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Discrimination of granulocyte colony-stimulating factor isoforms by high-performance capillary electrophoresis

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Abstract

Granulocyte colony-stimulating factor (G-CSF) is a glycoprotein which acts primarily to stimulate the proliferation, differentiation and activation of committed progenitor cells of the neutrophil–granulocyte lineage into functionally mature neutrophils. The traditional biological assays employed to detect G-CSF are a myeloid bone marrow colony assay, a factor-dependent cell line specific for G-CSF and commercially available immunoassays. However, these methods will not distinguish between glycosylated and non-glycosylated forms of the molecule. In this study high-performance capillary electrophoresis (HPCE) was used to analyse glycosylated and non-glycosylated recombinant human granulocyte colony-stimulating factor (r-met-hG-CSF). Glycosylated G-CSF preparations contained human serum albumin (HSA), added as a protein carrier. Glycosylated and non-glycosylated G-CSFs were prepared in 40 m*M* Na_2HPO_4 buffer, pH 2.5, containing hydroxypropylmethylcellulose (HPMC) or 50 m*M* Na_2HPO_4 buffer, pH 9.0. Glycosylated G-CSF could be separated into two distinct glycoform populations at the lower pH studied. Differences in migration time and peak shape between glycosylated and non-glycosylated G-CSF were demonstrated. HPCE analysis of G-CSF produced using a baculovirus expression vector system revealed a further distinct G-CSF glycoform and demonstrated the resolving power of the technique.

Keywords: Glycoproteins; Granulocyte colony-stimulating factor

1. Introduction

Granulocyte colony-stimulating factor (G-CSF) is a haematopoietic cytokine that stimulates and regulates the proliferation and differentiation of neutrophils [1]. It was initially purified from conditioned medium prepared from the lungs of mice injected with endotoxin and is a glycoprotein with a molecular weight of approximately 20 kDa [2]. It is currently commercially available for clinical use in two forms: non-glycosylated and glycosylated. The non-glycosylated form of the protein contains an extra methionine at its N-terminus, derived from expression in *E. coli* (r-met-hG-CSF) [3]. The glycosylated G-CSF produced in Chinese hamster ovary

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(CHO) cells possesses an *O*-linked carbohydrate chain attached to threonine-133 of the molecule. This sugar moiety accounts for approximately 4% of the total mass of the protein. Glycosylation has been reported to confer many advantages over non-glycosylation in terms of in vitro stability, although it does not appear to be essential for the biological activity of the G-CSF molecule [4].

The amino acid sequence of human G-CSF was originally deduced from a cloned cDNA sequence [5,6]. Further studies [7] revealed that there were two naturally occurring human G-CSF mRNAs encoding slightly different proteins. Differential splicing of the precursor mRNA gives rise to a predominant form of the molecule consisting of 174 amino acids (G-CSF 'b') and another minor form consisting of 177 amino acids (G-CSF 'a'). The molecules differ only in that three extra amino acids (Val, Ser and Glu) are inserted at the 35th amino acid residue in G-CSF 'a'. G-CSF 'b' has been reported to be 20 times more biologically active in terms of colony-stimulating activity in a myeloid bone marrow colony assay than G-CSF 'a' [7].

The functional activity of G-CSF can be determined using biological assays. A growth factor-dependent cell line assay, using GNFS-60 cells, can determine the proliferative activity of the G-CSF molecule [8]. The myeloid bone marrow colony assay evaluates both the proliferative and differentiation inducing properties of the molecule [9]. Nissen et al. [10], using the bone marrow colony assay, suggested that glycosylated rhG-CSF was more potent than r-met-hG-CSF. A collaborative study between the World Health Organisation (WHO) and the National Institute for Biological Standards and Control (NIBSC) used the growth factor-dependent cell line assay and an immunoassay to compare three different preparations of rhG-CSF derived from *E. coli*, yeast and CHO cells [11]. They found that recombinant preparations of the same mass varied considerably in their biological activity and that antibodies used in the immunoassay did not necessarily recognise different forms of G-CSF to the same extent. Thus, there are several caveats in using these assays to characterise G-CSF preparations.

Other analytical techniques, such as polyacrylamide gel electrophoresis (PAGE), are not sufficiently sensitive to resolve small differences in molecular weight. Therefore, the small difference in molecular weight between glycosylated and r-met-hG-CSF (4%) would be difficult to detect using these techniques. High-performance capillary electrophoresis (HPCE) has the advantage over other separation methods as it can detect on the basis of charge-to-mass ratio, enabling glycoforms of a molecule to be easily distinguished. Watson and Yao [12] showed that glycoforms of recombinant human G-CSF (rhG-CSF) could be successfully separated by HPCE using a 50 m*M* phosphate–50 m*M* borate buffer, pH 8.0, with and without 2.5 m*M* diaminobutane. G-CSF was undetectable below pH 5.0. In this paper we have evaluated HPCE, using a different buffer and pH system to that previously described, as a technique to discriminate between isoforms of G-CSF. One buffer contained a zeta potential inhibitor, hydroxypropylmethylcellulose (HPMC), that was included to increase the resolution of protein separation. Since traditional methods fail to be sufficiently sensitive to detect the small amino acid difference between G-CSF 'a' and 'b', it was postulated that HPCE might discriminate between 'a' and 'b'. The two HPCE buffering systems were then employed to monitor the expression levels of both G-CSF 'a' and 'b' from a baculovirus expression vector.

2. Experimental

2.1. Preparation of HPCE buffers

Two buffers were prepared for HPCE: (A) 50 m*M* Na_2HPO_4 were prepared in HPLC-grade water and the solution was adjusted to pH 9.0 using phosphoric acid; (B) 40 m*M* Na_2HPO_4 were prepared in HPLC grade water and the solution was adjusted to pH 2.5 using phosphoric acid. Hydroxypropylmethylcellulose (HPMC; Sigma) was added to a final concentration of 0.03%. Both buffers were filtered prior to use with a 0.22-μm syringe filter (Millipore).

2.2. Preparation of G-CSF

Glycosylated G-CSF, supplied lyophilised (Chugai Pharma), was reconstituted in sterile phosphate-buffered saline (0.01 *M*, pH 7.2; PBS; Life Technologies) to a concentration of 263 μg/ml. Prior to, and following reconstitution the vial was stored at

4°C. Human serum albumin (HSA) was present in the preparation at 1 mg/ml. Recombinant-met-hG-CSF, was supplied in 10 m*M* sodium acetate, pH 4.0, at a concentration of 300 μg/ml (Amgen). There was no carrier protein present in this r-met-hG-CSF preparation. This was also stored at 4°C. Dilutions were prepared from these stock solutions in both buffers at concentrations ranging from 100 to 10 μg/ml.

2.3. Preparation of HSA and spiking of recombinant-met-hG-CSF preparations

HSA was present in the glycosylated G-CSF preparation at a concentration of 1 mg per 263 μg G-CSF. HSA (Sigma) was prepared at 50 mg/ml in HPLC-grade water and was added to the r-met-h G-CSF dilutions (prepared in both buffers) so that the final HSA concentrations were equivalent to their glycosylated G-CSF counterparts.

2.4. Expression of G-CSF 'a' and 'b'

G-CSF 'a' and 'b' were expressed as glutathione *S*-transferase (GST) fusion proteins using a baculovirus expression vector (pAcSecG2T, Pharmingen). The fusion proteins were purified using glutathione sepharose 4B columns (Pharmacia) and cleaved using thrombin protease (500 units). Excess thrombin was removed from the G-CSF samples by

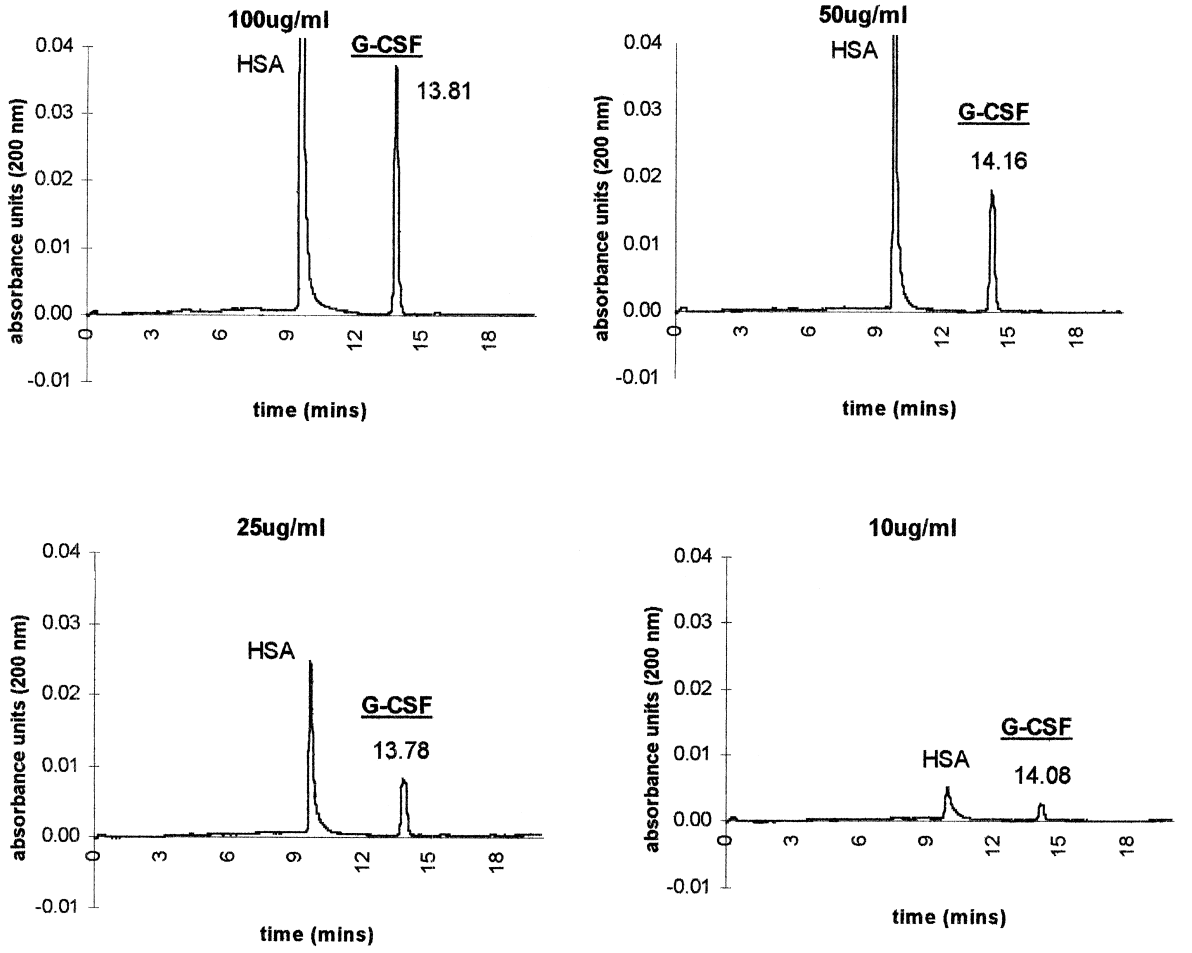

Fig. 1. Electropherograms of recombinant-met-hG-CSF (spiked with HSA). Conditions: 50 m*M* phosphate buffer, pH 9.0. G-CSF samples were electrophoresed at 10 kV for 30 min using a 57 cm×75 μm capillary.

incubation with 1 ml benzamidine sepharose (50% solution in PBS containing 0.5 *M* NaCl; Sigma) at room temperature for 30 min. Eluate containing G-CSF was separated from the sepharose matrix by centrifugation at 500 *g* for 10 min. The purified G-CSFs were subsequently desalted, freeze-dried and reconstituted in either 50 m*M* Na_2HPO_4 buffer, pH 9.0, or 40 m*M* Na_2HPO_4 buffer, pH 2.5, containing 0.03% HPMC.

2.5. Capillary electrophoresis

Capillary electrophoresis was carried out using a P/ACE system 2000 (Beckman Instruments, UK) using an eCAP capillary column, 57 cm long and 75 μm I.D. Samples were injected using nitrogen for 10 s (corresponding to 58.6 nl). UV detection was used at a wavelength of 200 nm. Recombinant hG-CSF samples were analysed under two differing sets of conditions. Recombinant hG-CSF was prepared in 40 m*M* Na_2HPO_4, pH 2.5, containing 0.03% HPMC and electrophoresed at 25 kV for 20 min at a constant temperature of 25°C. Alternatively, rhG-CSF was resuspended in 50 m*M* Na_2HPO_4, pH 9.0, and electrophoresed at 10 kV for 30 min with an initial ramp time of 2 min at a constant temperature of 25°C. Baculovirus expressed G-CSF 'a' and 'b' reconstituted in either buffer were electrophoresed

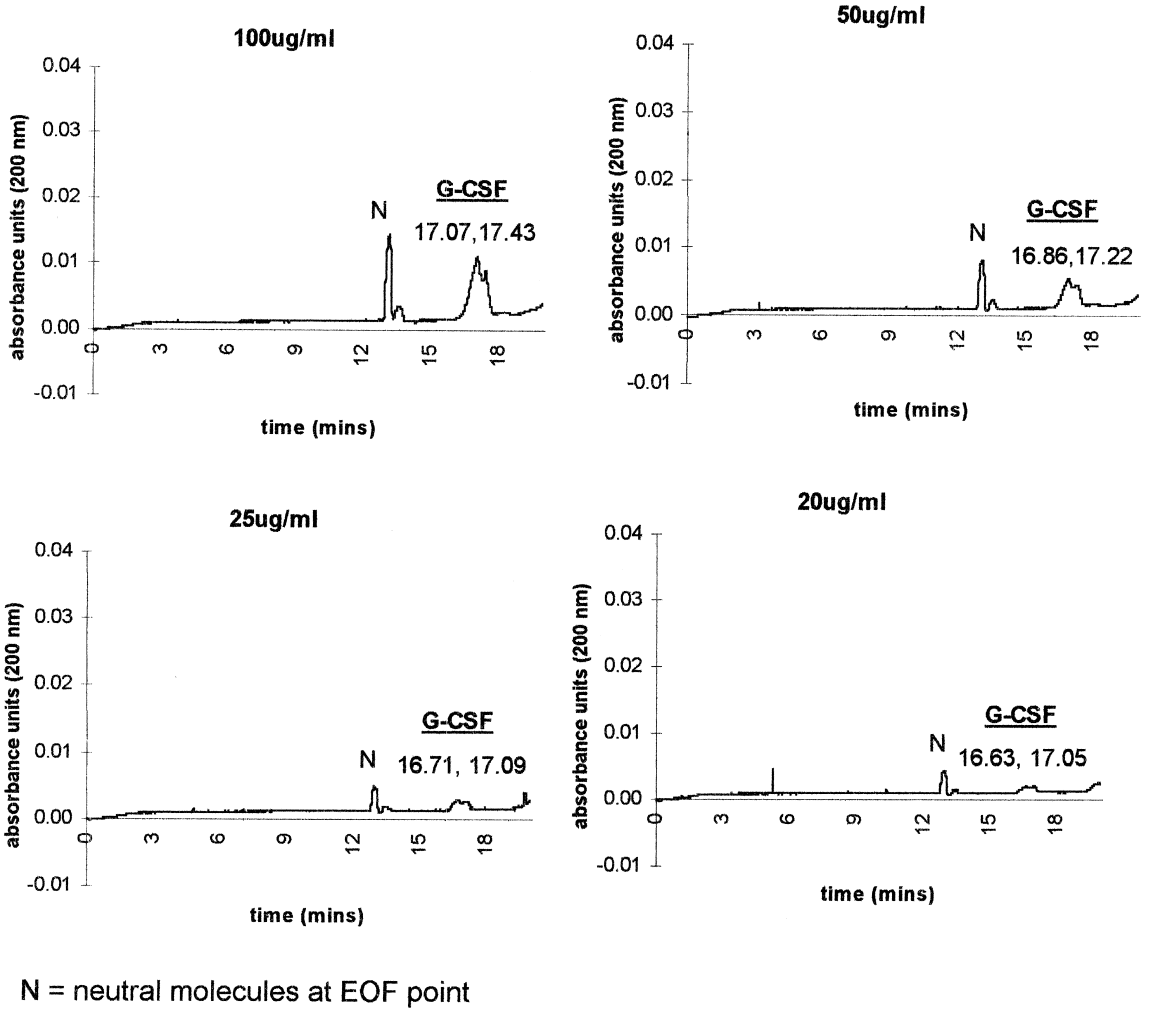

Fig. 2. Electropherograms of glycosylated rhG-CSF. Conditions: 50 m*M* phosphate buffer, pH 9.0. G-CSF samples were electrophoresed at 10 kV for 30 min using a 57 cm×75 μm capillary.

under identical conditions as the rhG-CSF samples. Between runs, the capillary column was washed for 2 min with 0.1 N NaOH followed by 2 min with HPLC-grade water. Data were analysed using the P/ACE system Gold software (version 8.01, Beckman Instruments, UK) after 24 and 34 min, respectively.

3. Results and discussion

Using HPCE, proteins can be separated on the basis of their different size-to-charge ratios and electrophoretic mobilities. The pH of the buffer solution influences the charge of the protein and the electroosmotic flow (EOF) within the capillary. To amplify differences in the electrophoretic migration, low concentrations of additives such as hydroxypropylmethylcellulose (HPMC) can be added to the running buffer. The resolution of protein separations can be significantly increased by the addition of low concentrations of this additive (0.01–0.03%). For example, Birrell et al. [13] added HPMC to 100 m*M* sodium phosphate buffer, pH 2.5, and found it significantly improved the separation of eminase. The EOF was reduced by working at a low pH and this ensured the separation was by the electrophoretic mobility of the analytes alone (reviewed in Ref. [14]). HPMC is known to decrease the zeta potential at the fused capillary wall and may assist electro-

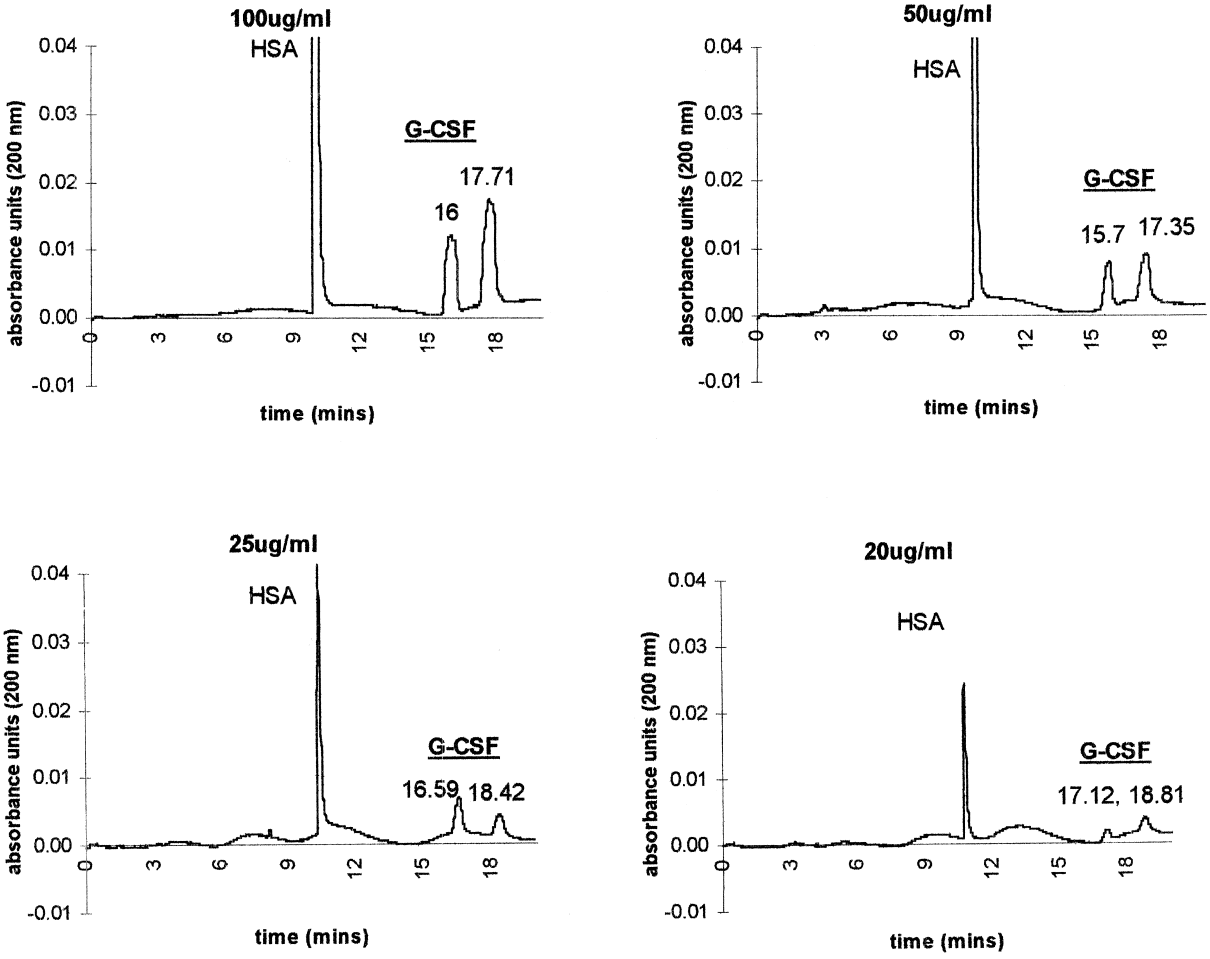

Fig. 3. Electropherograms of glycosylated G-CSF. Conditions: 40 m*M* phosphate buffer, pH 2.5, containing 0.03% HPMC. G-CSF samples were electrophoresed at 25 kV for 20 min using a 57 cm×75 μm capillary.

phoretic separations by minimising undesirable adsorption to the silica capillary [15].

In this study, the electrophoretic separation of rhG-CSF was assessed in buffers at two pH values. In phosphate buffer, pH 9.0, glycosylated G-CSF eluted as a doublet peak (Fig. 2) compared to r-met-hG-CSF that eluted as a single, sharp peak (Fig. 1). This may be due to the neuraminic acid residue present at threonine-133 on the glycosylated molecule. This has previously been reported by Watson and Yao [12]. Glycosylated G-CSF also eluted slightly later than its r-met-hG-CSF counterpart (Figs. 1 and 2), primarily due to glycosylation altering the mass-to-charge ratio of the G-CSF molecule.

In phosphate buffer, pH 2.5, containing HPMC, the doublet peaks present in the electropherograms were more distinct for the glycosylated G-CSF (Fig. 3) than their counterparts at pH 9.0 (Fig. 2). This could be due to the increased lability of the neuraminic acid residue at pH 2.5, which dissociates to produce two distinct peaks. To determine the lability of the neuraminic acid residue G-CSF was incubated with neuraminidase and re-run using both the low- and high-pH conditions. The neuraminidase appeared to elute at the same time as the G-CSF, thereby masking the presence of the G-CSF peaks. We were, therefore, unable to reliably prove/disprove the lability of neuraminic acid on threonine-133 of the molecule.

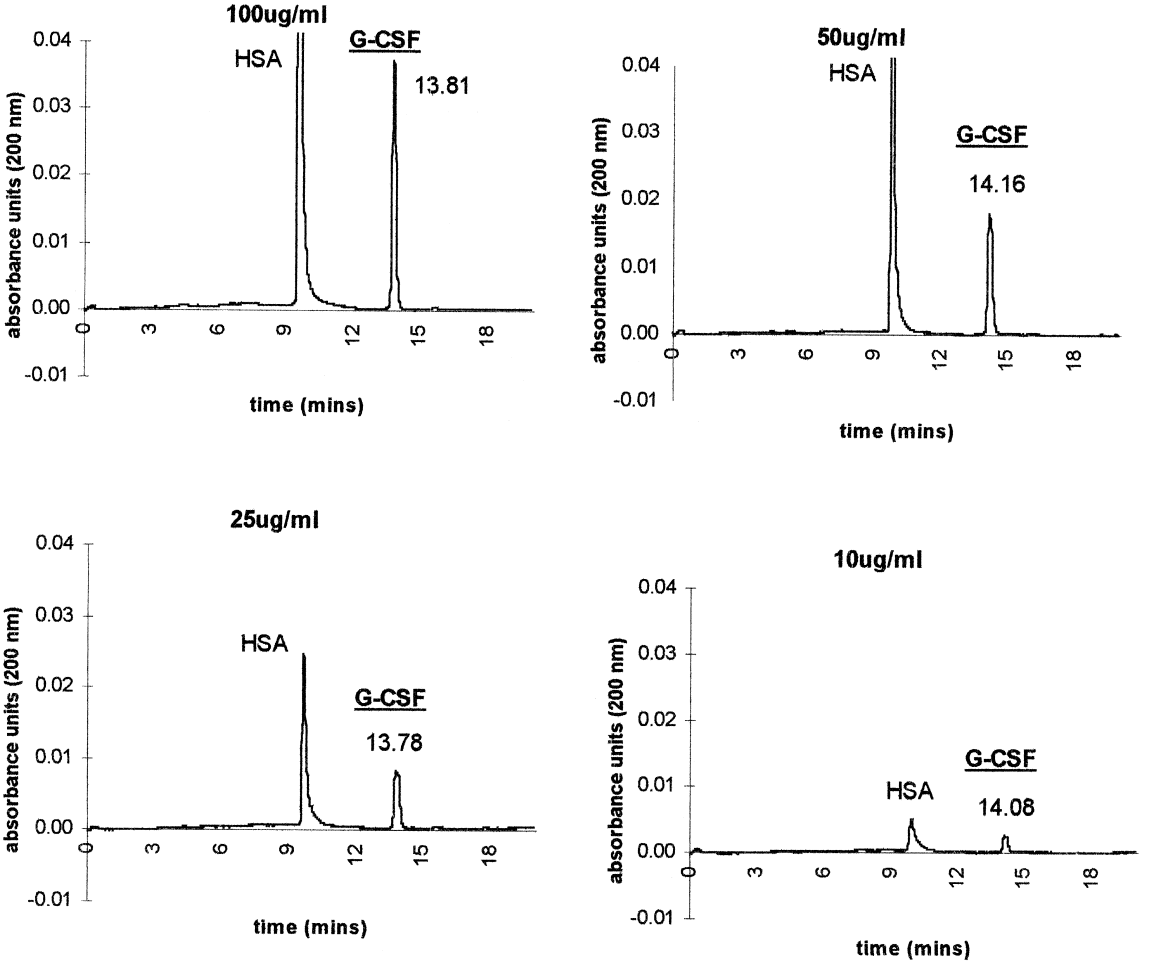

Fig. 4. Electropherograms of recombinant-met-hG-CSF. Conditions: 40 m*M* phosphate buffer, pH 2.5, containing 0.03% HPMC. G-CSF samples were electrophoresed at 25 kV for 20 min using a 57 cm×75 μm capillary.

The detection limit of G-CSF was also less for both glycoforms of G-CSF at the lower pH studied. Glycosylated G-CSF at pH 2.5 eluted slightly later than the r-met-hG-CSF (Figs. 3 and 4), again this is probably due to the presence of the neuraminic acid residue. The apparent difference in the glycoform ratios observed in Fig. 3 (25 μg/ml concentration) may be due to the adsorption of G-CSF to the sample container. There may also have been a delay in the analysis of the sample prior to electrophoresis as compared to the previous and subsequent G-CSF samples.

The addition of HSA to the r-met-hG-CSF reduced the limit of detection to 10 μg/ml at pH 2.5 as compared to 50 μg/ml without the presence of this additive. This can be observed in Table 1. The corresponding areas under the curve were also increased at both pH values for the r-met-hG-CSF at all concentrations.

The electropherograms observed in Fig. 5 show baculovirus expressed G-CSF 'a' and 'b', electrophoresed using 50 m*M* Na_2HPO_4 buffer pH 9.0. It proved impossible to detect both isoforms at pH 2.5, despite the extension of the electrophoresis run time to 30 min and the addition of HSA to the samples. This could be attributed to G-CSF adhering to the silica capillary wall despite the presence of HPMC and HSA.

The electropherograms of baculovirus expressed G-CSF isoforms (Fig. 5) differed from those obtained for the glycosylated rhG-CSF (Fig. 2), both in elution time and shape. Baculovirus expressed G-CSF gave a peak shape similar to that obtained for the r-met-h protein although glycosylation was shown to be present by biochemical analysis (data not shown). The pathways involved in the processing of glycosylated proteins in insect cells appear to differ from the mammalian pathways. Van Die et al. [16] studied the expression of glycosyltransferases involved in *N*-glycosylation. They found that insect cells were only capable of synthesising complex carbohydrate chains containing GalNAcβ1→4GlcNAc. Baculovirus infection of the insect cells, however, resulted in a decrease in the activity of β4-GalNAcT 24 h post-infection. The enzyme activity observed was considered to be responsible for the degradation of the oligosaccharide chains. Further reports have also confirmed that insect cells were unable to process complex carbohydrate structures [17,18]. The difference in the glycosylation process-

Table 1
Comparison of glycosylated and r-met-hG-CSF at two pH values

Concentration of G-CSF (μg/ml)	40 m*M* Na_2HPO_4 (pH 2.5) buffer+HPMC		50 m*M* Na_2HPO_4 (pH 9.0) buffer	
	Non-glycosylated G-CSF (AUC)	Glycosylated G-CSF+HSA (AUC)	Non-glycosylated G-CSF (AUC)	Glycosylated G-CSF+HSA (AUC)
(a) Glycosylated G-CSF and r-met-hG-CSF				
100	0.0232	0.0853	0.0360	0.0448
50	0.0082	0.0363	0.0135	0.0182
25		0.0196	0.0030	0.0070
20		0.0101		0.0051
Concentration of G-CSF (μg/ml)	40 m*M* Na_2HPO_4 (pH 2.5) buffer+HPMC		50 m*M* Na_2HPO_4 (pH 9.0) buffer	
	Non-glycosylated G-CSF+HSA (AUC)	Glycosylated G-CSF+HSA (AUC)	Non-glycosylated G-CSF+HSA (AUC)	Glycosylated G-CSF+HSA (AUC)
(b) Glycosylated G-CSF and r-met-hG-CSF both containing HSA				
100	0.0545	0.0853	0.0166	0.0448
50	0.0301	0.0363	0.0061	0.0182
25	0.0159	0.0196	0.0023	0.0070
20	0.0099	0.0101	0.0010	0.0051
10	0.0041			

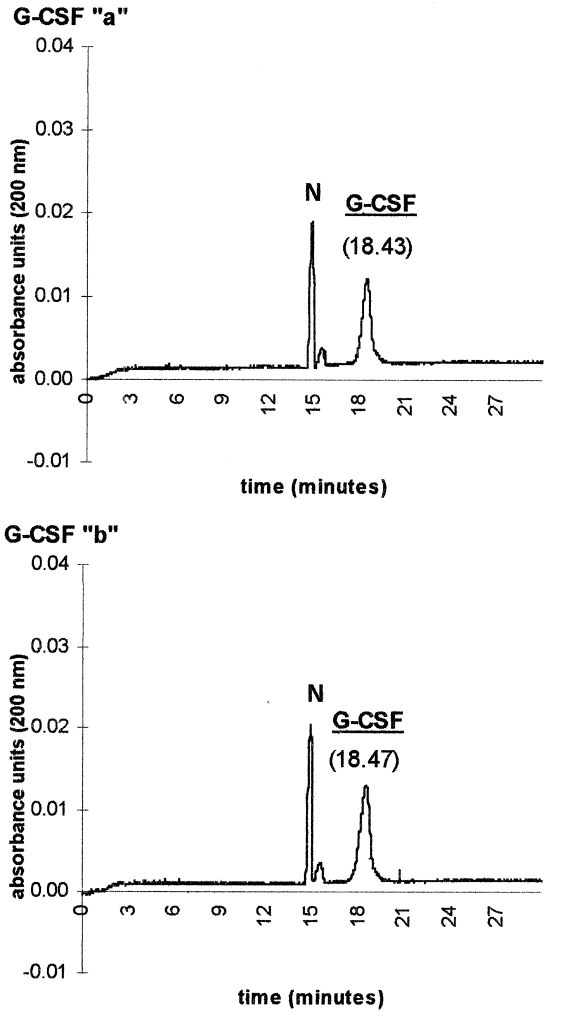

Fig. 5. Electropherograms of baculovirus-expressed glycosylated G-CSF 'a' and 'b'. Conditions: 50 m*M* phosphate buffer, pH 9.0. G-CSF samples were electrophoresed at 10 kV for 30 min using a 57 cm×75 μm capillary.

ing pathways could partly explain the absence of the doublet peaks observed in the baculovirus expressed G-CSF isoforms.

4. Conclusions

HPCE is now regarded as an established analytical technique in the pharmaceutical industry and most attention has been focused on its usefulness in the characterisation of biological molecules. HPCE has been shown to be a highly efficient, fast technique capable of separating protein glycoforms. The use of a low pH in conjunction with additives, such as HPMC, can reduce adsorption of protein to the silica capillary wall by charge repulsion, allowing a lower detection limit to be achieved. In comparison to other analytical techniques, HPCE can be automated and has a total analysis time of less than 40 min, allowing numerous samples to be screened in a short time period, dispensing with the need of continuous user intervention.

HPCE has been successfully used to analyse and characterise several recombinant proteins. For example, CZE was used to analyse and distinguish recombinant human erythropoietin (EPO) supplied by different drug manufacturers [19]. Yowell et al. [20,21] analysed granulocyte macrophage colony-stimulating factor (GM-CSF) using capillary isoelectric focusing to check concentrations of dosage forms, and they also used it to distinguish G-CSF from GM-CSF as the two molecules possess different p*I* values. It has been shown in this study that HPCE can, at high and low pH, reproducibly discriminate between glycosylated and r-met-hG-CSF. The use of a Na_2HPO_4 buffer, pH 2.5, supplemented with HPMC enabled a lower detection limit of the G-CSF to be achieved. Unfortunately, the three-amino acid difference between G-CSF 'a' and 'b' was insufficient to produce an elution time difference under the conditions optimised in this paper. However, HPCE revealed distinct differences between the baculovirus expressed products and the commercially available glycosylated rhG-CSF, possibly attributable to the insect cell glycosylation pathway differing from the mammalian pathway. In conclusion, HPCE was shown to be a useful technique in the monitoring of protein expression levels using a pH 9.0 buffer, capable of rapidly determining the presence and glycosylation status of G-CSF within a preparation.

Acknowledgements

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