METHOD FOR ANALYZING ACTIVATED POLYETHYLENE GLYCOL COMPOUNDS

BACKGROUND OF THE INVENTION

The instant invention relates to chemical analysis methods for analyzing polyethylene glycol compounds. More particularly, the instant invention relates to the analysis of polyethylene glycol compounds by liquid chromatography under "critical conditions". The polyethylene glycol compounds of the instant invention have been "activated" to facilitate chemical modification of physiologically active materials, which modified materials are applicable, for example, in drug delivery systems.

Biologically active compounds conjugated with polyoxyalkylenes can provide enhanced biocompatibility for the compound, See, for example, USP 5,366,735 and USP 6,280,745. A review of this subject by Zalipsky, in Bioconjugate Chem., 1995, 6, p150-165, identified polyethylene glycol as one of the best biocompatible polymers to conjugate with a biologically active compound (such as a drug, a protein, a peptide or an enzyme) to produce a conjugate having improved properties such as compatible solubility characteristics, reduced toxicity, improved surface compatibility, increased circulation time and reduced immunogenicity.

Polyethylene glycol (PEG) is a linear polyoxyalkylene terminated at the ends thereof with hydroxyl groups and generally represented by the formula: $HO(CH_2CH_2O)_nH$. Monomethoxy polyethylene glycol (mPEG) is generally represented by the formula: $CH_3O(CH_2CH_2O)_nH$. mPEG can be "activated" with a group "A" that will couple with a group of the biologically active material. Activated mPEG is generally represented by the formula: $CH_3O(CH_2CH_2O)_nA$. For example, trichloro-s-triazine activated mPEG will couple to an amine group of a biologically active material, as discussed by Henmanson in Chapter 15 of Bioconjugate Techniques (1996). Di-activated linear PEGs are also useful in formation of hydrogels.

More recently, so called "second generation" PEGylation chemistry has been developed to, for example, minimize problems of diol impurity contamination of mPEG, to increase the molecular weight of the PEG and to increase stability of the linker, see Roberts et al., Advanced Drug Delivery Reviews 54 (2002) p459-4. United States Patent 6,455,639

described an increased molecular weight mPEG having narrow molecular weight distribution.

Liquid chromatography under critical conditions has become an important method for polymer analysis, see, for example, Gorbunov et al., J. Chrom A, 955 (2002) 9-17. Liquid chromatography under critical conditions has been used to determine polyethylene glycol in mPEG (see, for example, Baran et al., J. Chrom. B, 753 (2001) 139-149; and Kazanskii et al., Polymer Science, Series A, Vol 42, No. 6 (2000), p585-595. However, the degree of resolution of the polyethylene glycol and mPEG peaks is poor when the molecular weight of the mPEG is 5,000 grams per mole or more (see Fig. 2 of the Kazanskii et al. reference). And, liquid chromatography under critical conditions has not been used to analyze activated mPEG.

SUMMARY OF THE INVENTION

The instant invention is the discovery that liquid chromatography under critical conditions (LCCC) can be used to analyze activated PEGs for residual unactivated PEGs and PEGs with different degrees of activation. For example, LCCC can be used to determine the relative amounts of non-activated mPEG alcohol and PEG diol, mono-activated mPEG and PEG diol, and di-activated PEG in an activated mPEG even when the molecular weight of the mPEG is 5,000 grams per mole or more. Furthermore, derivatization of the activated PEG can increase chromotagraphic resolution. Thus, for example, derivitization of a mono-activated mPEG containing di-activated PEG according to the instant invention can increase their chromatographic resolution. In addition, derivatization of the mono-activated mPEG containing di-activated PEG diol according to the instant invention can facilitate ability to detect the PEGs after they have been separated by chromatography.

Thus, the instant invention in one embodiment is a chemical analysis method comprising providing a sample of a composition comprising PEGs activated with activating group A and chromatographing the sample by liquid chromatograpy under critical conditions to determine the relative amounts of PEGs having various degrees of activation in the sample.

More specifically, the instant invention in one embodiment is a chemical analysis method for the determination of $RO(CH_2CH_2O)_nH$, $RO(C_2H_4O)_nA$, and $AO(C_2H_4O)_nA$ in a

mixture thereof, wherein R is hydrogen or an alkyl group, A is a functional group for coupling with a surface or a biologically active material or an other thing of use and n is an interger greater than 10, comprising the step of: chromatographing a sample of the mixture by liquid chromatography under critical conditions to determine the relative amounts of $RO(CH_2CH_2O)_nH$, $RO(C_2H_4O)_nA$, and $AO(C_2H_4O)_nA$ in the mixture.

In another embodiment the instant invention is a chemical analysis method for the determination of $RO(CH_2CH_2O)_nH$, $RO(C_2H_4O)_nA$, and $AO(C_2H_4O)_nA$ in a mixture thereof, wherein R is a hydrogen or an alkyl group, A is a functional group for coupling with a surface or a biologically active material or another thing of use and n is an interger greater than 10, comprising the steps of: (a) derivatizing the A groups of the mixture with a derivatizing agent to form a derivatized mixture comprising $RO(CH_2CH_2O)_nH$, $RO(C_2H_4O)_nAD$, and $DAO(C_2H_4O)_nAD$, wherein AD is the derivatized A group; and (b) chromatographing a sample of the derivatized mixture by liquid chromatography under critical conditions to determine the relative amounts of $RO(CH_2CH_2O)_nH$, $RO(C_2H_4O)_nAD$, and $DAO(C_2H_4O)_nAD$ in the derivatized mixture.

As an alternative embodiment the hydroxyl, R or A groups may occur more than twice and/or at locations other than at the end of PEG chains.

Thus, in another embodiment, the instant invention is a chemical analysis method comprising providing a sample comprising polyethylene glycol compounds of the formula $M[O(CH_2CH_2O)_nB]_x$, wherein

M is a small molecule or a polymer backbone with at least x locations for linking with the $O(CH_2CH_2O)_nB$ groups;

n is independently in each occurrence an integer of one or greater, preferably 5 or greater, more preferably 10 or greater and n is preferably less than 2500, more preferably less than 2000, and more preferably less than 1000;

B is independently in each occurrence hydrogen or A, wherein A is as defined above; and

x is an integer of 2 or more, preferably 3 or more;

and chromatographing a sample of the mixture by liquid chromatography under critical conditions to determine the relative amounts of the polyethylene glycols having various degrees of activation with group A. As a variant of this, prior to the step of chromatographing, the compounds may be derivatized with a group D.

In another embodiment, the method of this invention may be used to analyze mixtures of activated polyethyene compounds where the activating group is bonded as a pendant group to the polymer comprising PEG. Thus, according to this embodiment the sample comprises compounds of the formula: $BO[(CH_2CH_2O)_nM]_m(OCH_2CH_2)_pOB$ wherein

M is a small molecule that is attached to two PEGs and has a pendant group comprising B,

B is independently in each occurrence H or A,

m, n, and p are independently integers greater than 1,

A is as defined above. As a variant of this, the compounds may be derizatized with a group D.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a reproduction of a chromatogram showing the separation of mPEG, propionaldehyde diacetal activated mPEG and propionaldehyde diacetal activated diol;

Fig. 2 is a reproduction of a chromatogram showing the separation of mPEG alcohol, mesylate activated mPEG and mesylate activated diol; and

Fig. 3 is a reproduction of a chromatogram showing the separation of mPEG alcohol, para-nitrophenyl carbonate activated mPEG and para-nitrophenyl carbonate activated diol.

DETAILED DESCRIPTION

The compounds to be analyzed by one method of the instant invention are represented by the formulas I, II and III:

(I) $RO(C_2H_4O)_nA$;

(II) $AO(C_2H_4O)_nA$; and

(III) $RO(C_2H_4O)_nH$

wherein R represents (independently in each occurrence) a hydrogen or a C_{1-7} hydrocarbon group (usually a methyl group), n represents the average number of moles of C_2H_4O groups, e.g., from 10 to 2000 and A is the "activating" group. In many applications, the compound of formula I is the desired material. The compound of formula III is non-activated PEG which is unreactive. The compound of formula II is diactivated PEG which is produced from PEG diol impurity. Thus, the sample to be analyzed usually consists primarily of the

compound of formula I with relatively lower levels of the compounds of formulas II and III in a mixture. The relative amounts of the compounds of formula I, II and III are determined by chromatographing a sample of the mixture by liquid chromatography under critical conditions to determine the relative amounts of $RO(CH_2CH_2O)_nH$, $RO(C_2H_4O)_nA$, and $AO(C_2H_4O)_nA$ in the mixture.

It should be understood that the instant invention in its full scope includes PEG copolymers (for example, random or block copolymers comprising C_2H_4O groups) and any polymer topology including but not limited to linear, branched, comb and star topologies.

Activating group A may be any functional group known to be useful in activating PEGs for subsequent reaction, for example, coupling to physiologically active agents. Non-limiting examples of such activating groups include halides, sulfonates (e.g. mesylate), isocyanates, amines, hydrazines, aminooxys, thiols, carboxylic acids, esters, carbonates (e.g. para-nitrophenyl carbonate), amides, carbamates, aldehydes (e.g. propionaldehyde), acetals (e.g. propionaldehyde acetal), ketones, ketals, maleimide, vinyl sulfone, iodoacetamide, and disulfides (e.g. pyridyl sulfides).

It should be understood that a certain degree of experimentation is required to achieve liquid chromatography under critical conditions. However, reference to the literature will direct the person of ordinary skill in the art of liquid chromatography to the necessary conditions, see, for example, Gorbunov et al., J. Chrim A, 955 (2002) 9-17. Critical condition LC tends to separate polymers based on the composition of the end groups of the polymer and is (in theory) independent of the polymers molecular weight. For example, if a reverse phase column is operated under critical conditions, then polymers with hydrophobic end groups can be separated from polymers with hydrophilic end groups. The LCCC can be run isocractically, or initially isocratically followed by gradient operation. While it is desirable that conditions be critical, operation at conditions slightly off of critical will also provide some proportional determination of the various functionalized PEG in the sample.

EXAMPLE 1

0.1 gram of propionaldehyde diacetal activated 5,000 weight average molecular weight mPEG is mixed with 3 milliliters of water to produce a sample for injection. 5 microliters of the sample for injection is injected into a mobile phase of 52%A and 48%B (where A is 47% acetonitrile in water and B is 43% acetonitrile in water) at a mobile phase

flow rate of 0.75 milliliters per minute and flowed through a 5 micrometer packing diameter Supelco LC-18 reverse phase column at a column temperature of 30 degrees Celsius, the column having an internal diameter of 4.6 millimeters and a length of 250 millimeters, followed by an evaporative light scattering detector to produce the chromatogram shown in Fig. 1. The chromatogram of Fig. 1 shows a peak at about 3.8 minutes for mPEG, a peak at about 4.8 minutes for the activated mPEG and a peak at about 5.6 minutes for the activated diol. The relative amounts of the various PEGs is determined from the peak area and the response factor of the evaporative light scattering detector.

Evaporative light scattering detection is well-known in liquid chromatography, see, for example, Rissler, J. Chrom. A, 742 (1996) 45.

EXAMPLE 2

0.1 gram of mesylate activated 5,000 weight average molecular weight mPEG is mixed with 3 milliliters of water to produce a sample for injection. 5 microliters of the sample for injection is injected into a moblile phase of 52%A and 48%B (where A is 47% acetonitrile in water and B is 43% acetonitrile in water) at a mobile phase flow rate of 0.75 milliliters per minute and flowed through a 5 micrometer packing diameter Supelco LC-18 reverse phase column at a column temperature of 30 degrees Celsius, the column having an internal diameter of 4.6 millimeters and a length of 250 millimeters, followed by an evaporative light scattering detector to produce the chromatogram shown in Fig. 2. The chromatogram of Fig. 2 shows a peak at about 3.8 minutes for mPEG, a peak at about 4.4 minutes for the activated diol and a peak at about 4.9 minutes for the activated mPEG. The relative amounts of the various PEGs is determined from the peak area and the response factor of the evaporative light scattering detector.

EXAMPLE 3

0.1 gram of para-nitro phenyl carbonate activated 20,000 weight average molecular weight mPEG is mixed with 3 milliliters of water to produce a sample for injection. 5 microliters of the sample for injection is injected into a moblile phase of 52%A and 48%B (where A is 47% acetonitrile in water and B is 43% acetonitrile in water) at a mobile phase flow rate of 0.75 milliliters per minute and flowed through a 5 micrometer packing diameter Jupiter C-18 reverse phase column at a column temperature of 29 degrees Celsius, the

column having an internal diameter of 4.6 millimeters and a length of 150 millimeters, followed by an evaporative light scattering detector to produce the chromatogram shown in Fig. 3. The chromatogram of Fig. 3 shows a peak at about 3 minutes for mPEG, a peak at about 4.8 minutes for the activated mPEG and a small peak at about 8.5 minutes for activated diol. The relative amounts of the various PEGs is determined from the peak area and the response factor of the evaporative light scattering detector.

DERIVATIZED ACTIVATED mPEG

In another embodiment, the instant invention is a chemical analysis method for the determination of the above discussed activated PEGs comprising derivatizing the A group in the PEGS with a derivatizing agent D.

Thus, according to one embodiment the method comprises providing a sample of $RO(CH_2CH_2O)_nH$, $RO(C_2H_4O)_nA$, and $AO(C_2H_4O)_nA$ in a mixture, derivatizing the A groups of the mixture with a derivatizing agent to form a derivatized mixture comprising $RO(CH_2CH_2O)_nH$, $RO(C_2H_4O)_nAD$, and $DAO(C_2H_4O)_nAD$, wherein AD is the derivatized A group; and chromatographing a sample of the derivatized mixture by liquid chromatography under critical conditions to determine the relative amounts of $RO(CH_2CH_2O)_nH$, $RO(C_2H_4O)_nAD$, and $DAO(C_2H_4O)_nAD$ in the derivatized mixture. This embodiment of the instant invention is especially applicable when the activating group is hydrophilic, such as a group comprising, without limitation thereto, an aldehyde, a maleimide, an amine or a thiol. mPEGs activated with a hydrophilic group can be difficult to separate from mPEG alcohol, because the hydroxyl group of the mPEG alcohol is also hydrophilic. However, it has been discovered that if such activated mPEG is derivatized with a derivatizing agent which attaches a hydrophobic group to the A group of the activated mPEG, then the derivatized activated mPEG can be more readily separated from the non-activated PEG alcohol.

Similarly, mPEGs activated with a hydrophilic group can be difficult to separate from PEG diols activated with two hydrophilic groups, because the methyl group of the mPEG alcohol is not very hydrophobic. However, it has been discovered that if the A groups of the activated mPEG and the di-activated PEG are derivatized with a derivatizing agent which attaches a hydrophobic group to the A groups on both the activated mPEG and the di-

activated PEG, then the derivatized activated mPEG can be more readily separated from the derivatized di-activated PEG.

The derivatizing agent is therefore a group which reacts with the activating group A to alter the hydrophilicity/hydrophobicity of the group. Use of derivatizing agents where the derivatization is reversible back to the activating group A may also be desired. Non-limiting examples of suitable derivatizing agents includealcohols, amines, hydrazines (e.g. 1-(hydrazinocarbonylmethyl)pyridinium chloride and dinitrophenyl phenyl hydrazine, which react with PEG aldehydes (e.g. propionaldehyde), acetals, ketones, and ketals to form PEG hydrazones), aminooxys, thiols (e.g. naphthalene thiol, which reacts with PEG maleimide to form PEG thioethers), aldehydes, (e.g. aromatic aldehydes which react with PEG amines to form PEG imines), acetals, ketones, ketals, maleimides, vinyl sulfones, iodoacetamides, disulfides (e.g. dipyridyl disulfide, which react with PEG thiols to form PEG pyridyl disulfides), halides, sulfonates, isocyanates, carboxylic acids, esters, carbonates, amides, and carbamates.

The derivatizing agent most preferably imparts a detectable characteristic, e.g. ultra-violet (UV) chromaphore or fluorescent group, to the derivatized activated PEG (e.g. mPEG) to allow the derivatized activated PEG (e.g. mPEG) to be detected when it is eluted from the critical LC system. And, it should be understood that even when the activating group(s) A has(have) sufficient hydrophobic character to permit sufficient resolution of mono-activated (e.g. mPEG) from di-activated PEG in the critical LC chromatogram, it may never-the-less be desirable to derivatize the activated mPEG and di-activated PEG using a derivatizing agent that imparts sufficient detectable characteristic to be detected when it is eluted from the critical LC system.

CONCLUSION

In conclusion, it should be readily apparent that although the invention has been described above in relation with its preferred embodiments, it should be understood that the instant invention is not limited thereby but is intended to cover all alternatives, modifications and equivalents that are included within the scope of the invention as defined by the following claims.

WHAT IS CLAIMED IS:

1. A chemical analysis method for the determination of $RO(CH_2CH_2O)_nH$, $RO(C_2H_4O)_nA$, and $AO(C_2H_4O)_nA$ in a mixture thereof, wherein R is an alkyl group, A is a functional group for coupling with a surface or a biologically active material or other thing of use and n is an interger greater than 10, comprising the step of: chromatographing a sample of the mixture by liquid chromatography under critical conditions to determine the relative amounts of $RO(CH_2CH_2O)_nH$, $RO(C_2H_4O)_nA$, and $AO(C_2H_4O)_nA$ in the mixture.

2. The method of Claim 1, wherein R consists essentially of a methyl group.

3. The method of Claim 1 wherein A comprises an aldehyde group.

4. The method of Claim 3, wherein the aldehyde is propionaldehyde.

5. The method of Claim 1 wherein A is propionaldehyde diacetal.

6. The method of Claim 1 wherein A is mesylate.

7. The method of Claim 1 wherein A is para-nitrophenyl carbonate.

8. The method of Claim 1 wherein A comprises a maleimide group.

9. The method of Claim 1 wherein A comprises an amine group.

10. The method of Claim 1 wherein A comprises a thiol group.

11. The method of Claim 1 wherein A comprises a dithiol group.

12. The method of Claim 1 wherein the weight average molecular weight of the $RO(C_2H_4O)_nA$ is greater than 5,000 grams per mole.

13. The method of Claim 12 wherein the weight average molecular weight of the $RO(C_2H_4O)_nA$ is greater than 10,000 grams per mole.

14. A chemical analysis method for the determination of $RO(CH_2CH_2O)_nH$, $RO(C_2H_4O)_nA$, and $AO(C_2H_4O)_nA$ in a mixture thereof, wherein R is an alkyl group, A is a functional group for coupling with a surface or a biologically active material or other thing of use and n is an interger greater than 10, comprising the steps of: (a) derivatizing the A groups of the mixture with a derivatizing agent to form a derivatized mixture comprising $RO(CH_2CH_2O)_nH$, $RO(C_2H_4O)_nAD$, and $DAO(C_2H_4O)_nAD$, wherein AD is the derivatized A group; and (b) chromatographing a sample of the derivatized mixture by liquid chromatography under critical conditions to determine the relative

amounts of $RO(CH_2CH_2O)_nH$, $RO(C_2H_4O)_nAD$, and $DAO(C_2H_4O)_nAD$ in the derivatized mixture.

15. The method of Claim 14, wherein R consists essentially of a methyl group.

16. The method of Claim 14, wherein A comprises a carbonyl group.

17. The method of Claim 16, wherein A comprises an aldehyde group.

18. The method of Claim 17, whrein the aldehyde group is propionaldehyde.

19. The method of Claim 16, wherein the derivatizing agent is 1-(hydrazinocarbonylmethyl)pyridinium chloride.

20. The method of Claim 16, wherein the derivatizing agent is dinitro phenylhydrazine.

21. The method of Claim 14, wherein A is maleimide.

22. The method of Claim 21, wherein the derivatizing agent is thio-naphthalene.

23. The method of Claim 14, wherein A comprises an amine.

24. The method of Claim 23, wherein the derivatizing agent is an aromatic aldehyde.

25. The method of Claim 14, wherein A comprises a thiol group.

26. The method of Claim 25, wherein the derivatizing agent is ortho dipyridynyl disulfide.

27. The method of Claim 14, wherein the weight average molecular weight of the $RO(C_2H_4O)_nA$ is greater than 5,000 grams per mole.

28. The method of Claim 27, wherein the weight average molecular weight of the $RO(C_2H_4O)_nA$ is greater than 10,000 grams per mole.

29. A chemical analysis method comprising providing a sample of a composition comprising of PEGs activated with activating group A and chromatographing the sample by liquid chromatograpy under critical conditions to determine the relative amounts of PEGs having various degrees of activation in the sample.

30. The method of claim 29 wherein the sample comprises polyethylene glycol compounds

of the formula $M[O(CH_2CH_2O)_nB]_x$, wherein

M is a small molecule or a polymer backbone with at least x locations for linking with the $O(CH_2CH_2O)_nB$ groups;

n is independently in each occurrence an integer of one or greater, B is independently in each occurrence hydrogen or A; and

x is an integer of 2 or more,

and chromatographing a sample of the mixture by liquid chromatography under critical conditions to determine the relative amounts of the polyethylene glycols having various degrees of activation with group A.

31. The method of claim 29 wherein the sample comprises polyethylene glycols where the activating group A is pendant from the polymer chain.

32. The method of claim 31 wherein the sample comprises polymers of the formula:

$BO[(CH_2CH_2O)_nM]_m(OCH_2CH_2)_pOB$ wherein

M is a small molecule that is attached to two PEGs and has a pendant group comprising B,

B is independently in each occurrence H or A,

m, n, and p are independently integers greater than 1,

A is as defined above.

33. The method of any one of claims 29-32 further comprising derivatizing the A group with a derivatizing agent prior to chromatographing the sample.