Journal of Chromatography, 629 (1993) 398–400
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 744

Short Communication

Determination of N-hydroxysuccinimidyl-activated polyethylene glycol esters by gel permeation chromatography with post-column alkaline hydrolysis

Bhavana Shah and Eric Watson

Amgen Inc., Amgen Center, 1900 Oak Terrace Lane, Thousand Oaks, CA 91320-1789 (USA)

(First received September 4th, 1992; revised manuscript received November 9th, 1992)

ABSTRACT

An HPLC method is reported for the determination of N-hydroxysuccinimidyl-activated polyethylene glycol ester. The activated polyethylene glycol sample is first separated by size-exclusion chromatography on a polymeric column with tetrahydrofuran as the eluent, and after elution is subjected to post-column on-line hydrolysis with 0.1 *M* sodium hydroxide. Liberation of N-hydroxysuccinimide occurs rapidly and is monitored by UV detection at 266 nm. The amount released is determined from a standard curve generated from free N-hydroxysuccinimide and used to calculated the concentration of active ester initially present.

INTRODUCTION

Administration of proteins for therapeutic treatment is severely hampered by short half-lives in the circulatory system. It has been shown that the covalent attachment of polyethylene glycols, most often of M_r 5000, to enzymes and proteins has a major affect on their activity and physical properties. In particular, several reports have demonstrated the effect of increased circulating lives from the use of such polyethylene glycol (PEG) conjugates without any complications arising from immunogenicity [1,2].

Correspondence to: E. Watson, Amgen Inc., Amgen Center, 1900 Oak Terrace Lane, Thousand Oaks, CA 91320-1789, USA.

The protein–PEG adducts are typically prepared by reaction of the proteins with N-hydroxysuccinimidyl-activated PEG esters. These active esters are very unstable under aqueous conditions. There are few analytical methods available for determining the purity of these activated esters. The only previously reported method involved liberation of N-hydroxysuccinimide under alkaline conditions and following the increase in absorption at 266 nm [3]. This method provides only an estimate of the activated PEG ester since it will also determine any free N-hydroxysuccinimide and related degradation products present which may have been derived from the activated ester or reaction products. This report describes an on-line HPLC method with post-column hydrolysis for the determination of intact activated PEG ester.

0021-9673/93/$06.00

METHODS AND MATERIALS

Materials

N-Hydroxysuccinimide (NHS) and methoxypolyethylene glycolsuccinimidyl succinate (SS-MPEG) were obtained from Sigma (St. Louis, MO, USA). HPLC-grade tetrahydrofuran (stabilized with 0.029% butylated hydroxytoluene) was purchased from Fisher Scientific (Santa Clara, CA, USA).

Chromatographic separation

The chromatographic system consisted of a Kratos Spectroflow 400 system equipped with a Kratos URS 051 post column delivery pump. A single mixing coil, 2 ml, was found to be sufficient for complete hydrolysis and was used at room temperature. The liberation of NHS was monitored with a Shimadzu SPD-6A UV detector set at 266 nm.

Samples for analysis (0.25%) are prepared by weighing an amount of SS-MPEG into tetrahydrofuran. An aliquot, 100 μl, is injected into a gel permeation column (G2500HXL, 30.0 cm × 7.8 mm) obtained from Supelco (Bellefonte, PA, USA). The eluent is tetrahydrofuran at a flow-rate of 0.5 ml/min and the separated activated ester passes directly into the post-column system where it is hydrolyzed by 0.1 *M* sodium hydroxide delivered at 0.5 ml/min.

RESULTS

Hydrolysis of activated ester

Under base conditions, hydrolysis of the activated ester proceeds rapidly with the liberation of NHS, the λ_{max} of which is 266 nm. The effect of post-column mixing time and temperature on the liberation of NHS from SS-MPEG was evaluated. Comparison of the amount of NHS liberated from one and two 2-ml reaction coils showed no difference. Raising the temperature of the post-column reaction coil to 70°C had no effect on the amount of NHS liberated. Based on these results, a single 2-ml reaction coil at room temperature was used for all subsequent determinations. In a separate series of experiments, a comparison was made between 0.1 *M* sodium hydroxide and 0.1 *M* ammonium hydroxide for hydrolysing the activated ester. No difference in the amount of activated ester released occurred.

Analysis of active ester in SS-MPEG

Chromatography of PEG 5000 standard gave no response when analyzed in the current system. Fig. 1 shows the chromatogram obtained from 2 μg free NHS. The calibration plot curved toward the *y*-axis and was best fit with a non-linear model, $y = a_1x + a_2x^2$. For the standard curve shown in Fig. 2, the best fit for the standard curve was obtained from the binomial equation

$$y = 1.1391 \cdot 10^{-2} + 0.52097x + 1.9164 \cdot 10^{-2},$$
$$R^2 = 0.999.$$

Fig. 3 shows the chromatogram obtained from SS-MPEG when separated by size-exclusion chromatography and hydrolyzed by sodium hydroxide in the post-column reaction system. The major peak represents the M_r 5000 PEG ester while the peak that elutes ahead of it is considered to be a higher-molecular-mass PEG ester. The free NHS present was determined to be 0.2%. Determination of the amount of activated ester present in the original sample was based on the assumption that a single mol of NHS/mol activated ester was present in the original sample. The content of activated ester, SS-MPEG, M_r 5000 was found to be 63% (R.S.D. 2%, $n = 10$). The reported content by the supplier is >75%, which agrees with the results here when the higher-molecular-mass material is included.

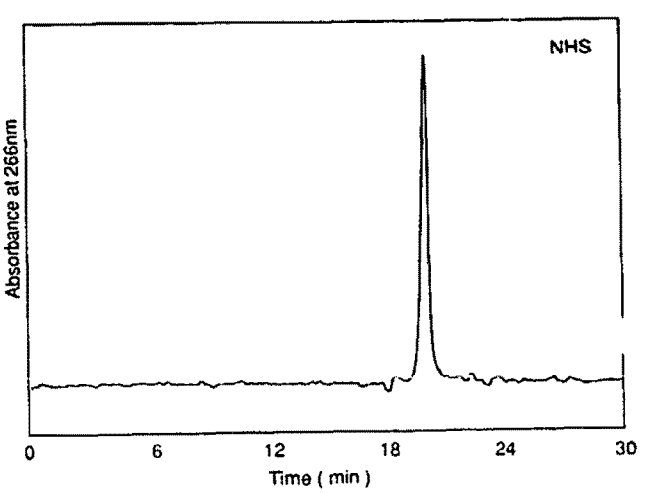

Fig. 1. Chromatogram from 2 μg free NHS.

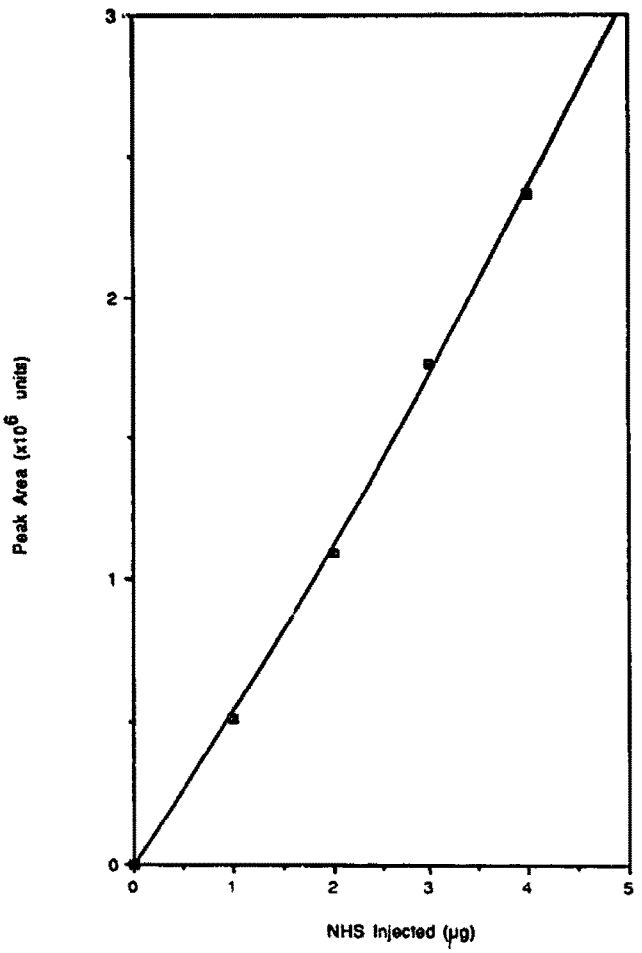

Fig. 2. Calibration plot obtained from NHS fitted to a binomial equation.

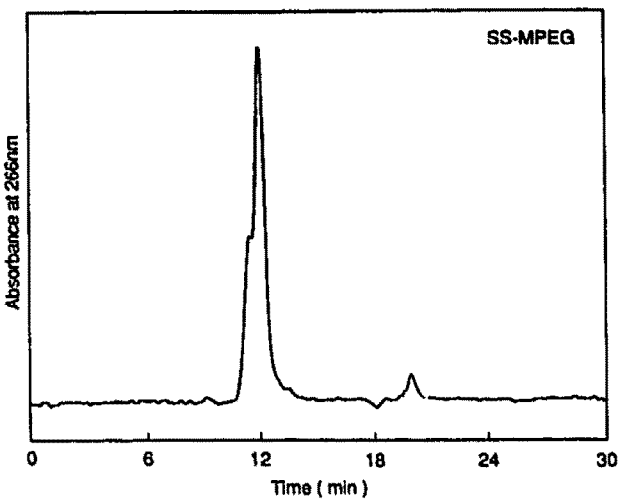

Fig. 3. Chromatogram obtained from SS-MPEG.

CONCLUSIONS

(1) Size-exclusion chromatography followed by post-column hydrolysis under basic conditions is a fast and relatively simple method for determining the purity of active succinimidyl esters.

(2) The technique provides a method that can be used to determine the stability, and monitor the purity of the ester produced by different processing steps.

(3) The overall method can also be applied without modification to determine the purity of other activated esters, *e.g.*, succinimidyl glutarate, succinimidyl carbonate and succinimidyl carboxymethyl.

(4) One potential drawback with the described procedure is that quantitation is carried out using free NHS as the standard. If experimental factors adversely affect the chromatography of NHS, *e.g.*, such as column adsorption, without a corresponding effect on the activated ester, then it is possible that variability would be introduced into the assay. To date, we have not observed this occurring, but it is a valid concern worth noting. This problem could be overcome by using a highly pure, characterized activated ester as the standard for quantitation.

REFERENCES

1 N. V. Katre, M. J. Knauf, D. P. Bell, P. Hirtzer, Z. Luo and J. D. Young, *J. Biol. Chem.*, 263 (1988) 15064–15070.
2 N. V. Katre, *J. Immunology*, 144 (1990) 209–213.
3 T. Miron and M. Wilchek, *Anal. Biochem.*, 126 (1982) 433–435.