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Full Length Research Paper

Effects of PEGylation on the Lowry method for the content determination of trichosanthin

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The current study was designed to establish a method for examining whether the content of PEG-TCS could be determined by the Lowry method. The research applicability of the Lowry method on the determination of PEGylated trichosanthin (PEG-TCS) protein content was proposed for the first time. Procedures were designed to determine whether residual PEG interfered with the result of protein content determination, PEG (≤20 µg/ml) interference was not apparent. The interference of a PEG-modified group was analyzed by utlra violet spectrophotometric colorimetry, and the differences in the rates were 5.0 and 6.2%. The linearity, precision, and recovery were determined. There is good linear relationship from 30 to 70 µg/ml PEG-TCS. The experiment results provided sufficient data to establish a quality control method for the determination of PEG-TCS content, and they were conducive to the determination method establishment of other PEGylated proteins for non clinical experiment application.

Key words: PEGylated trichosanthin, Lowry method, trichosanthin, methodology validation.

INTRODUCTION

Polyethylene glycol (PEG) modification can dramatically extend the circulation half-life of a protein in the blood, and improve the pharmacokinetics of a drug in the body (Veronese and Harris, 2002). PEGylated trichosanthin (TCS) which has therapeutic efficacy against AIDS, was developed by targeting modification of PEG Aldehyde (ALD-PEG) 20000 (Li-hua et al., 2005). The protein determination is very important for the quantification of PEGylated proteins, and the Lowry method (Lowry et al., 1951) has been recommended or used for this purpose (Hu et al., 2002; Baran et al., 2003; liang et al., 2010).However, Xing Wen Gong and co-workers found that the Lowry method gave overestimations with NHS (Xing et al., 2006).In this study, the applicability of the Lowry method for PEG-TCS with no NHS but ALD-PEG20000 was proposed, and the inspection and verification methods were established. The experiment results provided sufficient data to establish a quality control method for the determination of PEG-TCS content, and they were conducive to the determination methods establishment of other PEGylated proteins for non clinical experiment application.

MATERIALS AND METHODS

Chemicals and apparatus

Injections of PEG-TCS (1 mg/ml, batch numbers 20090818, 2009826, and 20100120) and TCS Solution (1.5 mg/ml) were provided by Anke Biotechnology (Group) Co., Ltd., Phenol reagent, bovine serum albumin standard, and mPEG-ALD (molecular weight, 20 000) were purchased from Sigma. A 752C-UV-Vis spectrophotometer was purchased from Shanghai Third Analysis Instrument Factory. All other instruments (IKA WORKS, pipette, scale pipette, centrifuge tubes, tubes, etc.) were purchased from local companies.

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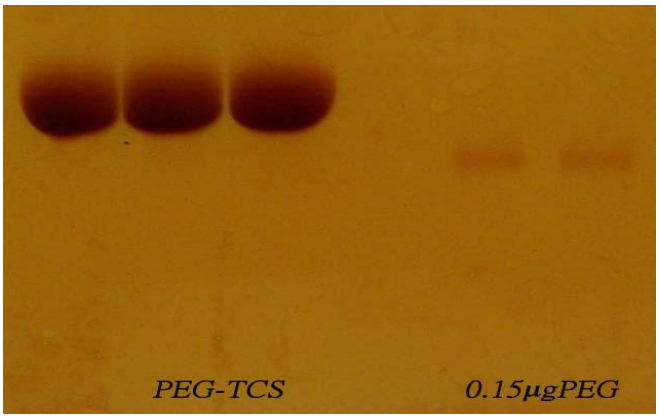

Figure 1. SDS-PAGE PEG dyeing atlas.

Reagent preparation

Alkaline copper solutions were prepared by sequentially mixing 0.2 mol/L sodium hydroxide, 4% sodium carbonate, 0.04 mol/L copper sulfate solution, and 2% potassium tartrate solution according at a 50:50:1:1 ratio. Bovine serum albumin used for calibration was from the China Institute for Drug Control, and was diluted precisely to 1 mg/ml with 0.02% NaN_3 solution as the standard protein stock solution. This solution was sub-packaged into 0.5 ml/branch and stored at -20°C while avoiding repeated freezing and thawing.

Experimental methods

Residue PEG SDS-PAGE determination

SDS-PAGE was performed as described by Laemli (1970), but the gels were stained in barium iodide (Baran et al., 2003). Take samples 1.0 mg/ml PEG-TCS 10 µl and 15 µg/ml PEG 10 µl , then the SDS-PAGE electrophoresis gel was dipped into fixed solution for 15 min, and then been rinsed for three times with injection water, and each time 2 min. After that, the rinsed gel was dipped into 0.1 mol/L perchloric acid solution for 15 min; added in 5% barium chloride solution 5 ml, 0.1 mol/L iodine solution 20 ml for 5 to 10 min without light. At last, the stained gel was rinsed with injection water for 3 times, and 5 min each time.

Residue PEG interference experiment

According to the PEG staining, results of electrophoresis measurements (Figure 1) displayed that the amount of residual PEG was much less than 15 µg/ml. Three concentrations of PEG solution were prepared: 20, 30 and 40 µg/ml. The PEG-TCS concentration was diluted to 100 µg/ml, according to UV absorption spectrometry. Thereafter, all PEG-TCS groups were mixed with 20, 30, and 40 µg/ml PEG, respectively, in equal volumes by adding each tube with 0.5 ml. The PEG-TCS concentration correspondingly reached 50 µg/ml (10 µg/ml PEG added), 50 µg/ml (15 µg/ml PEG added), and 50 µg/ml (20 µg/ml PEG added), respectively. The standard protein solution, which was diluted to 200 µg/ml, was added to standard 0, 0.1, 0.2, 0.3, 0.4 and 0.5 ml tubes, which were divided into three groups with 20, 30 and 40 µg/ml PEG, mixed in equal volumes. Distilled water was added to each tube to a total volume of 1 ml, and the tubes were subjected to the Lowry method.

PEGylation interference experiment

No PEG-TCS reference substance is presently available, and the PEG-TCS concentration cannot be accurately obtained. Consequently, a method for detecting the relative concentration to explore changes in the Lowry method measurement results before and after TCS PEGylation was designed and used to describe the PEGylation of interference on the determination of Lowry method. To prepare TCS and PEG-TCS, the OD was determined by UV absorption spectrometry and the samples were diluted to the same concentration. Using the same concentration of TCS and PEG-TCS in the Lowry method, the concentrations of c1 and C1 was obtained from the standard curve equation.

By two parallel measurements, the first relative deviation named RD1 was calculated. The samples were first measured by the Lowry method, and both stock solutions were diluted to 500 µg/ml. The same concentration of TCS and PEG-TCS was used in UV absorption spectrometry, and the concentration was calculated based on UV absorption, that is, c2 and C2, respectively.

By two parallel measurements, the second relative deviation named RD2 was calculated:

RD1 (%) = (c(—)1-C(—)1)/c(—)1 × 100%

RD2 (%) = (c(—)2-C(—)2)/c(—)2 × 100%

Recovery test

Given that no PEG-TCS national reference standard is currently available, the Pharmaceutical Quality Standard Analytical Method Validation Guidelines of the current edition of the Chinese Pharmacopoeia Appendix' was used in the subsequent description of recovery. If all preparation forms of the drug cannot be obtained, the recovery can be determined by adding known quantities of substances to the preparation. Therefore, a known amount of TCS can be added to PEG-TCS and the recovery of TCS can be determined. PEG-TCS interference should first be excluded in the TCS determination so the interference experiment can be reliable.

The TCS interference experiment in PEG-TCS was performed as follows: About 1 ml of TCS and 0.5 ml of PEG-TCS + 0.5 ml of TCS were as the first and second test samples. They were determined by the Lowry method, and the differences of the measured values were compared.

The recovery experiment of TCS in PEG-TCS was performed as follows: PEG-TCS was diluted to about 100 µg/ml. After quantification by the Lowry method, TCS was accurately diluted to 100 µg/ml. PEG-TCS and TCS at 1:1, 2:3, and 3:2 volume ratios were mixed. The TCS concentrations were 50, 60 and 40 µg/ml. Correspondingly, the PEG-TCS concentrations were 50, 40 and 60 µg/ml, respectively. The recovery of TCS in PEG-TCS was calculated after the determination.

Linear study

PEG-TCS diluted to 100 µg/ml was collected from 0.3, 0.4, 0.5, 0.6 and 0.7 ml twice by parallel measurements.

Repeatability (precision)

The measurement of PEG-TCS samples was repeated five times.

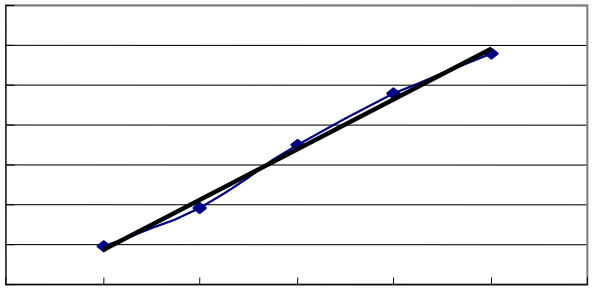

Figure 2. PEG-TCS standard linear equation.

RESULTS AND DISCUSSION

Residue PEG SDS-PAGE determination

Three batches of samples of polyethylene glycol staining results are shown as follows: there is no band in the position of free PEG, and the two samples on the right are 15 μg/ml PEG, that is, the reference substance band. Reference Roche Ltd. pegylated interferon α-2a injection of free the mPEG content standards, PEG-TCS injection of the free PEG content using polyethylene glycol staining should be less than 15 μg/ml.

In this study, the MW of TCS is 27 KD while the MW of mPEG-ALD is 20KD. Its big hydrodynamic volume caused the high position of mPEG in Figure 1. Amphiphilic of SDS-PAGE made mPEG not to be fully wrapped by SDS as other proteins, but part of the diffusion, even with the same amount of shallow strip.

SDS-PAGE is always a very important and conventional method whether it is for PEG modification reaction process monitoring, or for PEGylated proteins quality control after the separation, and SDS-PAGE is also essential to the protein drugs detection method in the Pharmacopoeia. SDS-PAGE staining method for PEGylated protein systems mainly has three kinds: barium iodide staining, coomassie brilliant blue staining and silver staining. Wherein, coomassie brilliant blue staining and silver staining mainly dye the protein-containing parts (including the unmodified protein, protein modification), while barium iodide staining can dye PEG parts (including the PEGylated protein, free PEG).

Residue PEG interference experiment

The absorption of PEG is free at 605 nm, and the three concentrations of PEG were added to the standard protein to determine the non-interference in the concentration. The concentration of PEG (≤20 μg/ml) within PEG-TCS interference was not evident. The results are shown in Table 1. The standard linear equations of the proteins are shown in Table 2.

PEGylation interference experiment

RD_1 (%) = (73.48 - 70)/70 × 100% = 5.0%

RD_2 (%) = (429.4-402.65)/429.4 × 100% = 6.2%

Recovery test

The TCS recovery of the interference of PEG-TCS is shown in Table 3. The recoveries of TCS were 97.5, 104 and 105%. The RSD was 4.1%, as shown in Table 4.

The OD values were substituted into the respective standard curve equation, and the concentrations of TCS (C1, C2, and C3) were measured. The recoveries were (48.73/50) × 100% = 97.5%, (62.4/60) × 100% = 104%, and (42.01/40) × 100% = 105%.

Linear study

The linear equation of PEG-TCS was Y = 0.0025X + 0.0005, R^2 = 0.9998, R ≥ 0.999, shown in Figure 2. In the Lowry method experiment, when the phenol reagent stock solution was doubly diluted, the sensitivity increased.

Repeatability (precision)

The PEG-TCS samples were tested five times, and the OD values were 0.128, 0.134, 0.129, 0.130, and 0.132. The RSD was 1.8%; therefore, the precision passed.

Sample determination

Three batches of PEG-TCS samples (20090818, 20090826, and 20100120) were determined. The contents were 1.08, 0.98 and 1.01 mg/ml, respectively.

Conclusions

The concentration of PEG (≤20 μg/ml) within the PEG-TCS interference is not significant. According to the PEG staining results of electrophoresis measurements, the amount of residual PEG is much less than 15 μg/ml. This finding is verified by the amount of residual PEG using the Lowry method in the determination of PEG-TCS concentration with no interference. The TCS and PEG-TCS differences in the rate are within reasonable limits (≤15%). Therefore, the protein concentration of PEG-TCS can be measured by Lowry method.

Methodology validation reveals that PEG-TCS has no effect on the determination of TCS recovery as qualified

Table 1. PEG interference results for sample determination.

Sample	0	0.1	0.2	0.3	0.4	0.5	PEG	PEG-TCS
Standard protein (OD value)	-	0.049	0.090	0.121	0.158	0.170	0.001	0.126
Protein (add 10 μg/ml PEG)	-	0.041	0.084	0.142	0.168	0.226	0.003	0.131
Protein (add 15 μg/ml PEG)	-	0.044	0.088	0.127	0.159	0.226	0.002	0.136
Protein (add 20 μg/ml PEG)	-	0.046	0.093	0.131	0.172	0.226	0.002	0.130

Table 2. Standard linear equations.

Sample	Linear equation of standard protein		PEG-TCS (μg/ml)	RSD
Standard protein + PEG-TCS	Y = 0.0017X + 0.0117	R^2=0.975	67.24	5.9%
Standard protein+PEG-TCS+10 μg/ml PEG	Y = 0.0022X - 0.0019	R^2=0.994	60.4	
Standard protein+PEG-TCS+15 μg/ml PEG	Y = 0.0022X - 0.0008	R^2=0.991	62.18	
Standard protein+PEG-TCS+30 μg/ml PEG	Y = 0.0022X + 0.0009	R^2=0.998	58.62	

RSD, Relative Standard Deviation.

Table 3. TCS interference results.

Sample	0	0.1	0.2	0.3	0.4	0.5
TCS (100 μg/ml) OD value	-	0.018	0.037	0.058	0.074	0.091
0.5 ml PEG-TCS (100μg/ml) and TCS (100 μg/ml) OD value	-	0.015	0.031	0.055	0.079	0.088

Note: T test reveals two kinds of state of the measured TCS concentration; P ≥ 0.05, no significant difference.

Table 4. Results of the call back.

Standard protein (μg/ml)	Standard protein-containing (50 μg/ml PEG-TCS)	50 μg/ml PEG-TCS + 50 μg/ml TCS	Standard protein-containing (40 μg/ml PEG-TCS)	40μg/ml PEG-TCS + 60 μg/ml TCS	Standard protein-containing (60 μg/ml PEG-TCS)	60 μg/ml PEG-TCS + 40 μg /ml TCS
0	0	OD1 = 0.099	0	OD2 = 0.098	0	OD3 = 0.094
20	0.045	Y = 0.0025X + 0.005	0.038	Y = 0.0019X + 1E-04	0.035	Y = 0.0016X + 0.0028
40	0.080	R^2 = 0.9978	0.072	R^2 = 0.9983	0.065	R^2 = 0.997
60	0.123	C1 = 48.73	0.116	C2 = 62.4	0.102	C3 = 42.01
80	0.165		0.154		0.126	
100	0.196		0.185		0.156	

by TCS exploration. PEG-TCS has a good linear relationship within the 30 to 70 µg/ml concentration range. Based on the aforementioned results, three batches of PEG-TCS samples are successfully determined.

Xing et al. (2006) indicated that Lowry method for the determination of PEGylated proteins had error and suggested using Bradford method instead. They concluded that this only depends on the fact that the Lowry method was interfered by N-hydroxysuccinimide (NHS) which is produced when using SC-PEG5000 as PEG reagent; however, they neglected the NaCl, Na_2SO_4 and $(NH_4)_2SO_4$ (used in ion-exchange, size-exclusion, and hydrophobic interaction chromatography, respectively), which interfered in the determination of Bradford method. Furthermore, presently, site-PEGylation has more advantage but NHS is more popular just as the site-PEGylation process of first modifying TCS using mPEG-ALD 20K.

In this study, taking the PEG-TCS as an example, a method is designed to examine whether site-PEGylation interfered with protein content determination in the Lowry method, and a methodology validation is proposed.

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