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Multi-step purification strategy for RANTES wild-type and mutated analogues expressed in a baculovirus system

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Abstract

RANTES (regulated on activation, normal T cell expressed and secreted), a C-C chemokine, is one of the major HIV-suppressive factors produced by $CD8^+$ T cells. Wild-type RANTES and genetically modified analogues were expressed in a baculovirus system and purified from cell culture supernatants employing a multi-step strategy based on affinity and RP-HPLC. Quantification and purity control of the final proteins were carried out by capillary electrophoresis using the synthetic or the recombinant wild-type RANTES as a reference. The procedure here reported requires only three days to obtain 0.016–0.270 mg of the pure and characterised proteins, starting from 370–900 ml of culture media, and is suitable for the analysis of a large number of RANTES analogues

Keywords: RANTES mutagenesis; Purification; Baculovirus; Chemokines

1. Introduction

Chemokines (chemotactic cytokines) constitute a large superfamily of proteins active in the physiological and pathological trafficking of hematopoietic cells: its members are able to induce chemotaxis and activation of leukocytes [1,2]. RANTES (regulated on activation, normal T cell expressed and secreted), a member of the C-C chemokine family (in which in the N-terminal conserved two cysteines, in position 10 and 11, are contiguous) is produced by different cells including $CD8^+$ T cells, and is responsible for chemotaxis of monocytes, lymphocytes and eosinophils [3,4]. Moreover, RANTES may play an important role in the generation of inflammatory infiltrates [5] and in the mechanism of eosinophil recruitment from mucous membranes. Recent investigations demonstrated that RANTES is also a potent inhibitor of infection with human immunodeficiency virus (HIV), being able to compete with the virus for interaction with CCR5 receptor [6–9]. Many studies have been focused on the production of genetically mutated RANTES analogues maintaining its antiviral effect, but with reduced proinflammatory activity [10,11]. With this aim, three mutated analogues of the wild-type molecule R1($S^1 \rightarrow C$, $S^5 \rightarrow C$), R5 ($S^1 \rightarrow C$) and R23 ($R^{17} \rightarrow E$), were expressed employing a baculovirus system in *Trichoplusia ni* insect cells.

The aim of our study was to set up a multi-step procedure for purification of RANTES wild-type (WT) and genetically mutated analogues. With respect to other methods [12,13], our strategy aimed at

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reducing the time of execution and obtaining proteins with a higher recovery rate and purity grade. Ion-exchange chromatography or preparative reversed-phase high-performance liquid chromatography (RP-HPLC), frequently used as intermediate steps [12,14], were replaced with a simple centrifugal ultrafiltration step. In addition to the enzyme-linked immunosorbent assay (ELISA) quantification, the purified proteins were analysed by capillary electrophoresis (CE) in order to characterise their purity.

2. Experimental

2.1. Chemicals

All the chemicals and solvents employed were of analytical grade. Disodium hydrogen orthophosphate anhydrous (Na_2HPO_4), sodium chloride (NaCl) and acetonitrile (CH_3CN) were all from BDH (Poole, UK). Trifluoracetic acid (TFA) was from Fluka (Buchs, Switzerland) and orthophosphoric acid (H_3PO_4) from Merck (Darmstadt, Germany).

2.2. Standard proteins

Chemically synthesised human RANTES was kindly donated by Dr. Corradin (Dictagene, Lausanne, Switzerland). Human recombinant RANTES expressed in *E. coli* by recombinant DNA technology was donated by Dr. Proudfoot (Serono, Geneva, Switzerland). The lyophilised products were resuspended in water to a final concentration of 100 μg/ml, and aliquots were stored at −20°C.

2.3. Production of mutated RANTES

RANTES complementary DNA (cDNA), encompassing its natural signal peptide sequence, was derived by polymerase chain reaction (PCR) amplification from reverse-transcribed total RNA extracted from in vitro-activated primary human T cells. The different mutations were introduced into the wild-type gene by overlap-extension techniques. Insect cells (High Five) derived from *Trichoplusia ni* (Invitrogen, San Diego, CA, USA), cultured in serum-free medium, were infected at a multiplicity of infection (MOI) of 10; culture supernatants were collected 72 h later [15].

2.4. Purification strategy

The purification strategy required the same HPLC apparatus for affinity and RP chromatography. The HPLC system was a Beckman System Gold (Beckman Instruments, Palo Alto, CA, USA) assembled with two dual-piston pumps (Model 126) and a variable double-beam UV detector (Model 168). The whole apparatus was computer controlled with the System Gold Software for storage and handling of data.

Cell supernatants from WT and RANTES analogues (R1, R5 and R23) were loaded via the HPLC pump to an HiTrap Heparin affinity column (5 ml, 34 μm particle size, 3 mg antithrombin III/ml gel binding capacity) (Pharmacia, Uppsala, Sweden) previously conditioned with 10 mmol l^{-1} Na_2HPO_4, pH 7.3 (10 ml). To avoid backpressure problems, the column was directly connected to the outlet check valve. Sample reservoirs and the column were kept on ice to avoid protein denaturation and the sample was allowed to recirculate two times. After a wash with 25 ml of 10 mmol l^{-1} Na_2HPO_4, pH 7.3, elution of the sample was carried out with a linear NaCl gradient (166 min) from 100% of 0.4 mol l^{-1} to 100% of 2 mol l^{-1} in 10 mmol l^{-1} Na_2HPO_4, pH 7.3 at a flow-rate of 0.3 ml/min. Temporised fractions (3 ml) were collected during the elution and absorbance was monitored at 280 nm.

Fractions identified positive by ELISA (R&D System, Minneapolis, MI, USA) were pooled (9–27 ml) and ultrafiltered (Centricon-3, 3000 M_r cut-off, Amicon, Beverly, MA, USA) for 5 h at 3000 *g*. The retentate (0.69–3.6 ml) was injected (200 μl at a time) in RP-HPLC column (Vydac C4 214TP, 25 cm×4.0 mm, 5 μm, 30 nm) (Vydac, Hesperia, CA, USA) and eluted with a gradient of CH_3CN in 0.1% aqueous TFA at 1 ml/min (0–100% CH_3CN in 60 min). Analysis was monitored at 215 nm and RANTES peak was collected, dried under vacuum (Savant Instruments, Hicksville, NY, USA) and resuspended in water for CE and ELISA quantification.

2.5. CE quantification

The analysis was carried out employing a P/ACE 5010 equipped with UV detector and computer controlled by the System Gold Software (Beckman). A coated capillary (CElect P150, Supelco, Bellefonte, PA, USA) [27 cm (20 cm to the detector)×50 μm I.D.×363 μm O.D.] and 150 mmol l^{-1} H_3PO_4, pH 2.5 as running buffer, were employed. The samples were injected in pressure mode ($3.4 \cdot 10^{-3}$ MPa) for 5–10 s (9.8–19.5 nl). A voltage of +9 kV was applied and the UV detector set at 214 nm.

Quantification of purified RANTES was referred to the synthetic standard RANTES and to the RANTES obtained from recombinant DNA. The mean response factors (peak area corrected for the t_M/ng), obtained from 10 injections of the two standards, were 1.267±0.05 and 1.269±0.2, respectively (not significantly different). Standard solutions were injected three times a day and the mean area corrected for the t_M/ng factor used to quantify RANTES purified from baculovirus.

2.6. Mass spectrometry

Mass spectrometric analysis was executed with a mass spectrometry matrix assisted laser desorption ionisation time-of-flight (MS–MALDI-TOF) Voyager DE system (PerSeptive Biosystems, Framingham, MA, USA). Light from a nitrogen laser (337 nm) was focused on to the sample target with an extraction voltage of 20 kV. Desorbed ions were accelerated to 25 kV and drifted down a 1.2 m effective pathlength flight tube. The laser desorption matrix was an α-cyano-4-hydroxicinnamic acid (Sigma, St. Louis, MO, USA), the mass analyser operated in scan mode and signals from multiple (256) laser shots were summed at a repetition rate of 3 Hz.

2.7. Quantification of total proteins

Total protein amount was determined by the method of Bradford [16] in aliquots drawn from cell supernatants, ELISA-positive fractions eluted from the Heparin column, retentate from ultrafiltration and fractions collected from RP-HPLC. Following the standard procedure of Bio-Rad Protein Assay (Bio-Rad, Munich, Germany), a dye-binding assay, samples were read at 595 nm at the end-point reaction.

2.8. Biological activity

The biological activity of purified RANTES WT and analogues was assessed by the intracellular Ca^{2+} mobilisation test, using CCR5-transfected U87 cells and by chemotaxis of primary human monocytes according to standard protocols [17].

2.9. Statistical analysis

The Student *t*-test was employed to evaluate the differences between ELISA and CE quantification: $P \leq 0.05$ was considered significantly different.

3. Results

The procedure here reported was initially applied to the purification of RANTES WT. Fig. 1 shows the chromatographic profiles related to each step of this purification process. Fractions collected from heparin (90–130 min) and positive ELISA, were resolved from the important peak corresponding to all the non-specific proteins contained in the culture media (0–70 min) (Fig. 1A). Fig. 1B shows the RP-HPLC analysis of RANTES (t_R=22.19±0.06, RSD=0.27%, n=18) after ultrafiltration.

Fig. 1C shows the CE analysis of the protein collected from RP-HPLC: only one peak was observed (t_M=3.842±0.045, RSD=1.17%, n=5) which was perfectly superimposed to the peak of RANTES recombinant standard. A further confirmation of the efficacy of the purification strategy was obtained by mass spectrometric analysis on the final product, resulting in a single peak at m/z 7849 (Fig. 1D), being the theoretical RANTES WT m/z 7847.

The purification yield after each step of the procedure was assessed as ratio between RANTES, quantified by RANTES-specific ELISA, and total protein content, by the dye-binding method of Bradford. The results obtained are summarised in Table 1. The first step by Heparin column increased the RANTES WT purity from 0.13%, in the cell supernatant (900 ml), to 14.5% in the fractions collected. After RP-HPLC the total protein recovered corres-

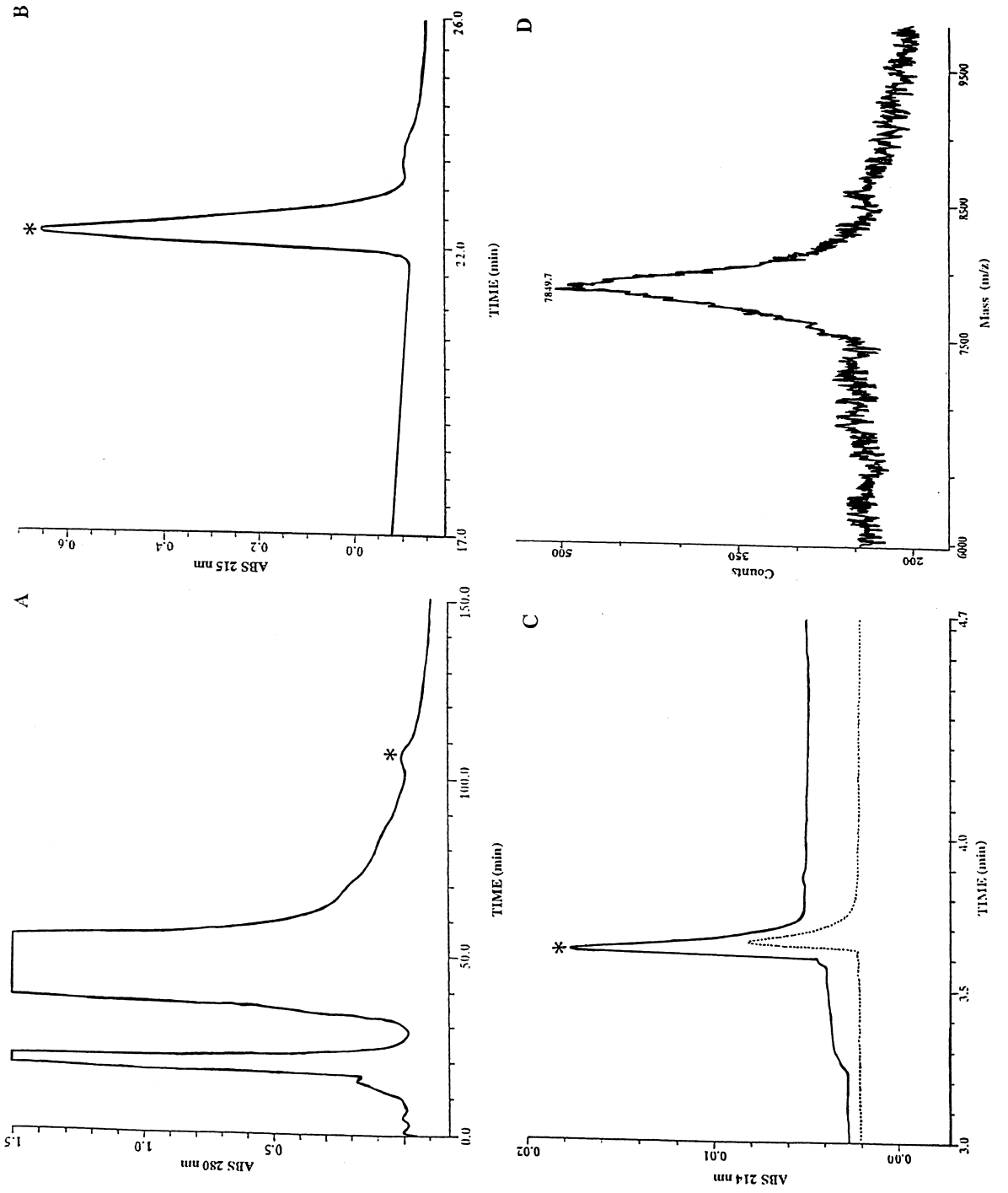
A
ABS 280 nm
0.0
0.5
1.0
1.5
50.0
100.0
150.0
TIME (min)
*
B
ABS 215 nm
0.0
0.2
0.4
0.6
17.0
22.0
26.0
TIME (min)
*
C
ABS 214 nm
0.00
0.01
0.02
3.0
3.5
4.0
4.7
TIME (min)
*
D
Counts
200
350
500
7849.7
6000
7500
8500
9500
Mass (m/z)

Table 1
RANTES purification strategy from baculovirus

	Step	Volume (ml)	RANTES (ELISA) (mg)	Protein amount[a] (mg)	Purity by ELISA[b] (%)	Purification fold	Yield (%)	RANTES (CE) (mg)	Purity by CE[c] (%)
WT	Supernatant	900	0.77	612	0.13	1	100		
	Heparin	12	0.45	3.1	14.5	111	58		
	Ultrafiltration	3.6	0.33	1.7	19.4	149	43		
	RP-HPLC	0.75	0.27	0.27	100	769	35	0.270	100
R1	Supernatant	400	0.060	270	0.022	1	100		
	Heparin	12	0.046	0.78	5.89	267	77		
	Ultrafiltration	0.69	0.038	0.345	11.0	500	63		
	RP-HPLC	0.2	0.031	0.046	67	3045	52	0.023	50
R5	Supernatant	600	0.15	554	0.020	1	100		
	Heparin	27	0.092	1.2	8.0	400	61		
	Ultrafiltration	1.68	0.036	0.39	9.2	460	24		
	RP-HPLC	0.1	0.026	0.046	57	2850	17	0.018	39
R23	Supernatant	370	0.074	310	0.023	1	100		
	Heparin	9	0.045	1.57	2.9	125	61		
	Ultrafiltration	0.98	0.019	0.4	4.8	208	25		
	RP-HPLC	0.1	0.016	0.039	41	1782	22	0.019	48

[a] Total protein amount was quantified by a dye-binding assay (Bio-Rad Protein assay).
[b] Purity was assessed as a ratio between ELISA quantification and total proteins amount by the dye-binding method.
[c] Purity was assessed as a ratio between CE quantification and total proteins amount by the dye-binding method.

ponded only to WT full-length RANTES. Moreover, as expected, the amounts of RANTES quantified by ELISA and CE were consistent. Recovery of RANTES WT, evaluated step-by-step by ELISA, showed that the greatest loss occurred in the first heparin step (42%) while in the other steps, the loss accounted for about 10% of the total.

The same purification protocol was applied to the mutated RANTES R1, R5 and R23. Fig. 2 shows the chromatograms related to R1 purification: the RP-HPLC chromatogram (Fig. 2A) showed the presence of two unresolved peaks which were collected together and later appeared well separated in the CE electropherogram (Fig. 2B). We compared the CE profile of R1 to that of the purified WT (Fig. 2B, dotted line), which corresponded to the major peak of the electrophoretic pattern ($t_M = 3.695 \pm 0.039$ min, RSD = 1.05%, $n = 5$). MS analysis of this sample also showed two peaks, the major one with the theoretical m/z value of full-length R1 (m/z 7866), and the minor (m/z 7652) supposed to be a truncated form lacking the two amino acids at the N-terminus (S-P) (Fig. 2C). To confirm which of the CE peak corresponded to the protein of interest, another set of experiments was carried out on R40 ($I^{15} \rightarrow A$) displaying the same behaviour of R1. Again, R40 showed, by CE, two well defined peaks: the second one had the same t_M of WT and corresponded, in the MS analysis, to the theoretical MW of full-length RANTES. The two unresolved peaks by RP-HPLC

Fig. 1. Profiles related to RANTES WT purification. (A) Heparin affinity chromatography. RANTES WT (*) was eluted by a NaCl gradient from the HiTrap Heparin column and the ELISA-positive fractions corresponded to the 90–130 min time range. (B) RP-HPLC analysis. Retentate from ultrafiltration was injected into a C_4 Vydac column eluted with a CH_3CN gradient and RANTES WT peak (*) at t_R 22.19 min was collected. (C) CE analysis. RANTES WT (*, continuous line) was analysed in a coated capillary (CElect P150) employing 150 mmol l^{-1} H_3PO_4 as running buffer at +9 kV. The peak (t_M 3.842 min) was perfectly superimposable to that of the standard RANTES obtained from chemical synthesis or recombinant DNA technology (dotted line) analysed in the same conditions. (D) MS analysis. The RANTES WT spectrum was obtained under the conditions reported in Section 2. The theoretical RANTES WT m/z was 7847.

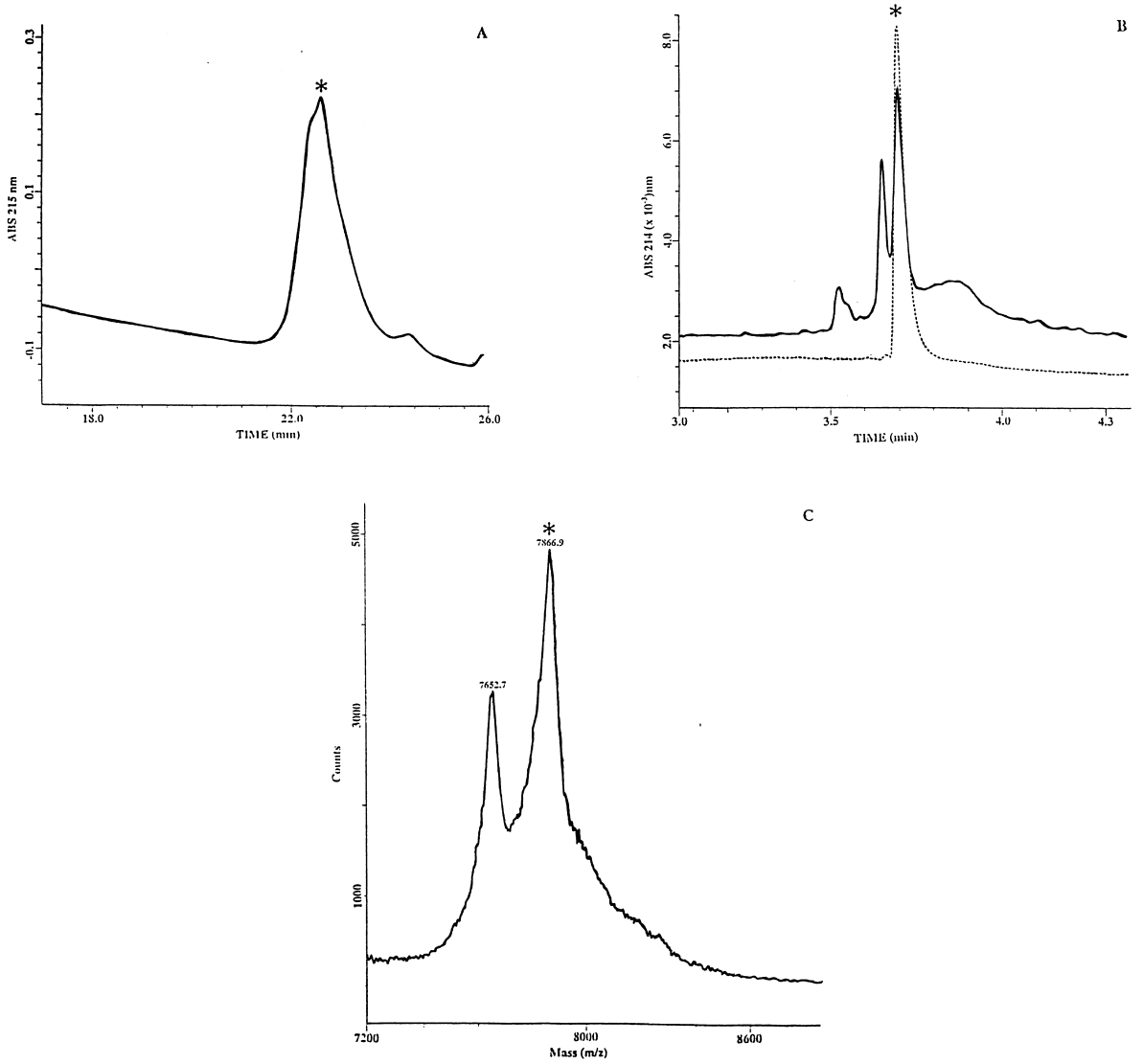

Fig. 2. Profiles related to R1 purification. (A) RP-HPLC analysis. RANTES collected from affinity chromatography and ultrafiltration was injected into the C_4 column and eluted with a CH_3CN gradient. The profile shows two unresolved peaks having t_R 22.3 and 22.7min, respectively. (B) CE analysis. The electropherogram (continuous line) shows three resolved peaks and the major one (*) (t_M 3.695 min) was perfectly superimposable to that of the purified RANTES WT (dotted line). (C) MS analysis. Mass spectrum shows the presence of the expected R1 peak (m/z 7866) together with a peak (m/z 7652) corresponding to a truncated form lacking in two amino acids (S-P) at the N-terminus.

were collected separately by slowing the RP-HPLC gradient (15–45% CH_3CN in 120 min). In the second peak with t_R=22.7 min, MS analysis confirmed the presence of the full-length mutated protein, while CE on this fraction showed only one peak corresponding to the second one of the original R40 CE profile (data not shown).

The RP-HPLC and CE profiles of the other two

mutated analogues (R5 and R23) were similar to the R1 and R40 ones, showing a major peak corresponding to RANTES, together with other minor peaks of related proteins. As seen for R1, quantification of RANTES peak was done by comparison with the synthetic or recombinant WT protein and positive identification was confirmed, also in these cases, by MS analysis (data not shown).

Table 1 reports the results related to R1, R5 and R23 purification at the end of the whole procedure. The three mutated proteins were recovered with a purity of 67%, 57% and 41% for R1, R5 and R23 respectively. The recovery of R1 after each purification step was comparable to that of the WT, with a final yield of 52%. R5 and R23, instead, showed a lower final recovery (17 and 22%, respectively) with a major loss of protein during the ultrafiltration step (about 40%). This finding was probably due to the higher protein concentration in the original culture media of R23 and R5 (0.84 and 0.92 mg/ml, respectively) compared to that of the WT and R1, which could have slowed down the ultrafiltration step. Moreover, while for RANTES WT ELISA and CE results were in agreement, values obtained by the two methods for mutated analogues were significantly different ($P<0.001$). For R1 and R5, the concentration measured by CE was lower than by ELISA due to the presence of the truncated form which was probably revealed by the immunoassay. On the contrary, the R23 quantification by ELISA was underestimated with respect to the CE one probably due to ELISA inadequacy in detecting RANTES analogues mutated at particular sites (from amino acid positions 14 to 17). As a consequence, the final protein purity calculated on the basis of RANTES CE, taking into account only the peak corresponding to the t_M of synthetic RANTES, was lower than the purity calculated on the basis of ELISA for R1 and R5, but was higher in the case of R23 due to the underestimation by ELISA (Table 1).

The biological activity of RANTES WT after purification was comparable to that of synthetic RANTES. All the three mutants showed variable levels of functional impairment in a calcium mobilisation assay using U87-CCR5 cells. In a monocyte chemotaxis assay, R5 and R23 were less active than the WT, while R1 was equally effective.

4. Discussion

The aim of this project was to set up a rapid and inexpensive purification strategy for RANTES able to yield a good purity grade and recovery rate. The whole procedure should have been suitable for the purification of different kinds of genetically mutated RANTES. Based on these goals, we set up a three-step strategy that proved to be cost-effective (the same HPLC apparatus for affinity and RP chromatography), time-saving (three days starting from culture medium to purified protein) and suitable for the purification of RANTES analogues. Evaluation of the final protein amount was carried out also by CE, a more suitable method for quantification of mutated analogues with respect to the immuno assays commonly used and specifically built for wild-type RANTES assessment.

The first observation is that our procedure gave a better recovery of RANTES WT and R1 (35 and 52%, respectively) with respect to a previously described one [14]. Moreover, even if the R5 and R23 purifications lead to low final yields (17 and 22%, respectively), these values were still better than those obtained by the previously published procedure [14]. We have hypothesised that the presence of high protein concentration in the culture media of these mutated analogues could have slowed down the ultrafiltration step, increasing the possibility of a non-specific bond of RANTES to the filter membrane or to the reservoir walls, decreasing its concentration in the retentate. To improve the recovery in purification, it might be useful to standardise the proteins contents in the culture medium.

The second interesting result was the good correspondence between ELISA and CE in the quantification of RANTES WT, which gave a further validation of the purification procedure and a confirmation of CE accuracy. By contrast, the significant differences observed in the analysis of the mutated proteins may be due to the inadequacy of ELISA to reveal molecules bearing mutations at particular sites. This observation was generally confirmed by CE quantification of RANTES proteins mutated at different positions (data not shown). On these proteins the ELISA assay always underestimated the concentration with respect to CE. We suppose that

this might be due to mutations affecting the sites recognised by the antibodies of the immuno assay. These findings emphasise the need for a combined use of different quantitative assays when dealing with mutagenized proteins.

The last important observation, further underlining the importance of CE coupled to other analytical techniques used for protein purification, was the high similarity of the electropherograms of R1 and R5 with their mass spectrometric traces, additionally confirmed by MS analysis of the peaks collected separately by HPLC.

In conclusion, the three-step purification procedure reported here proved to be advantageous in terms of final recovery and protein purity, allowing an accurate quantification and a complete characterisation of the final products also in the presence of mutagenized molecular forms.

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