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Report

Determination of Degree of Substitution of Formyl Groups in Polyaldehyde Dextran by the Hydroxylamine Hydrochloride Method

Huiru Zhao[1] and Ned D. Heindel[1,2]

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Colorimetric or potentiometric titration of the aldehyde residues in polyaldehyde dextran by the hydroxylamine hydrochloride/sodium hydroxide method has been found to be a convenient and accurate method to determine formyl content. Nitrogen combustion analyses on the isolated oximes confirmed the titrametric results.

KEY WORDS: polyaldehyde dextran; formyl content; hydroxylamine titration; drug conjugations.

INTRODUCTION

Recently, conjugates of polyaldehyde dextran (PAD) have been avidly exploited in pharmaceutical research for delivery/controlled release of drugs or toxins and as drug-bearing spacers in immunoconjugates such as those transporting doxorubicin and cytosine arabinoside (1–5). Studies from our laboratory have involved ellipticine and CI-921 conjugates of PAD (6). Researchers have found that this multivalent carrier can decrease the cytotoxicity of certain anticancer drug-ligands, can increase the metabolic stability of a drug or an enzyme, and can amplify the number of the drug molecules conjugated to the desired antibody with minimum distortion of the binding avidity of the antibody (1–5). The conjugation of a drug or a toxin to PAD has been based upon the reaction between amino groups or hydrazide groups in the drug or toxin and aldehyde groups in PAD to form Schiff bases or hydrazones.

The techniques for preparation and purification of PAD are well described in the literature but there is a paucity of analytical techniques for assessing the number of formyl groups introduced by the traditional periodate oxidation. Accurate drug loading can be achieved by controlling the ratio of coupling ligand to available aldehydes but only if the degree of substitution of the latter is accurately known (6). Published techniques which would seem suitable for formyl-group quantification include titrations with 3,5-dinitrosalicylic acid/Rochelle salt (DNSA) (7) or $NaHSO_3$ (3) and colorimetric analyses with Purpald reagent (8) or *N*-(2,4-dinitrophenyl)-β-alanylglycylglycine hydrazide reagent (9). The first two assays presume quantitative oxidation of all the formyls, while the latter two methods presume quantitative reaction between the aldehyde groups in PAD and a hydrazide functionality in a chromogenic reagent.

Each method has some serious limitation in precise determination of aldehyde content in PAD. The DNSA method is suitable for aldehyde concentration in compounds with fewer than six carbons (10). In the case of the formyl-containing polymer, it gives false-positive results. The second technique requires the use of an oxidatively labile titrant ($NaHSO_3$) which must be restandardized before each titration. The latter two assays, carried out in basic media, have been shown to be adequate for quantifying the aldehyde concentration in small molecules where Beer's law applies. In our hands, however, Purpald reagent's rate of reaction and the color of its chromophore product were highly variable with structural features in the aldehyde. When Purpald was reacted with butyraldehyde, the recommended calibration standard, the purple product is indeed formed rapidly with maximum absorbance at 535 nm. This λ_{max} was, in fact, invariant for most aldehydes. However, in the case of isobutyraldehyde, glucose, or PAD, a yellow chromophoric product was formed with a λ_{max} of 400 nm. Thus, this method lacks suitable universal standard. The reagent required for the hydrazide titration, *N*-(2,4-dinitrophenyl)-β-alanylglycylglycine hydrazide, is not widely available.

In this study, we have applied the hydroxylamine hydrochloride titration to the determination of degree of substitution of aldehydes in PAD, which proved to be reliable without using a standard. The analytical reaction is as follows:

$$\text{dextran–(CHO)}_n + H_2N\text{–}OH \cdot HCl = \text{dextran–(CH=N–OH)}_n + H_2O + HCl \quad (1)$$

$$HCl + NaOH = NaCl + H_2O \quad (2)$$

When hydroxylamine hydrochloride in methyl orange indicator is reacted with the aldehydes in PAD at pH 4, a dextran polyoxime is produced, releasing an HCl equivalent for each formyl residue. The dextran polyoxime so produced can be isolated and analyzed for nitrogen content by combustion as a confirmation of the titration results.

EXPERIMENTAL

Materials

Dextran T-40 (MW 40 KDa) was provided by Pharmachem Inc., Bethlehem, PA. Purpald was obtained from Sigma. The polyaldehyde dextrans (PAD) with various degrees of formyl content were synthesized by dextran oxidation using differing ratios of periodate (11). All other chemicals were of analytical or reagent grade.

Analytical Procedures

Preparation of 0.25 N *Hydroxylamine Hydrochloride Solution.* Hydroxylamine hydrochloride, reagent grade, 17.500 g dried to constant weight, was dissolved in 150 ml of distilled water to which was added 6.0 ml of methyl orange reagent (0.05%). The solution was diluted to a volume of 1 liter and the pH adjusted to 4.0.

Determination of Aldehyde Concentration in PAD. The PAD analytes, dried to constant weight with sample sizes ca. 0.100 g, were dissolved in 25 ml of 0.25 *N*-hydroxylamine hydrochloride-methyl orange solution. Each mixture was al-

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[1] Department of Chemistry, Lehigh University, Bethlehem, Pennsylvania 18015.

[2] To whom correspondence should be addressed.

lowed to stand for 2 hr and was then titrated with standardized sodium hydroxide solution until the red-to-yellow end point was achieved by matching the color of the sample solution with that of a blank one. Alternatively, the change of pH with volume of added standard base was recorded (Fig. 1) so that equivalent volume could be determined by the first derivative of the titration curve (Fig. 2). Thereby, degrees of substitution of –CHO functions were calculated based on the sample weights (grams) taken as indicated: 0.126 g for $L = 94$; 0.060 g for $L = 160$; 0.104 g for $L = 209$; 0.095 g for $L = 274$; and 0.091 g for $L = 292$.

As a check on the accuracy of the titration, the weighed samples were converted to their oximes, recrystallized from water, dried to constant weight, and analyzed. The aldehyde content in these PADs was calculated from the combustion results for percentage nitrogen.

The degree of aldehyde substitution was calculated by

$$\frac{(\text{vol in ml}_{\text{NaOH}} \times N_{\text{NaOH}}) \times 10^{-3}\ \text{mol}}{\text{weight of sample (g)} \times (\text{dextran MW})} = \frac{\text{mol of CHO}}{\text{mol of dextran}}$$

RESULTS

Table I shows the results of the hydroxylamine hydrochloride titration of the polyaldehyde dextrans with differing degrees of aldehyde substitution to the colorimetric end points or the potientiometric end points (by first derivative of the titration curve for pH vs volume). Table I also shows the determination of aldehyde load by nitrogen combustion analysis of the precipitated PAD oximes. The degrees of aldehyde substitution calculated from these three assay methods are in excellent internal agreement. We analyzed PADs having aldehyde content from 90 to 285 formyl residues per mol of dextran. Marked deflections in the PAD titration curves (Fig. 1) and in the first derivatives (Fig. 2) are evident no matter what aldehyde content exists in the samples. Values are replicable with CV of 0.85% and the titration is equally applicable to PADs of both higher load and lower load.

DISCUSSION

The degree of substitution of aldehyde in PAD has been determined by the hydroxylamine hydrochloride titration method (13). The accuracy of the assay was checked by combustion analyses for percentage nitrogen in the isolated, purified oximes of the PAD. Minor variations were observed in the results obtained by titration from those obtained by oxime combustion analyses. As shown in Table I, the load of aldehyde determined from the titration data is greater than the load from combustion analyses in samples 1, 3, and 4. Is this difference statistically acceptable? The data were analyzed by F-test and t-test statistics (14). When the precision of the data in each set was checked by F test, the variance for the hydroxylamine titration was 74.4, whereas the variance for nitrogen combustion analysis was 74.5. Thus $F(5,5) = S_1^2/S_2^2 = 0.998$. Employing the F-distribution table, we find $F(5,5)_{0.05} = 5.05$, thereby demonstrating that the precision of the two data sets is comparable.

In further support we calculated the $t(n_1 + n_2 - 2)$ to probe whether these two methods show different results and thereby determined $t(10)_{\text{sample}} = 0.12$. From the t distribution table we find $t(10)_{0.05} = 2.228$. Since the measured variation in our study is less, it can be concluded that the differences between the results obtained by these two methods are insignificant. Moreover, one can rely on the results obtained from hydroxylamine hydrochloride titration alone.

A possible explanation for the higher formyl values obtained in the titration may be residual periodate in the sample solution, which oxidatively consumes hydroxylamine and raises apparent –CHO values. Alternatively, there could be real loss of aldehyde content in PAD, which is known to undergo autodecomposition on standing, in the process of isolating the dextran polyoxime (12). In the hydroxylamine titration, the equivalence point can be detected by visual or potentiometric methods. While the latter technique is presumably more accurate, the colorimetric/visual approach for defining the endpoint is more facile and equally accurate in routine use.

For accurate analyses it is essential to dissolve the PAD sample completely in the hydroxylamine hydrochloride solution. While dextran itself is readily water soluble, PADs containing high aldehyde content become much less soluble. Furthermore, a minimum of 2 hr of incubation of the PAD and hydroxylamine hydrochloride is required for complete oxime formation. Reactions which were sealed and retained for up to 2 months before titration did not yield higher formyl

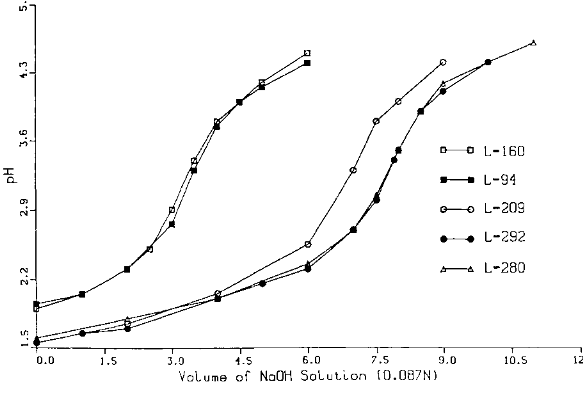

Fig. 1. Hydroxylamine hydrochloride potentiometric titration of various polyaldehyde dextrans.

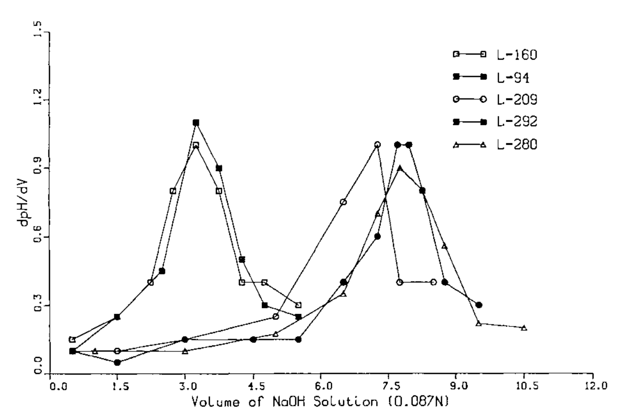

Fig. 2. Hydroxylamine hydrochloride potentiometric titration (first derivative).

Table I. Aldehyde Loads by Hydroxylamine Titration or Nitrogen Analyses

Sample No.	Load of aldehyde by titration[a]			N% (found) in oxime	Load of aldehyde by combustion[b]
	Visual	Potentiometric	Average		
1	93.6	94.0	94	2.75	81
2	165.2	160.0	163	5.51	166
				5.33	161
3	216.5	209.0	213	6.52	201
4	274.5	292.0	283	8.52	267
5	285.6	274.0	280	8.97	282

[a] Aldehyde analyses (formyls/mol of dextran) are the averages of three duplicate runs. Standard deviations were < ±3% except for Run 5, which was ±5%. Potentiometric titration values were obtained from the first derivative of the titration curves.

[b] Nitrogen analyses were performed by Dr. Paul Winslow, Quantitative Technologies, Bound Brook, NJ, on a Perkin Elmer Model 2400 analyzer. Calibration variations observed on test standards on the Model 2400 analyzer are ±0.3% over the range of absolute values in these samples, which corresponds to ±9 formyl units per dextran (15).

numbers upon titration. The hydroxylamine titration assay was replicable with a coefficient of variation of 0.85%.

Representative sampling is a key factor in the titration assay. In these studies we found that a sample weight of more than 0.050 g generates the most significant shift in the equivalent point, thereby improving the sensitivity of the detection. We have already shown that these PADs with known degrees of formyl substitution are useful for conversion into drug hydrazide conjugates of desired load by careful control of the reacting drug/formyl ratio (6).

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