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Development of a routine analysis method for liposome encapsulated recombinant interleukin-2

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Abstract

This paper describes the development of an isocratic reversed-phase high-performance liquid chromatographic method for the routine analysis of recombinant interleukin-2 (rIL-2) in liposome samples. The chromatographic system employed a C_4 column maintained at 30°C eluted with 52.5% (w/w) acetonitrile in water, containing 100 m*M* $NaClO_4$ and 10 m*M* $HClO_4$. To remove phospholipid interference the chromatographic method was combined with a lipid-extraction procedure. No significant loss of rIL-2 was noted upon inclusion of this extraction step. The protein eluted from the column with a capacity factor (k') of 5.8. The method was validated for robustness, linearity, precision and reproducibility. It was shown that the method was linear over a sample concentration range of 1–100 μg/ml. Upon assessment of the intra-day and inter-day precision, the relative standard deviations (RSD) were within the range of the methodical error (approximately 5%), except at the lower concentration of 10 μg/ml, where the intra-day RSD was relatively high (17.8%). The recovery of rIL-2 upon liposome preparation and subsequent analysis of the samples was in the range 94±9%. The results indicate that the method is suitable for routine quantitation of rIL-2 in liposomal samples. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

In the last two decades, several cytokines have attracted considerable research attention for use in anticancer immunotherapy. Interleukin-2 (IL-2), in particular, has been investigated extensively because of its immune-stimulating properties, which may be employed to enhance the immune response against tumour cells. The production of recombinant interleukin-2 (rIL-2) cleared the way for large-scale application, boosting the research in this field. Unfortunately, the results obtained so far are disappointing: systemic administration of rIL-2 elicits severe toxicity, while the antitumour efficacy remains limited [1,2]. Alternative routes of administration (subcutaneous injection or constant infusion) reduce the intensity of the therapy-associated toxicity, but do not increase the antitumour efficacy of rIL-2 [3]. Administration of low doses of rIL-2 directly into the tumour was shown to induce antitumour activity in preclinical and clinical experiments [4–13].

Liposomes have been employed as delivery sys-

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tems for rIL-2 in systemic as well as in locoregional rIL-2 immunotherapy. As liposomes may function as a depot releasing entrapped rIL-2 over a prolonged period of time, they may offer a means to enhance the therapeutic efficacy of rIL-2. Enhancement of the therapeutic efficacy of rIL-2 by liposomal delivery has been well established in several animal tumour models [14–20]. Another area where rIL-2 liposomes have been employed is as an adjuvant in anticancer vaccination. Liposomal rIL-2 was shown to be significantly more potent than the free cytokine in several anticancer vaccination studies [21–23].

For the quantitation of cytokines a number of methods have been employed, including absorbance and fluorescence measurements, chemical reactions yielding coloured or fluorescent end-products [24,25], bioassays [26], enzyme-linked immunosorbant assays (ELISA) [27] and several chromatographic methods [28,29]. Some of these methods (chemical assays, absorbance and fluorescence measurements) have the drawback of being non-specific, thus being unsuitable for determining the amount of a specific protein in a protein mixture. Protein-specific methods such as ELISA or bioassays have a high sensitivity but usually a low reproducibility, and are expensive and/or laborious, thus not allowing a routine sample screening in a normal laboratory setting. Finally, most of the available techniques do not provide pharmaceutically relevant information on the presence of degradation products such as oxidised or reduced species.

In this paper we focus on the development of an isocratic high-performance liquid chromatography (HPLC) method for the analysis of rIL-2 in liposome samples. Chromatography of rIL-2 using the HPLC technique may yield information on the presence of rIL-2 degradation products and offer detection of rIL-2 in protein mixtures, with the prerequisite that the proteins have different retention times. Additionally, HPLC offers high accuracy and reproducibility. A drawback of HPLC analysis of rIL-2 is that the method is relatively insensitive compared to, for example, bioassay or ELISA. HPLC methods for analysis of rIL-2 employing gradient elution from a C_4 reversed-phase column have already been described [29–31]. However, isocratic elution HPLC offers some advantages compared to gradient elution HPLC in terms of the simplicity of the required equipment and the ease of automation of the system. To estimate the elution properties of rIL-2 from the column, rIL-2 was first chromatographed using a gradient system. Thereafter, this system was converted to an isocratic setting.

Considering the detection wavelength and the physico-chemical properties of phospholipids it could be expected that phospholipids interfere in the HPLC method, preventing the analysis of rIL-2 in liposome samples. Therefore, the chromatographic method was combined with an extraction step for removing phospholipids from liposome samples. The procedure was validated for precision, selectivity, linearity within the required detection range, and robustness. The detection limits were not investigated as the samples were expected to have concentrations in the range of approximately 2.5–100 μg/ml.

2. Materials and methods

2.1. Chemicals

Proleukin® (des-alanyl, 125-ser recombinant interleukin-2, rIL-2) was kindly donated by Chiron (Amsterdam, The Netherlands). Reconstitution of the freeze-dried protein was performed according to the manufacturer's instructions, resulting in a 1 mg/ml rIL-2 solution. Egg-phosphatidylcholine (EPC), egg-phosphatidylglycerol (EPG), disteaorylphosphatidylcholine (DSPC) and dipalmitoylphosphatidylglycerol (DPPG) were gifts from Lipoid (Ludwigshafen, Germany). Cholesterol was obtained from Sigma (St. Louis, MO, USA). Methanol p.a., chloroform p.a., $NaClO_4$, $HClO_4$ and glucose were obtained from Merck (Darmstadt, Germany). Acetonitrile (gradient quality) was obtained from Biosolve (Barneveld, The Netherlands). All other reagents used were of analytical grade. Throughout the experiments water purified by the reversed-osmosis technique was used.

2.2. Liposome preparation

Liposomes were prepared by the film method. Liposomes were composed of EPC and EPG (9:1 molar ratio), or DSPC, DPPG and cholesterol

(10:1:10 molar ratio). The total lipid concentration was typically 40 m*M*. A lipid film was prepared by dissolving the appropriate amounts of lipids in chloroform and methanol and subsequently evaporating the solvent under reduced pressure using a rotary evaporator. The film was kept under a nitrogen atmosphere for at least 15 min and hydrated with water, a 5% glucose solution or 100 μg/ml rIL-2 in 5% glucose. In the case of gel-state liposomes (DSPC–DPPG–cholesterol, 10:1:10 molar ratio) the temperature was raised to 60°C during the hydration process to facilitate liposome formation. After the lipid film was hydrated with a solution containing rIL-2, the non-entrapped rIL-2 was removed by ultracentrifugation as described elsewhere [14].

2.3. Lipid extraction

As phospholipids interfere in the chromatographic analysis, a phospholipid extraction was performed according to the method of Bligh and Dyer [32]. Liposomes were disrupted by addition of 225 μl methanol and 125 μl chloroform to a 100 μl sample. After mixing, phase separation was achieved by addition of 125 μl 0.1 *M* HCl and 125 μl chloroform. The samples were thoroughly mixed and centrifuged at 7500*g* for 5 min. Thereafter, the upper water–methanol layer, containing the protein, was collected and analysed by HPLC.

2.4. Gradient HPLC system

The HPLC configuration included two Model 510 pumps controlled by an automated gradient controller, and a U6K injection device (all from Waters Associates, Milford, MA, USA). The detection was performed at 205 nm with a Model 783A UV detector (Applied Biosystems, NJ, USA) coupled to a Kipp DB 40 recorder (Kipp and Sons, Delft, The Netherlands). A Phenomenex W-Porex RP4 column (150×4.6 mm) was used with 5 μm particle size and 300 Å pore size (Phenomenex, Torrance, CA, USA). The mobile phases consisted of 40 and 70% (w/w) acetonitrile in water, respectively, containing 100 m*M* $NaClO_4$ and 10 m*M* $HClO_4$. The mobile phase was degassed by ultrasonication and pumped at a flow-rate of 1 ml/min. Upon injection a linear gradient of 40–70% acetonitrile was developed in 10 min.

2.5. Isocratic HPLC system

The chromatographic system consisted of a Spectroflow 400 solvent delivery system (Applied Biosystems), a Wisp 710B autosampler (Waters Associates), and a 783A UV detector operating at 205 nm (Applied Biosystems). Data were collected using a SP4000 integrator (Spectra Physics, Fremont, CA, USA) with the program Winner on Windows (Thermo Separation Products, Riviera Beach, FL, USA). Samples were applied in an injection volume of 100 μl to a Phenomenex W-Porex C_4 column maintained at 30°C in a water bath. The mobile phase consisted of 52.5% (w/w) acetonitrile in water, containing 100 m*M* $NaClO_4$ and 10 m*M* $HClO_4$, and was pumped at a flow-rate of 1 ml/min. With this isocratic system recombinant IL-2 eluted from the column with a retention time of approximately 8.5 min.

2.6. Calibration curve

A calibration curve was constructed by admixing aliquots of rIL-2 with empty liposomes (i.e. devoid of rIL-2, EPC–EPG molar ratio, 9:1) to achieve final rIL-2 concentrations between 1 and 100 μg/ml. At each concentration, two independent samples were analysed in duplicate after extraction of the phospholipids. Peak areas were determined and plotted versus the amount of rIL-2 injected. The data were fitted using linear regression.

2.7. Validation

The effect of the *column temperature* on the rIL-2 retention was investigated by heating the column in a water bath. The capacity factor (k') was calculated using $k' = (t_r - t_m)/t_m$, where t_r represents the rIL-2 retention time and t_m the void volume time (typically 1.8 min). To investigate whether the isocratic method could separate rIL-2 *degradation products* from the native protein, oxidised and reduced rIL-2 were analysed. Oxidation of rIL-2 methionine residues was performed by incubation of 100 μg/ml rIL-2 with 0.5% H_2O_2 in 0.1 *M* HCl for 5 or 25 min at

room temperature [33]. Reduction of the single disulphide bridge of the rIL-2 molecule was performed by incubation of 100 μg/ml rIL-2 with 20 m*M* DTT for 20 min at room temperature [29]. Native, oxidised and reduced rIL-2 were applied in the isocratic RP-HPLC system.

To investigate whether *loss of rIL-2* occurred upon lipid extraction of liposome samples, the peak area was plotted versus the injected amount of rIL-2 for both non-liposomal (extracted) and liposomal standards (non-extracted). The data were fitted with linear regression and the equations compared.

To assess the *linearity*, calibration curves were constructed and fitted by linear regression.

For evaluation of the *precision*, standards containing 10, 50 or 100 μg/ml were applied to the chromatographic system six consecutive times on one day, or six times over a period of 11 days. The mean peak concentration and relative standard deviation (RSD) were calculated.

To investigate the *reproducibility*, several curves constructed by two operators were compared.

The *recovery* of rIL-2 was evaluated after preparation of rIL-2 liposomes and removal of non-entrapped rIL-2 by ultracentrifugating twice and collecting the fluid containing non-liposomal rIL-2. The resulting fractions (liposomes, washing fluid 1 and 2) were analysed using isocratic HPLC and the recovered amount of rIL-2 was calculated and expressed as a percentage of the amount of rIL-2 initially added during the film hydration step in the liposome preparation procedure.

3. Results and discussion

The aim of these experiments was to develop an isocratic HPLC analysis method for rIL-2 in liposome samples. Several papers have already focused on the analysis of rIL-2 via RP-HPLC [29–31]. However, in these experiments the protein was eluted from the column by development of an acetonitrile gradient. For practical reasons, gradient elution is not the method of choice for analysis of large series of samples. The isocratic HPLC method developed here had to comply with the following demands: (1) easily automated for the routine analysis of large quantities of samples; (2) capable of analysing samples containing rIL-2 in the concentration range 5–100 μg/ml; (3) capable of analysing the rIL-2 content of samples containing liposomes, without interference of phospholipids.

3.1. Development of an isocratic HPLC method

Under the experimental conditions described, gradient elution of rIL-2 applied on a reversed-phase C_4 column caused elution of the protein at an acetonitrile concentration of 63%. This system could be adapted to an isocratic setting by fixing the concentration of acetonitrile to 52.5%. The column was heated to 30°C in a water bath to avoid changes in retention time by variation of the environmental temperature. This system was further validated and used for analysis of rIL-2.

3.2. Validation of the isocratic HPLC method

The isocratic system for analysis of rIL-2 was validated. Firstly, the *influence of temperature* on the rIL-2 retention was investigated. The rIL-2 retention time was highly affected by alteration of the column temperature. The data were fitted with a linear regression, obtaining the equation $k' = -0.24(\pm 0.004)T - 13(\pm 0.1)$, where k' is the capacity factor and T the temperature in degrees Celsius. The correlation coefficient was 0.99. When stabilising the column temperature in a water-bath at 30°C, the capacity factor (k') of rIL-2 was constant.

Oxidation of methionine residues to methionine sulfoxides is among the first processes occurring in the degradation of most proteins [34]. Detection of rIL-2 degradation products is therefore pharmaceutically relevant, although the oxidation of the four methionine residues of rIL-2 does not seem to alter the protein's bioactivity [35]. Reduction of the rIL-2 disulphide bridge, however, completely abolishes the bioactivity [36]. The *separation capability* of the method was investigated by inducing oxidation or reduction of the protein. Fig. 1A shows a chromatogram of native rIL-2 (peak A). Recombinant IL-2 was oxidised with hydrogen peroxide in an acidic medium, a method proven to oxidise methionine residues exclusively [33,35]. After 25 min of incubation several peaks with a shorter retention time than native rIL-2 were observed (Fig. 1B, peaks B and

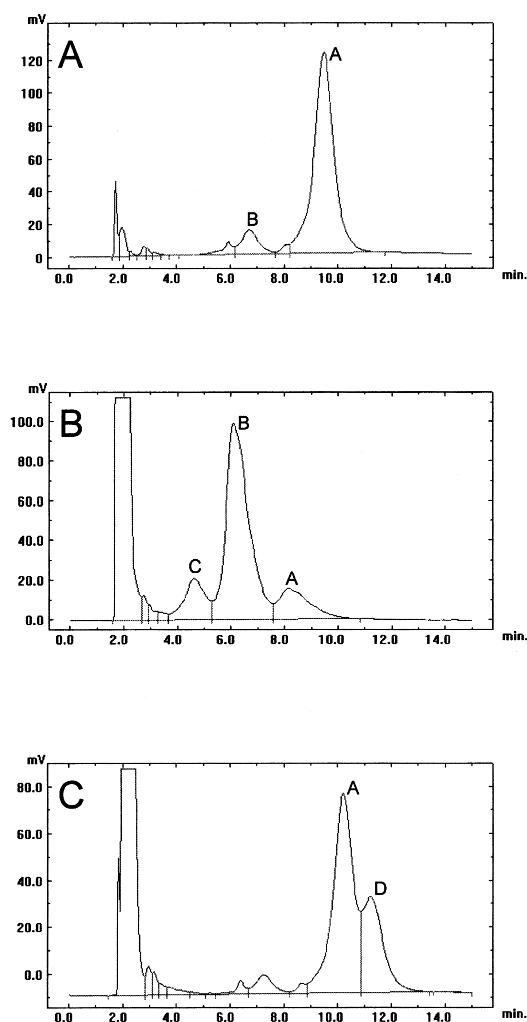

Fig. 1. Isocratic RP-HPLC chromatogram of native and degraded rIL-2. (A) Native, (B) oxidised, (C) reduced rIL-2. Units of vertical axis are mV detector output.

C), whereas the area of the native rIL-2 peak was reduced to 19% of the original peak area (Fig. 1B peak A versus Fig. 1A peak A). The total area of the peaks after the void volume (t_r>4 min) remained the same (110% of peak area of native rIL-2). The occurrence of several peaks with shorter retention times than native rIL-2 was also observed when analysing oxidised rIL-2 using gradient RP-HPLC [35]. The decrease in retention time upon oxidation implies an increase of hydrophilicity as a consequence of the formation of methionine sulfoxides.

Reduction of the rIL-2 disulphide bond with DTT resulted in the occurrence of a shoulder on the native rIL-2 peak with a longer retention time (Fig. 1C, peak D). Baseline separation of the native and reduced species could not be achieved. The longer retention time of the reduced product, implying a higher lipophilicity, can be attributed to a loss of integrity of the protein by cleavage of the disulphide bond, resulting in a further unfolding of the protein in the mobile phase. The peak area of the rIL-2 peak was reduced by 29% compared to the native form, whereas the peak area of the combined peaks after the void volume (t_r>4 min) remained the same (100% of peak area of native rIL-2).

The above results indicate that the analytical method described here may be of value for indication of the occurrence of oxidation or reduction products. The occurrence of the latter species, however, is not very likely in practice. Using the described method, the peaks resulting from oxidation of rIL-2 cannot be attributed to the oxidation of a specific methionine as shown with gradient HPLC. Also, exact quantitation of the oxidation products is difficult as some of them partially overlap other oxidised rIL-2 species. Further studies using CNBr cleavage techniques may be of use for the identification of the oxidation products.

Upon *injection of liposome samples* into the chromatographic system, several large interfering peaks in the chromatogram of rIL-2 were observed, probably due to the presence of phospholipids (EPC and EPG). Therefore, the analysis procedure was extended with the implementation of a phospholipid-extraction step. The method of Bligh and Dyer [32] was chosen as it offers the possibility of using small sample volumes in combination with a low dilution factor. Inclusion of this procedure in the analysis protocol completely prevented the occurrence of phospholipid-derived peaks in the chromatograms.

To assess whether rIL-2 was lost during the phospholipid-extraction procedure, the peak area was plotted versus the injected amount of rIL-2 for lipid-containing samples which were extracted using the procedure described, and for non-extracted samples containing only rIL-2. In both cases the data could be fitted using linear regression, yielding correlation coefficients of 0.999. The obtained equations were:

non-extracted free rIL-2: $Y = 271 \times 10^4(\pm 2.8 \times 10^4)X - 2.3 \times 10^4(\pm 3.4 \times 10^4)$

extracted rIL-2 liposomes: $Y = 280 \times 10^4(\pm 3.2 \times 10^4)X - 13.3 \times 10^4(\pm 11.8 \times 10^4)$

[Y = slope($\pm$SD)X + intercept($\pm$SD), where Y represents the peak area and X the amount of rIL-2 applied onto the column (μg); both $n=6$]. The regression standard deviations were 21.2×10^4 and $7.4\times x10^4$ for non-extracted free rIL-2 and for extracted rIL-2 liposomes, respectively.

It is clear that rIL-2 is recovered almost quantitatively after the extraction procedure. As the extraction method combines the complete removal of phospholipids from the sample together with a minimal loss of rIL-2, we conclude that it is very suitable for application together with the chromatographic assay. The resulting *graphs of the peak area versus the amount of injected rIL-2* constructed by analysis of rIL-2 standards admixed with empty liposomes were linear in the range of interest (0.1 up to 2.2 μg injected rIL-2, corresponding to rIL-2 sample concentrations of 5–100 μg/ml). A small fraction of the rIL-2 may irreversibly adsorb to the HPLC column, as indicated by the tendency of the calibration curves to intersect the x-axis.

Precision parameters of the method were determined by repetitive measurement of standards on one day (inter-day precision) and over a period of 11 days (intra-day precision). The relative standard deviations were within the range of the methodical error (approximately 5%) except at the lower concentration of 10 μg/ml (Table 1). In this case the intra-day RSD is high (17.8%). This may be caused by adsorption of the cytokine to container walls and/or components of the HPLC system.

The reproducibility of the method was investigated by comparison of a number of calibration curves over the range 5–100 μg/ml rIL-2 (concentration before lipid extraction) recorded by two operators. The equations of linear regression of the data were:

Operator 1: $Y = 273 \times 10^4(\pm 2.2 \times 10^4)X - 2.8 \times 10^4(\pm 2.8 \times 10^4)$ ($n = 30$)

Operator 2: $Y = 264 \times 10^4(\pm 3.1 \times 10^4)X - 7.8 \times 10^4(\pm 2.8 \times 10^4)$ ($n = 20$)

[Y = slope($\pm$SD)X + intercept($\pm$SD), where Y represents the peak area and X the amount of rIL-2 applied onto the column (μg)].The average slopes are similar. However, there was a significant difference between the calculated intercepts ($p<0.01$). This may be caused by a difference in the degree of adsorption of the rIL-2 during the handling by the two operators.

To demonstrate the general validity of the developed rIL-2 analysis method for lipid-containing samples the *recovery* of rIL-2 was determined after liposome preparation and subsequent analysis of the samples. Recombinant IL-2 liposomes were prepared and washed twice by ultracentrifugation. The amount of rIL-2 in the liposomes and washing fluids was determined with the isocratic RP-HPLC method and the total recovery calculated. When rIL-2 was encapsulated in fluid-type liposomes composed of EPC and EPG, a recovery of 94±9% was obtained (mean±SD of seven experiments). With liposomes composed of solid-phase lipids in combination with relatively high amounts of cholesterol (DSPC–DPPG–cholesterol liposomes), 96±7% was recovered. This indicates that, during the whole procedure (liposome preparation, removal of unentrapped rIL-2, removal of the phospholipids and HPLC analysis), the average loss of rIL-2 is less than 10%, irrespective of the phospholipids used.

Table 1
Precision parameters for the analysis of rIL-2 in samples containing liposomes

Concentration (μg/ml)	Intra-day precision (RSD)[a]	Inter-day precision (RSD)[b]
10	4.9	17.6
50	1.7	5.0
100	1.2	4.1

The intra-day precision was determined by comparison of peak areas of a standard sample injected six times on one day. The inter-day precision of the method was determined by comparison of peak areas of six standards injected over an 11-day period.

[a]Relative standard deviation of six independent measurements of one sample on 1 day.

[b]Relative standard deviation of six independent measurements over an 11-day period.

4. Conclusion

These studies focused on the development of an isocratic HPLC method for the quantitation of rIL-2 in liposomal samples. In comparison with the traditional assays for cytokines (e.g., bioassays, ELISA), the presented isocratic HPLC method offers a high sample throughput and good analytical qualities. The method is indicative for the occurrence of methionine oxidation and reduction of the rIL-2 disulphide bridge. Furthermore, the analytical procedure can be combined with a phospholipid extraction, thus allowing the quantitation of rIL-2 in lipid-containing samples. The method is suitable for the analysis of rIL-2 in liposome-containing samples as was demonstrated by a high rIL-2 recovery after liposome preparation and subsequent quantitation of rIL-2.

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