Degradation of O^6-Benzylguanine in Aqueous Polyethylene Glycol 400 (PEG 400) Solutions: Concerns with Formaldehyde in PEG 400

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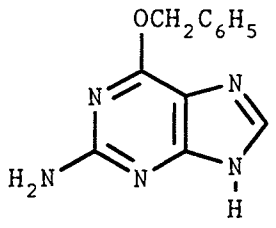
The degradation of O^6-benzylguanine (BG) in aqueous polyethylene glycol (PEG) 400 solution at room temperature had been investigated using chromatographic and spectrometric methods. The degradation of BG in this solvent appeared to arise from a reaction between BG and formaldehyde. The formaldehyde was present as an impurity in PEG 400 and probably formed through air oxidation of PEG 400. The major product of this reaction was believed to be a methylene-bridged compound containing two BG molecules. This was probably produced via an intermediate imine, a schiff base between one BG molecule and formaldehyde. This degradation reaction was the only observable reaction in the 40% PEG/water solvent (pH 8.0) i.e. degradation of the drug via hydrolysis was minimal under these conditions.

KEY WORDS: O^6-benzylguanine; polyethylene glycol; hydrolysis; cross-linked; formaldehyde; autoxidation.

Introduction

O^6-benzylguanine (NSC-637037, I) is an effective substrate for depleting the mammalian DNA repair protein, O^6-alkylguanine-DNA alkyltransferase. The depletion of alkyltransferase by O^6-benzylguanine has chemotherapy potential since it appears that therapeutic effectiveness of certain alkylating agents such as 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) is greater in cells lacking alkyltransferase (1,2). Furthermore, treatment with O^6-benzylguanine may provide valuable information on the role of the DNA repair protein in carcinogenesis and mutagenesis (2). Further evaluation of the clinical potential of this poorly water soluble agent (110 μg/mL) required the development of a parenteral dosage form containing 1–3 mg/mL.



I

It has been shown that O^6-benzylguanine degrades exclusively through hydrolysis under acidic conditions (3). The site of bond cleavage for hydrolysis was assigned to the benzylic carbon-oxygen bond leading to formation of guanine and benzyl alcohol as the major products. A transition state with considerable positive charge development at the benzylic carbon atom was suggested for this reaction. The purpose of the present study was to evaluate the stability of O^6-benzylguanine under neutral and basic pH-conditions and explore various strategies for development of a dosage form with the desired stability and concentration. An injectable formulation of O^6-benzylguanine in PEG 400 (40%), 0.05 M phosphate buffer, pH 8.0 (60%) was chosen for development. The chemical reactivity of O^6-benzylguanine in this solvent was studied further after initial studies showed the potential of precipitate formation not attributable to a polymorph or hydrate of O^6-benzylguanine or the very insoluble guanine degradation product.

The autoxidation reaction of polyethylene glycols is known to produce peroxides, aldehydes and carboxylic acids (4-6). The reactive organic peroxide intermediates have been implicated in the degradation of several drugs in formulations containing polyethylene glycols (7,8). This study attempted to identify the precipitate formed in O^6-benzylguanine formulations in aqueous PEG 400 and relate the degradation product(s) to the contaminants in PEG 400.

Experimental

Materials

O^6-benzylguanine was obtained from National Cancer Institute, Bethesda, MD. All other reagents were analytical grade and were used as received.

Chromatographic analysis of O^6-benzylguanine

The analysis of O^6-benzylguanine and its degradation products was accomplished using a LC system consisting of a Shimadzu LC-6A pump, a Rheodyne 7125 injection valve fitted with 20 μL loop, a Shimadzu SPD-6A UV detector set at 280 nm, and a 150 mm × 4.6 mm column packed with 5 μm ODS-Hypersil (Shandon). The mobile phase for O^6-benzylguanine analysis consisted of 50 parts of 0.05 M phosphate buffer, PB (pH 7.0), 50 parts of methanol and 1 mM tetrabutyl ammonium dihydrogen phosphate (TBA) and the flow rate was 1.5 mL/min. The mobile phase used for the analysis of the degradation product(s) obtained in aqueous PEG 400 (pH 8.0) consisted of 30 parts of 0.05 M phosphate buffer (pH 7.0), 70 parts of methanol and 1 mM TBA and the flow rate was 1.5 mL/min. Samples, if necessary, were diluted with the mobile phase prior to analysis. Experimental details for the kinetic measurements had been described previously (3).

Formaldehyde and peroxide Analysis

A colorimetric assay was employed for the analysis of formaldehyde content in PEG 400. This method was based on the formation of diacetyldihydrolutidine (λ_{max}, 412 nm) from formaldehyde and acetyl acetone in the presence of an

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excess of ammonium salt (9). The hydroperoxide content of PEG 400 was determined by the classical iodimetric method which involved the titration of iodine liberated from potassium iodide by peroxides with thiosulphate (10).

Long-Term Stability Studies

O^6-benzylguanine samples at 3 mg/mL in 40% PEG 400/ 0.05 M PB (pH 8.0) were sealed in glass ampules and stored at room temperature and 50°C. The ampules were drawn periodically and the samples analyzed for O^6-benzylguanine and its degradation products. At variable time intervals, a precipitate was noted in some ampules.

The precipitate was collected from the bottom of the ampules stored at room temperature and washed with PEG 400-water mixture followed by a washing with water. The precipitate was then dried under vacuum at ambient temperature. A known quantity of the precipitate was dissolved in DMSO and diluted in the mobile phase for HPLC analysis.

Mass spectrometry and NMR analysis

Fast Atom Bombardment mass spectra (FAB-MS) were obtained on an Autospec-Q mass tandem hybrid spectrometer (VG Analytical LTD, Manchester, UK) equipped with an Opus data system. The mass spectra were recorded in continuum mode while scanning 1200-100 amu at 15 sec/dec. Ionization was achieved using a cesium gun operated at 20 ke V energy and 1-2 μA emission. The sample in DMSO was added to dithiotheritol/dithioerytheritol (3:1) as the matrix. FAB collision activated decomposition (CAD) spectra were acquired after collision of the m/z 495 ion in the quadrupole collision cell. Argon was the collision gas and the pressure was set to attenuate the beam by 60%.

Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker AM-500 instrument in deutrated dimethyl sulfoxide. O^6-benzylguanine (I): NMR (^{1}H, 500 MHz, δ): 12.45 (s, 1H, N^9-H), 7.86 (s, 1H, C^8-H), 7.40 (m, 5H, Ar-H), 6.26 (s, 2H, NH_2), 5.50 (s, 2H, Ar-Ch_2). Precipitate (11): NMR (^{1}H, 500 MHz, δ): 12.58 (s, 2H, N^9-H), 7.86 (s, 2H, C^8-H), 7.40 (m, 10H, Ar-H), 7.08 (t, 2H, N^2-H), 5.51 (s, 4H, $ArCH_2$) 4.93 (t, 2H, N^2-Ch_2-N^2).

Results and Discussion

Solubility Studies

Equilibrium solubility of O^6-benzylguanine in various solvents was determined at 25°C after rotation in constant temperature bath for 24 h. Minimal degradation was observed over this period. The aqueous solubility of O^6-benzylguanine was only 110 μg/mL. The desired solubility of 1–3 mg/mL was achieved by the use of co-solvent systems, 40% propylene glycol/10% ethanol/water (3.4 mg/mL) and 40% PEG 400/water (5.0 mg/mL).

Chemical Stability

The kinetics of hydrolytic degradation of O^6-benzylguanine was investigated at 50 ± 0.2° C in aqueous solutions at pH values ranging from 1 to 8.5. O^6-benzylguanine degraded via apparent first-order kinetics at all pH values. A partial pH-rate profile between pH values 1 and 5 has been reported earlier (3). The pH of maximum hydrolytic stability was found to be in a range between pH 8.0 and pH 8.5. At pH 8.0 and 50°C, the apparent first-order rate constant was approximately 1.5×10^{-6} min^{-1}, corresponding to a half-life of 321 days.

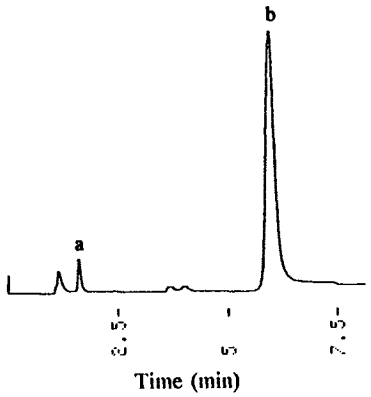
Parenteral formulation of O^6-benzylguanine

Based on the solubility data, a ready for use formulation of O^6-benzylguanine in 40% PEG 400 (Aldrich, Batch 1)-water at a concentration of 3 mg/mL was selected for further evaluation. As mentioned earlier, polyethylene glycols are known to undergo autoxidation in the presence of oxygen producing reactive organic peroxides. These hydroperoxides further degrade to give short-chain products such as carboxylic acids and aldehydes. The apparent pH of an aqueous solution of polyethylene glycol may vary anywhere from 5.5 to 3.5 depending upon how much autoxidation polyethylene glycol has undergone during storage (8). In order to suppress the acid-catalyzed hydrolysis of O^6-benzylguanine, pH of the formulation was adjusted to 8.0 with 0.05 M phosphate buffer. As one would expect on the basis of pH stability data, O^6-benzylguanine was found to be very unstable in aqueous PEG 400 (Aldrich, Batch 1) solution in the absence of any pH control (half-life of 8 days at 50°C).

During long-term stability studies on O^6-benzylguanine formulation (3 mg/mL) in 40% PEG 400/ 0.05 M phosphate buffer, pH 8.0 a slight loss (about 2%) was observed in the drug concentration during the first 14 days of storage at room temperature. This loss, which was not accompanied by formation of guanine and benzyl alcohol (normal products of benzylguanine hydrolysis) was initially believed to be a quantitation error. However, it became apparent that a reaction/s other than hydrolysis may be involved in the degradation of O^6-benzylguanine in PEG formulations, when very minute quantities of a white precipitate started to appear in the formulations stored at room temperature. It was possible to re-dissolve the precipitate by incubating the formulation at 50°C for a few hours which would explain why the formulations stored at 50°C remained precipitate free. HPLC analysis of the degraded sample in which precipitate had been re-dissolved did not show any evidence of the formation of degradation products. A loss in drug concentration was still observed. These results indicated that the precipitate was probably a slow forming product/s of a reaction other than hydrolysis and this product/s did not elute from the column under the conditions employed for O^6-benzylguanine analysis.

Product analysis

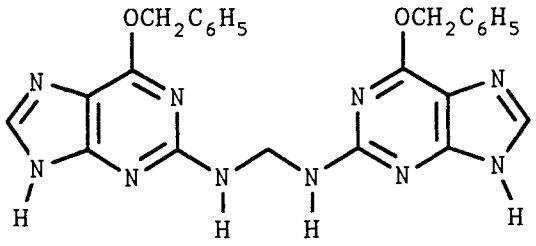
The concentration of the organic modifier (methanol) in the HPLC analysis was raised from 50% to 70% for the analysis of the isolated precipitate and the degraded O^6-benzylguanine sample. The chromatogram of the precipitate under new elution conditions consisted of a major late eluting peak (b) corresponding to the major degradation product, a small peak (a) due to O^6-benzylguanine appearing close to the solvent front and two other minor peaks (Fig. 1). The HPLC analysis of the degraded O^6-benzylguanine formulations in which precipitate had been re-dissolved by heating to 50°C also showed similar peaks (data not shown).



1. The HPLC chromatogram of the isolated precipitate showing degradation product peak, b, and O^6-benzylguanine peak, a. Mobile phase: 70 parts methanol, 30 parts 0.05 M PB, pH 7.0 and 1 mM TBA, Flow rate: 1.5 mL/min.

FAB-MS of a standard O^6-benzylguanine sample produced intense quasi-molecular ion peak, MH^+ at m/z 242. FAB-MS of the slow forming precipitate obtained from the aqueous PEG 400 O^6-benzylguanine formulation produced a 495 ion which was accompanied by a +22 adduct (m/z 517) when NaOAc was added to the sample/matrix system. A molecular weight of 494 was assigned to the degradation product.

Formaldehyde is a well known denaturing and interstrand cross-linking agent for nucleic acids (11–13). It has been shown previously that formaldehyde is formed during radiochemically initiated autoxidation of polyoxyethylene molecules (14) and air autoxidation of diethylene glycol (6). Based on FAB-MS data and the evidence that formaldehyde was present in the formulations as a contaminant of PEG 400 (see the following section), a structure in which amino functionalities of two O^6-benzylguanine molecules were cross-linked by a single methylene group was suggested for the major degradation product (II). The other possible products of this reaction include the hydroxy methyl derivative ($-NCH_2OH$), the Schiff base intermediate, and the corresponding derivatives of guanine. Similar methylene bridged products containing purines and cytosine have been isolated from formaldehyde-treated ribonucleic acid and deoxyribonucleic acid (11). The proton NMR spectra of the precipitate was also consistent with the proposed structure (see the experimental section). The NMR spectra for the degradation product was only slightly different from the O^6-benzylguanine spectra. It consisted of an extra peak due to the methylene group and one less amino proton per O^6-benzylguanine residue. The other major ions in the FAB-MS spectra of the precipitate had masses of 242 and 254 which could be rationalized at MH^+ and $(M+12)H^+$ fragments of structure II.



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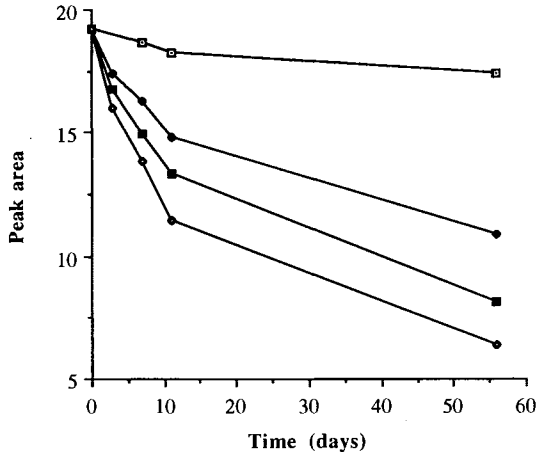
Colorimetric estimation of Formaldehyde in PEG 400

Different batches of PEG 400 used in this study were analyzed for their formaldehyde content using a colorimetric assay described by Nash (9). The conditions of this assay were mild enough to allow for formaldehyde determination in PEG 400, which is easily oxidized under the strong conditions used by some of the more popular methods of formaldehyde analysis (15). The results of formaldehyde analysis are reported in Table I. Assuming that the interference from other aldehydes was minimal (9), it can be concluded that most samples of PEG 400 used during initial formulation development work had significant levels of formaldehyde. The peroxide content of these PEG samples was also fairly high indicating that the samples had undergone extensive auto-oxidation during long-term storage (Table I). Freshly opened bottles of PEG 400 obtained from various suppliers contained only traces of formaldehyde (<3 ppm). It was pos-

Table I. Formaldehyde and peroxide content of various batches of PEG 400 obtained from various suppliers

Supplier/ lot #	Batch	# of months on shelf	Formaldehyde (μg/mL)	Peroxide (μeq. thiosulphate/ mL PEG)
Aldrich (06011MX)	1	10	194	4.0
Fisher (911150)	1	unknown	83	8.2
Union Carbide (UCC-B18224)	1	fresh	2.20	Traces
Aldrich (05105PZ)	2	fresh	0.62	n.d.
Fisher (933384)	2	fresh	0.50	n.d.

n.d. not determined.



2. Loss of O^6-benzylguanine with time from O^6-benzylguanine formulations (≈3 mg/mL) in 40% PEG 400/buffer (pH 8.0) containing varying amounts of formaldehyde; (1) □, no added formaldehyde; (2) ◆, 0.12 mg/mL formaldehyde; (3) ■, 0.24 mg/mL formaldehyde; (4) ◇, 0.48 mg/mL formaldehyde. The samples were stored at room temperature in sealed ampules.

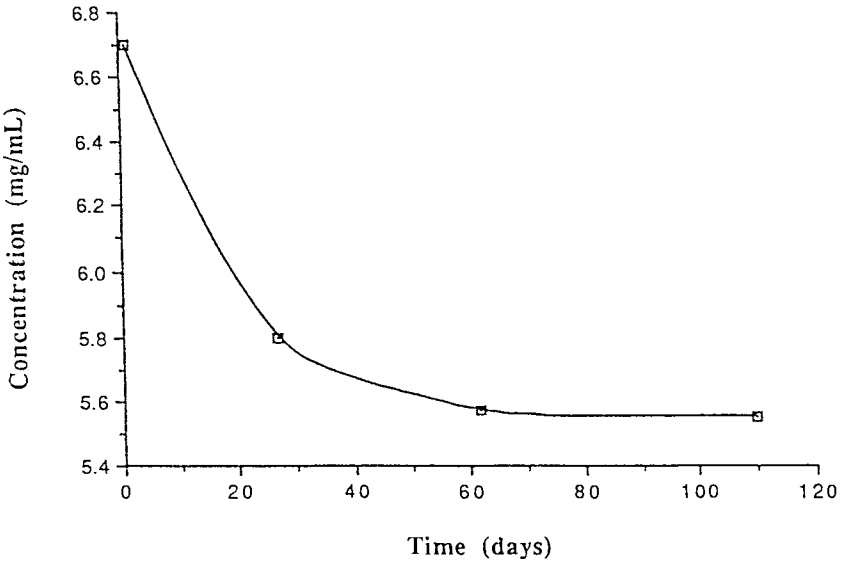
sible to slow down the autoxidation and maintain low levels of formaldehyde in the opened bottles of PEG by storing the PEG samples under argon in a refrigerator. For a given drug concentration, a relationship was found between the formaldehyde content of PEG and the time it took for the precipitate to first appear in the formulation (data not shown).

Controlled formaldehyde addition

In order to confirm that formaldehyde was involved in the degradation of the drug as suggested, controlled amounts of formaldehyde were added to the O^6-benzylguanine formulations in PEG 400 (Union Carbide, Batch 1)-phosphate buffer. The drug was rapidly lost from the solutions containing formaldehyde (Fig. 2). The rate at which drug was lost was proportional to the amount of formaldehyde present in the sample. A white precipitate was seen within a few days in all samples containing added formaldehyde. The reversed-phase HPLC analysis of this precipitate indicated that it was similar but not as pure as the slow forming precipitate seen earlier in the formulations. FAB-MS spectrum of this precipitate was quite similar to the one isolated in the earlier study with significant m/z 495 signal. FAB CAD was used to compare the 495 ion of the formulated precipitate obtained from the formaldehyde-spiked sample with the 495 ion of the slow forming precipitate from the stability samples. The CAD spectra from these two 495 ions were virtually identical producing product ions at m/z 404, 344, 254, 242 and 91.

Finally, in order to see if formaldehyde was continuously generated in the formulation during its storage, O^6-benzylguanine samples were prepared in glass ampules at 6 mg/mL in 60% PEG 400 (Aldrich, Batch 1)-40% 0.05M PB, pH 8.0. A higher concentration of PEG 400 was used in order to keep the degradation product in solution. The stability curve for this sample is shown in Fig. 3. As shown in the figure, the drug concentration slowly reached a plateau after a relatively steep initial drop. The observed loss might be attributed to the formaldehyde which was initially present in the formulation. Upon storage in the ampules, further oxidation of PEG 400 was considerably slowed down due to lack of oxygen and hence slowing any further supply of formaldehyde. The presence of water in the formulation is also shown to prevent further autoxidation of polyethylene glycols (7). On going studies are assessing the rate of formaldehyde production in pure PEG and PEG/water mixtures.

In conclusion, the stability of O^6-benzylguanine in aqueous PEG 400 solutions is compromised due to the presence of formaldehyde in PEG 400. O^6-Benzylguanine undergoes a reaction with formaldehyde to form a cross-linked product. This study also emphasizes the need to use only the high quality polyethylene glycols in the formulations and to store polyethylene glycols properly to prevent autoxidation. Although the instability of the drugs in polyethylene glycols is usually attributed to the presence of peroxides, reaction of



3. Stability profile of a O^6-benzylguanine sample in 60% PEG 400 (Aldrich, batch 1)-buffer (pH 8.0). The samples were stored at room temperature in sealed ampules.

drugs with autoxidation products such as formaldehyde should be considered.

References

1. Dolan, M. E., Mitchell, R. B., Mummert, C., Moschel, R. C. and Pegg, A. E., Effect of O^6-benzylguanine analogs on sensitivity of human tumor cells to the cytotoxic effects of alkylating agents. *Cancer Res.* 51(1991) 3367–3372.
2. Dolan, M. E., Moschel, R. C. and Pegg, A. E., Depletion of mammalian O^6-alkylguanine-DNA alkyltransferase activity of O^6-benzylguanine provides a means to evaluate the role of this protein in protection against carcinogenic and therapeutic alkylating agents. *Proc. Natl. Acad. Sci USA* 87 (1990) 5368–5372.
3. Safadi, M., Bindra, D. S., Williams, T., Stella, V. J., Kinetics and mechanism of the acid-catalyzed hydrolysis of O^6-benzylguanine. *Int. J. Pharm.*, 90 (1993), 239–246.
4. Hamburger, R., Azaz, E., Donbrow, M., Autoxidation of polyoxyethylenic non-ionic surfactants and of polyethylene glycols. *Pharm. Acta Helv.* 50 (1975) 10–17.
5. LLyod, W. G., Inhibition of polyglycol autoxidation. *J. Chem. Engng. Data*, 6 (1961), 541–547.
6. LLyod, W. G., The low temperature autoxidation of diethylene glycol. *J. Amer. Chem. Soc.*, 78, (1956), 72–75.
7. McGinity, J. W., Hill, J. A., Lavia, A. L., Influence of peroxide impurities in polyethylene glycols on drug stability. *J. Pharm. Sci.*, 64 (1975), 356–357.
8. Johnson, D. M., Taylor, W. F., Degradation of fenprostalene in polyethylene glycol 400. *J. Pharm. Sci.*, 73, (1984), 1414–1417.
9. Nash, T., The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem.*, 55 (1953), 416–421.
10. Azaz, E., Donbrow, M., Hamburger, R., Assay of micro-scale amounts of hydroperoxide and of iodine in aqueous non-ionic surfactant solutions by a spectrophotometric method. *Analyst*, 98 (1973), 663–672.
11. Chaw, Y. F. M., Crane, E., Lange, P., Shapiro, R., Isolation and identification of cross-links from formaldehyde-treated nucleic acids. *Biochem.*, 19 (1980), 5525–5531.
12. Huang, H., Solomon, M. S., Hopkins, P. B., Formaldehyde preferentially interstrand cross-links duplex DNA through deoxyadenosine residues at the sequence 5′-d(AT). *J. Am. Chem. Soc.*, 114, (1992), 9240–9241.
13. McGhee, J. D., von Hippel, P. H., Formaldehyde as a probe of DNA structure. I. Reaction of exocyclic amino groups of DNA bases. *Biochem.*, 14 (1975), 1281–1296.
14. Goudet, J. C., Crouzet, C., Marchal, J., Mechanism of the monomolecular decomposition of peroxy radicals formed on poly(oxyethylene) during autoxidation initiated in dilute chloroform or aqueous solutions studied by gamma-irradiation at 25 deg. *Kinet. Mech. Polyreactions, Int. Symp. Macromol. Chem.*, 5 (1969), 249–254.
15. Kersey, R. W., Maddocks, J. R., Johnson, T. E., The determination of small amounts of formaldehyde in air. *Analyst*, 65 (1940), 65–69.

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