triammonium citrate, an inexpensive salt with a net charge of −3 at pH 7.0 was used to displace IL-1β(163–171) (net charge −2 at the same pH). The high charge density of the citrate can perhaps also explain the displacement of the peptide impurity (Glu)IL-1β(163–171), which has the same net charge as the displacer.

It seems pertinent in this discussion to point out the remarkably mild conditions of the IE-DC experiments. Using water as the carrier and 50 m*M* ammonium citrate as the displacer, the desired product and some of the related impurities are collected in water and recovered simply by lyophylization, with clear advantages in terms of ease and the cost of product recovery.

CONCLUSIONS

As large amounts of the synthetic peptide IL-1β(163–171) are used for biological and clinical studies in this institute, DC has been adopted as a technique which is easy to scale-up, has an efficient use of the stationary phases and equipments and uses a reduced volume of eluent.

About 35.0 g of crude IL-1β(163–171), prepared by solid-phase synthesis on a Merrifield-type resin and used as such after acidic cleavage from the solid support, have been efficiently purified in a single chromatographic DC run.

In the RP mode, the superiority of DC compared to the classical preparative RP-HPLC was demonstrated by the purity of peptide obtained (>95%), the chromatographic yield (>80%), the load capacity (45 mg/g of stationary phase) and the considerably reduced consumption of solvents.

In the IE mode, DC exhibited a remarkably convenient feature, namely, the use of low concentrations (50 m*M*) of the inexpensive salt ammonium citrate as the eluent, the collection of highly concentrated fractions (up to 30 mg/ml) in water and the simple final recovery of pure IL-1β(163–171) by lyophylization.

The feasibility of using DC in series in the RP and IE modes has been demonstrated, with a evident improvement both in the purity (>98%) and total chromatographic recovery (>90%) of the target peptide.

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