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Simultaneous determination of O^6-benzylguanine and 8-oxo-O^6-benzylguanine in human plasma by reversed-phase high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic assay for the quantification of O^6-benzylguanine (O^6BG) in human plasma was modified to include the metabolite, O^6-benzyl-8-oxo-guanine (8-oxo-O^6BG). O^6-(*p*-Chlorobenzyl)guanine was used as the internal standard. Plasma samples were extracted with ethyl acetate and chromatographed on a C_{18} base-deactivated reversed-phase column. Separation was accomplished by gradient elution with mobile phases consisting of acetonitrile and phosphate buffer, pH 3.60. Eluted compounds were observed with diode array detection at 288 nm (O^6BG) and 292 nm (8-oxo-O^6BG). Standard curves were linear from 12.5 ng/ml to 1000 ng/ml, with an average regression coefficient of 0.999 (n=5) for both compounds. The lowest limit of quantitation was 25 ng/ml, with a signal-to-noise ratio of 8:1. The within-day relative standard deviations for O^6BG quality control samples (n=18) with concentrations of 735 ng/ml, 305 ng/ml and 38 ng/ml were 2.4%, 4.2% and 5.3%, respectively. The within-day relative standard deviations for 8-oxo-O^6BG quality control samples (n=18) at concentrations of 735 ng/ml, 420 ng/ml and 42 ng/ml were 2.2%, 4.0% and 7.1%, respectively. The day-to-day relative standard deviations for the same control specimens were 3.1%, 4.8% and 7.1% for O^6BG, respectively, and 2.3%, 4.7% and 11.0% for 8-oxo-O^6BG, respectively. This method was applied to plasma samples obtained from patients in a clinical trial of O^6-benzylguanine. O^6-Benzyl-8-oxo-guanine was identified in patient plasma specimens by liquid chromatography–electrospray mass spectrometry by comparison with spectral data acquired from reference material. Published by Elsevier Science B.V.

Keywords: O^6-Benzylguanine; 8-oxo-O^6-Benzylguanine

1. Introduction

Resistance of tumor cells to chloroethylating agents correlates with cellular capacity for elimination of O^6-methylguanosine from damaged DNA [1]. This repair activity is attributed to the protein O^6-alkylguanine DNA alkyltransferase (AT) [1], which functions by removing small alkyl or functionalized small alkyl moieties from O^6 of guanosine in chemi-

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cally damaged DNA [2]. O^6-Benzylguanine (O^6BG) is an active and virtually irreversible inhibitor of AT [3] which was tolerated well by rhesus monkeys in preclinical studies [4]. This knowledge has led to cancer treatment experiments which involve AT depletion by O^6BG and subsequent administration of cytotoxic agents.

Phase I clinical trials of O^6BG are in progress. A high-performance liquid chromatography (HPLC) method for quantitation of O^6BG in human plasma for our clinical trial was reported previously [5]. The method does not resolve some O^6BG metabolites found in earlier animal studies of O^6BG metabolism [6] (Fig. 1). At the outset of the initial clinical trial a probable metabolite peak was observed in the plasma extract chromatograms. This peak coeluted with some animal metabolites of O^6BG. To identify the unknown peak, the previously developed separation conditions were modified to achieve resolution of the metabolites and O^6BG. Herein is described a modified HPLC method that resolves O^6BG and several oxidized and acetylated metabolites of O^6BG. With the aid of improved separation conditions, spectrophotometric data, and liquid chromatography–diode array detection–electrospray mass spectrometry (LC–DAD–ES-MS) data, the probable human metabolite peak was identified as O^6-benzyl-8-oxoguanine (8-oxo-O^6BG). Under the improved separation conditions, 8-oxo-O^6BG as well as O^6BG could be quantified.

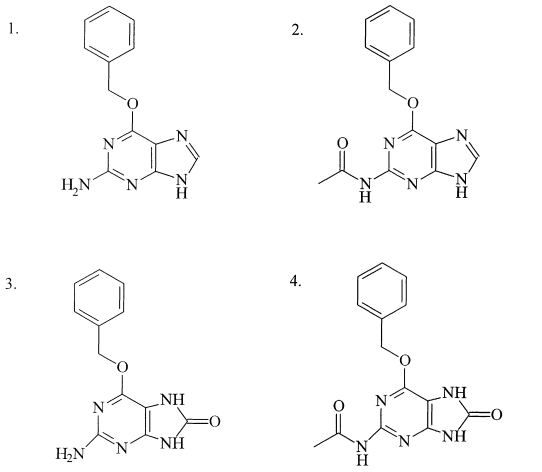

Fig. 1. Chemical structures of O^6-benzylguanine and some of its metabolites [6]. 1: O^6-Benzylguanine, 2: N^2-acetyl-O^6-benzylguanine, 3: O^6-benzyl-8-oxo-guanine, 4: N^2-acetyl-O^6-benzyl-8-oxo-guanine. The acetylated metabolites have not been observed in this clinical trial of O^6-benzylguanine.

2. Experimental

2.1. Materials

All reagents used in the mobile phase were purchased from Fisher Scientific (Pittsburgh, PA, USA). Ethyl acetate (Fisher Scientific) and sodium hydroxide pellets (Aldrich, Milwaukee WI, USA) were used in the extraction procedure. Dry grade compressed nitrogen (Gas Technics, Cleveland, OH, USA) was used for sample evaporation. A Milli-Q water purification system (Millipore, Milford MA, USA) was used to produce HPLC grade water. O^6BG, O^6-(*p*-chlorobenzyl)guanine (pCl-O^6BG), and the rat and mouse metabolites 8-oxo-O^6BG, N^2-acetyl-O^6-benzylguanine (N^2-acetyl-O^6BG) and N^2-acetyl-O^6-benzyl- 8-oxoguanine (N^2-acetyl-8-oxo-O^6BG) were supplied by Dr. Robert Moschel, Frederick Cancer Research and Development Center (Frederick, MD, USA).

2.2. Equipment

The liquid chromatograph consisted of a 1050 series quaternary pump, automatic liquid sampler and diode array detector purchased from Hewlett-Packard (HP) (Avondale, PA, USA). A Gateway 2000 P4D-66 microcomputer running under Microsoft Windows 3.11 (Microsoft, Redmond, WA, USA) and HP LC ChemStation software version A.03.02 were used for collection and processing of chromatographic data. The chromatographic separation was performed on a Hypersil BDS C_{18}, 10× 0.4 cm I.D., 3 μm particle size column purchased from HP Analytical Direct (Wilmington, DE, USA). The extraction was aided by the use of a multi-tube vortexer (Glas-col, Terre Haute, IN, USA). All sample evaporation steps were carried out with an Evapo-Rac sample concentration unit (Cole-Parmer Instruments, Chicago, IL, USA). An

HP Model 8452 spectrophotometer was used to record UV spectra of O^6BG, 8-oxo-O^6BG, N^2-acetyl-8-oxo-O^6BG and N^2-acetyl-O^6BG at several solution pH values. The mobile phase buffer was prepared with a Model 8010 pH meter, VWR Scientific (Greenbelt, MD, USA). The pH meter was calibrated with IUPAC-traceable calibration buffers obtained from Radiometer Analytical (Westlake, OH, USA).

2.3. Chromatographic conditions

Three mobile phases were used for gradient elution of O^6BG, 8-oxo-O^6BG and pCl-O^6BG (internal standard). Mobile phase A consisted of sodium phosphate buffer, pH 3.60. Mobile phases B and C consisted of acetonitrile–phosphate buffer, pH 3.60 (30:70, v/v) and (60:40, v/v), respectively. The phosphate buffer was prepared by dissolving 13.8 g ($1\cdot10^{-1}$ mol) of sodium phosphate (monobasic, monohydrate) in 2000 ml of water to obtain a 0.05 *M* solution prior to pH adjustment. The pH was then adjusted to 3.60 with 85% phosphoric acid (w/w). Mobile phase A was prepared by vacuum filtration of 800 ml of the phosphate buffer through a 0.45 μm nylon membrane. Mobile phases B and C were prepared similarly by vacuum filtration of 700 ml of the phosphate buffer and 300 ml of acetonitrile for mobile phase B, and 200 ml of phosphate buffer and 300 ml of acetonitrile for mobile phase C.

Initially, the pump was set to deliver 50% of mobile phase A and 50% of mobile phase B at a flow-rate of 1.0 ml/min. Immediately after injection, the concentration of mobile phase B was increased linearly to 100% during the next 10.0 min. The concentration of mobile phase C then was increased linearly to 100% during the next 3.0 min. The initial conditions then were established, and these conditions were sustained for 7.0 min to allow column equilibration. The total analysis time required was 20.0 min.

O^6BG was detected at 288 nm, followed by a change in the detection wavelength at 6.7 min after injection to 292 nm for detection of 8-oxo-O^6BG. The detection wavelength was reset to 288 nm at 8.5 min after injection for detection of the internal standard.

2.4. Liquid chromatograph–diode array detector–electrospray mass spectrometer

The chromatographic component of the LC–DAD–ES-MS instrument consisted of an HP 1100 series quaternary gradient pump, a HP 1100 automatic liquid sampler and an HP 1050 series diode array detector. The chromatograph was controlled and chromatographic data were acquired by a Gateway 2000 P5-166 microcomputer running under Microsoft Windows 95 and HP version A.04.02 LC-ChemStation software. The mass spectrometer was a LCQ ion trap instrument obtained from Finnigan MAT (San Jose, CA, USA). This instrument was controlled and mass spectral data were acquired by a Gateway 2000 P5-120 microcomputer running under Microsoft Windows NT Workstation 4.0 and Finnigan MAT LCQ Navigator instrument control software.

2.5. LC–DAD–ES-MS chromatographic conditions

Plasma sample extract separations were achieved on a 10×0.4 cm I.D. DBS Hypersil-C_{18} column (3 μm particle size, HP Analytical Direct). HPLC–ES-MS of patient specimen extracts was carried out with a ternary eluent system containing volatile buffer salts. Mobile phase A consisted of 0.04 *M* ammonium acetate in water, pH 3.60. This was prepared by combining 6.2 g ($8.0\cdot10^{-2}$ mol) of ammonium acetate with 2000 ml of water and adjusting the solution pH to 3.60 with glacial acetic acid. Mobile phase B consisted of 0.04 *M* ammonium acetate in water pH 3.60 and acetonitrile (70:30, v/v). This was prepared by combining 700 ml of mobile phase A with 300 ml of filtered acetonitrile. Mobile phase C consisted of 0.04 *M* ammonium acetate in water (pH 3.60) and acetonitrile (40:60, v/v). This was prepared by combining 400 ml of mobile phase A with 600 ml of filtered acetonitrile.

The initial mobile phase composition was A–B–C (35:65:0, v/v/v). These conditions were maintained for 5.0 min after sample injection, after which the mobile phase proportions were varied linearly during the next 2 min to final elution conditions of A–B–C (0:0:100, v/v/v). At 7.01 min after injection the eluent component proportions were reset to A–B–C (35:65:0, v/v/v), and the column was allowed to

equilibrate for 4 min before subsequent sample injection.

Plasma specimens were extracted and concentrated for analysis as described under Section 2.8. They were reconstituted in 40 μl of acetonitrile–mobile phase A (50:50, v/v), and sample aliquots of 10 μl were injected into the chromatograph.

2.6. LC–DAD–ES-MS instrumental conditions

The eluent flow-rate was 1.0 ml/min, and the diode array detector acquired spectra over the wavelength range of 200–450 nm at a rate of 1.2 Hz. The chromatographic eluent initially was diverted from the spectrometer ion source before sample injection. At 3.50 min after sample injection, the eluent flow was restored to the spectrometer ion source. The mass spectrometer was operated in positive ion mode, with an inlet capillary temperature of 220°C, and capillary and spray potentials of 3.0 V and 4200 V, respectively. Nitrogen was used as the ion source sheath and auxiliary gases. Helium at about 10^{-3} Torr (1 Torr=133.322 Pa) pressure was used as the ion trap damping gas. The resonance excitation RF voltage applied to the mass analyzer in the MS–MS experiments was 1.75 V. An ion isolation width of 2 a.m.u. was used in these experiments. The mass spectrometer acquired spectra over the ion mass range 65–500 m/z at about 0.4 Hz.

2.7. Extraction efficiency

The extraction efficiency for 8-oxo-O^6BG with ethyl acetate was investigated using external standardization as described previously [5].

2.8. Sample processing

Patient samples and quality control samples were stored at −70°C. Before extraction, the samples were thawed on ice, gently vortex-mixed, and 500 μl was removed. The remainder was returned immediately to the freezer. A typical daily sample set consisted of eight calibration standards, a plasma zero, a plasma blank, duplicate high, medium and low concentration quality control (QC) plasma samples and some patient samples.

Plasma samples (500 μl) were dispensed into 13×100 mm borosilicate glass tubes. The extraction was carried out with 100 μl of 0.10 *M* NaOH, 50 μl of internal standard (500 ng/ml in methanol), and 3.0 ml of ethyl acetate. The samples were vortex-mixed for 1.5 min and centrifuged for 5.0 min at 1760 *g* to facilitate phase separation. The organic layers were transferred to 12×75 mm borosilicate glass tubes and evaporated to dryness under a stream of nitrogen. The resulting residues were reconstituted in 40 μl of a solution containing acetonitrile–0.05 *M* phosphate buffer, pH 3.60, (50:50, v/v) (reconstitution solvent), and 10 μl was injected into the chromatographic system. Linear regression of the standard curve was calculated from the peak height ratios of O^6BG or 8-oxo-O^6BG calibration standards and the internal standard. Quantitative values for the samples were obtained by interpolation from the regression line.

2.9. Method validation: linearity, day-to-day and within-day precision

Calibration standards ranging in concentration from 12.5 ng/ml to 1000 ng/ml were prepared in drug free plasma. The calibration standards were processed, and linear regression was calculated as described in Section 2.8.

High, medium and low concentration QC plasma samples were prepared by diluting methanolic standard solutions (1.0 mg/ml) in 50 ml of drug-free plasma. Dilutions were prepared with final concentrations of 735 ng/ml of both O^6BG and 8-oxo-O^6BG, 305 ng/ml of O^6BG and 420 ng/ml of 8 oxo-O^6BG, and 38 ng/ml of O^6BG and 42 ng/ml of 8-oxo-O^6BG. Each quality control specimen was transferred in 3.0 ml volumes to plastic tubes for storage at −70°C. Six samples of each concentration were thawed on ice, extracted and chromatographed on three separate days (n=18). Quantitation of the QC samples was carried out as described in Section 2.8.

2.10. Identification of O^6-benzyl-8-oxo-guanine in plasma specimens

Extracts of patient plasma specimens were prepared as described in Section 2.8 and subjected to LC–DAD–ES-MS analysis as described in Section

2.5. Extracts of quality control specimens which contained 305 ng/ml of O^6BG and 420 ng/ml of 8-oxo-O^6BG were prepared similarly and analyzed to acquire reference spectra. Spectra obtained during elution of patient plasma sample extract constituents were compared with reference spectra obtained during chromatographic analysis of the quality control specimen extracts.

3. Results and discussion

3.1. Identification of O^6-benzyl-8-oxo-guanine

At the outset of the O^6BG clinical trial a probable metabolite peak was observed in chromatograms of human plasma extracts. This compound was identified as O^6-benzyl-8-oxo-guanine on the basis of its chromatographic retention behavior and ultraviolet absorption spectrum in relation to those of authentic 8-oxo-O^6BG [5]. 8-Oxo-O^6BG is an active metabolite of O^6BG produced by mice, rats [6] and rhesus monkeys [4]. Consequently, this work was undertaken to confirm this identification with supplemental spectroscopic data, and to extend the O^6BG measurement method to include determination of 8-oxo-O^6BG.

An eluent system containing volatile buffer salts was developed for application to experiments with a liquid chromatograph–diode array absorbance detector–electrospray ion trap mass spectrometer instrument. A spectrophotometric chromatogram obtained after injection of an extract of a plasma specimen obtained from a patient who received a 1 h infusion of 23.5 mg/m^2 O^6BG appears in Fig. 2. The chromatographic peaks at 4.3 min and 4.8 min correspond with elution of O^6BG and 8-oxo-O^6BG, respectively. During this experiment, ions of nominal m/z 258 (corresponding with the protonated 8-oxo-O^6BG molecular ion) were trapped, subjected to collision induced fragmentation and the product fragment ions were analyzed to produce the mass spectrum shown in Fig. 3. The m/z 258 extracted ion chromatogram obtained from this data set exhibited one peak at 4.8 min (data not shown). A mass spectrum obtained in a similar MS–MS experiment with trapped m/z 258 ions after injection of a plasma specimen which contained 305 ng/ml of O^6BG and 420 ng/ml of 8-oxo-O^6BG reference materials is shown in Fig. 4. The m/z 258 extracted ion chromatogram obtained from this data set also showed a single peak at 4.8 min. The signals in these mass spectra at nominal m/z 230 probably correspond with loss of carbon monoxide from the protonated molecular ion. The peak at m/z 180 corresponds arithmetically with loss of the elements of benzene. The peak at m/z 173 could arise from loss of CH_2N_2

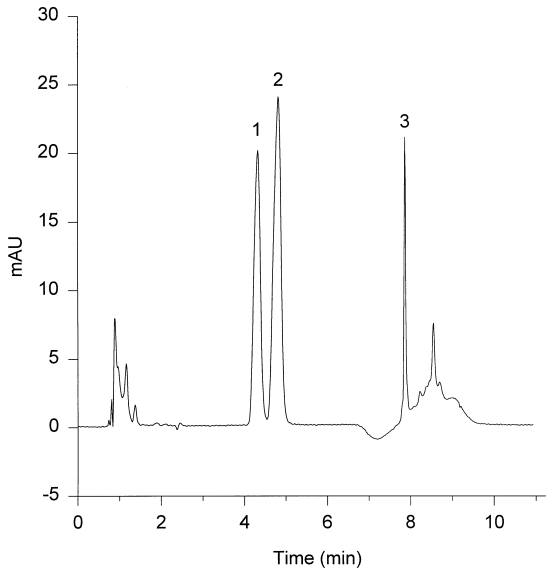

Fig. 2. This 288 nm extracted wavelength chromatogram was obtained during HPLC–DAD–ES-MS analysis of an extract of a plasma specimen obtained from a patient volunteer. Peaks 1 and 2 at 4.3 min and 4.8 min correspond with elution of O^6BG and 8-oxo-O^6BG, respectively. The separation was achieved on a 10×0.4 cm I.D. BDS Hypersil-C_{18} (3 μm particle size) column with a ternary eluent system. Mobile phase A consisted of 0.04 *M* ammonium acetate in water, pH 3.60. Mobile phase B consisted of 0.04 *M* ammonium acetate in water, pH 3.60–acetonitrile (70:30, v/v). Mobile phase C consisted of 0.04 *M* ammonium acetate in water, pH 3.60–acetonitrile (40:60, v/v). The initial mobile phase composition was A–B–C (35:65:0, v/v/v). These conditions were maintained for 5.0 min after sample injection, after which the mobile phase proportion was varied linearly for 2.0 min to final elution conditions of A–B–C (0:0:100, v/v/v). At 7.01 min after injection the eluent component proportions were reset to A–B–C (35:65:0, v/v/v), and the column was allowed to equilibrate for 4.0 min before subsequent sample injection. The eluent was pumped at 1.0 ml/min, and the DAD acquired spectra at 1.2 Hz over the wavelength range 200–450 nm.

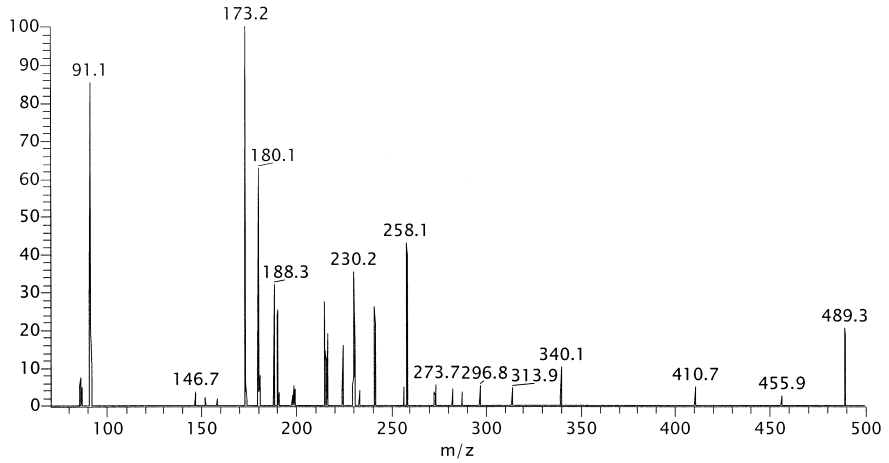

Fig. 3. This mass spectrum was obtained 4.8 min after sample injection during the HPLC–DAD–ES-MS experiment shown in Fig. 2. Ions of nominal m/z 258 (corresponding with the protonated 8-oxo-O^6BG molecular ion) were trapped, subjected to collision induced fragmentation, and the product fragment ions were analyzed. Signal intensity units are arbitrary.

and CHNO to give a protonated $C_{10}H_8N_2O$ fragment. The prominent peak at m/z 91 is a characteristic mass spectral feature of benzylic ethers. O^6BG was identified in patient plasma specimens in separate similar MS–MS experiments with trapped m/z 242 ($[M+H]^+$) ions. The m/z 242 extracted ion chromatograms obtained from these data sets showed a single chromatographic peak at 4.3 min.

3.2. Extraction

The extraction efficiency for 8-oxo-O^6BG was determined by external standardization. Results for the extraction of 160 ng/ml 8-oxo-O^6BG in plasma were 82±8% (n=10). Results for the extraction efficiency studies of O^6BG and pCl-O^6BG were reported previously [5].

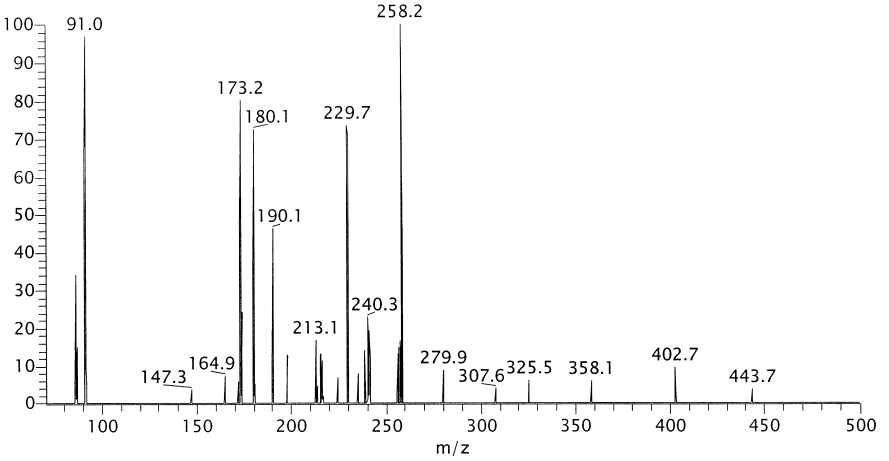

Fig. 4. This mass spectrum was obtained in a similar MS–MS experiment with trapped m/z 258 ions after injection of a plasma specimen which contained 305 ng/ml of O^6BG and 420 ng/ml of 8-oxo-O^6BG reference materials. Experimental conditions were as described in Fig. 3. Signal intensity units are arbitrary.

3.3. Chromatographic conditions

Chromatographic conditions used to quantitate O^6BG were reported previously [5]. These elution conditions did not separate some animal metabolites of O^6BG. The metabolites were separated on a reversed-phase BDS column by a modified mobile phase without methanol. However, this mobile phase alteration resulted in partial co-elution of an endogenous plasma compound with O^6BG. UV maximum absorbance data for O^6BG and the animal metabolites were determined separately over a pH range of 1.0 to 7.0 in mobile phase buffer (data not shown). Observed hypsochromic shifts in wavelengths of spectral maxima were assumed to result from changes in the protonation state of the compounds. Changes in maximum absorbance wavelength for the comparatively acidic metabolite conjugate acids occurred below pH 3.0. The absorbance maximum change for the comparatively less acidic O^6BG conjugate acid occurred between pH 3.0 and pH 4.0. Mobile phase buffers of pH 3.50, 3.55, 3.60 and 3.75 were investigated for optimal separation of O^6BG from the interfering plasma compound. Retention of the metabolites was unaffected at these pH values. O^6BG retention was increased with increasing mobile phase pH, and it was better separated from the interfering compound. However, at pH 3.75 O^6BG co-eluted with the acetylated metabolites. The optimum pH for resolution of the interfering plasma sample constituent from O^6BG was 3.60. At this mobile phase pH, O^6BG and all the metabolites were resolved (Fig. 5).

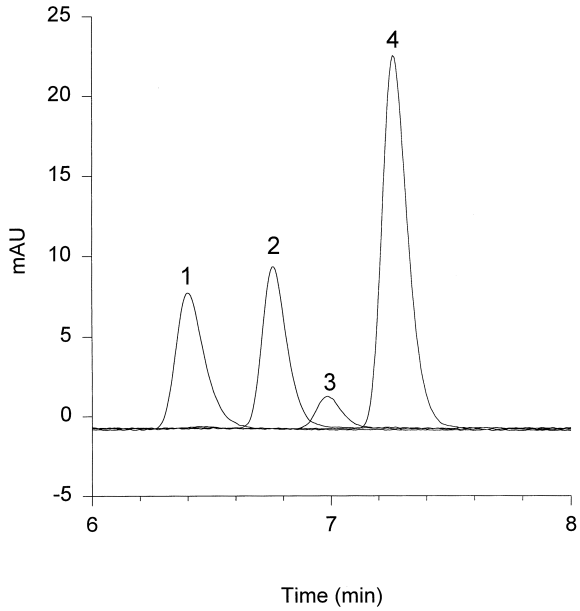

Fig. 5. Overlaid chromatograms of O^6BG (1), N^2-acetyl-8-oxo-O^6BG (2), N^2-acetyl-O^6BG (3) and 8-oxo-O^6BG (4) are shown in this Figure. Standard solutions of reference materials were diluted to approximately 10 μg/ml in reconstitution solvent, and 10 μl of each solution was injected into the chromatographic system. This separation was achieved on a Hypersil BDS C_{18}, 10×0.4 cm I.D., 3 μm particle size column. Three eluents were used for ternary gradient development of these chromatograms. Mobile phase A consisted of 0.05 *M* sodium phosphate buffer, pH 3.60. Mobile phase B consisted of acetonitrile–phosphate buffer, pH 3.60 (30:70, v/v). Mobile phase C consisted of acetonitrile–phosphate buffer, pH 3.60 (60:40, v/v). Initial chromatographic conditions were A–B–C (50:50:0, v/v/v). Immediately after injection, the concentration of mobile phase B was increased linearly to proportions of A–B–C (0:100:0, v/v/v) during the next 10.0 min. The concentration of mobile phase C then was increased linearly to proportions of A–B–C (0:0:100, v/v/v) during the next 3.0 min. The initial conditions of A–B–C (50:50:0, v/v/v) then were established, and these conditions were sustained for 7.0 min to allow column equilibration. The eluent was pumped at 1.0 ml/min throughout the chromatographic experiment. O^6BG was detected at 288 nm, followed by a change in the detection wavelength at 6.7 min after injection to 292 nm for detection of 8-oxo-O^6BG. The detection wavelength was reset to 288 nm at 8.5 min after injection for detection of the internal standard.

3.4. Method validation

Linearity of the O^6BG and 8-oxo-O^6BG standard curves was validated from 12.5–1000 ng/ml with an average regression coefficient of 0.999 (n=5) for both compounds. Average y-intercepts of 0.007 (n=5) for O^6BG, and 0.009 for 8-oxo-O^6BG (n=5) were determined. The lowest limit of quantitation was 25 ng/ml with a signal-to-noise ratio of 8:1 for both compounds. Six replicates of high, medium and low concentration QC samples were assayed each day for three days (n=18) for within-day and day-to-day precision studies. The within-day relative standard deviations (R.S.D.s) for QC samples with O^6BG concentrations of 735 ng/ml, 305 ng/ml and 38 ng/ml were 2.4%, 4.2% and 5.3%, respectively. The within-day R.S.D.s for 8-oxo-O^6BG QC samples at concentrations of 735 ng/ml, 420 ng/ml and 42

ng/ml were 2.2%, 4.0% and 7.1%, respectively. The day-to-day R.S.D.s for the same QC samples were 3.1%, 4.8% and 7.1% for O^6BG, respectively, and 2.3%, 4.7% and 11.0% for 8-oxo-O^6BG, respectively. The R.S.D.s for collected data ($n=29$) for 38 ng/ml O^6BG and 42 ng/ml 8-oxo-O^6BG QC samples were 10.3% and 16.5%, respectively. Collected data ($n=30$) for 305 ng/ml O^6BG and 420 ng/ml 8-oxo-O^6BG showed R.S.D.s of 5.8% and 6.9%, respectively. The R.S.D.s for collected data ($n=27$) for the 735 ng/ml quality control samples were 4.8% for O^6BG and 5.1% for 8-oxo-O^6BG.

3.5. Interfering drugs

Patients participating in the Phase I clinical trial were prescribed several drugs in addition to O^6BG and cytotoxic agents. These drugs were investigated for chromatographic interference, and these results were reported previously [5]. The same compounds and several other drugs were investigated for chromatographic interference under the modified conditions described in this paper. The additional compounds were alprazolam, amitriptyline, diazepam, diphenhydramine, enalipril, hydrochlorothiazide, lanoxin, lisinopril, lorazepam, meperidine, naproxene, quinidine and terazosin. Those compounds that coeluted or nearly coeluted with O^6BG, 8-oxo-O^6BG or pCl-O^6BG were extracted and chromatographed as described in Section 2.8. Terazosin was the only compound listed above that was extracted and interfered with the quantification of O^6BG.

3.6. Method application

The chromatographic procedure described in this paper was developed for identification and quantification of a probable metabolite peak that was seen in patient plasma samples. Once this compound was identified as 8-oxo-O^6BG, plasma concentrations of

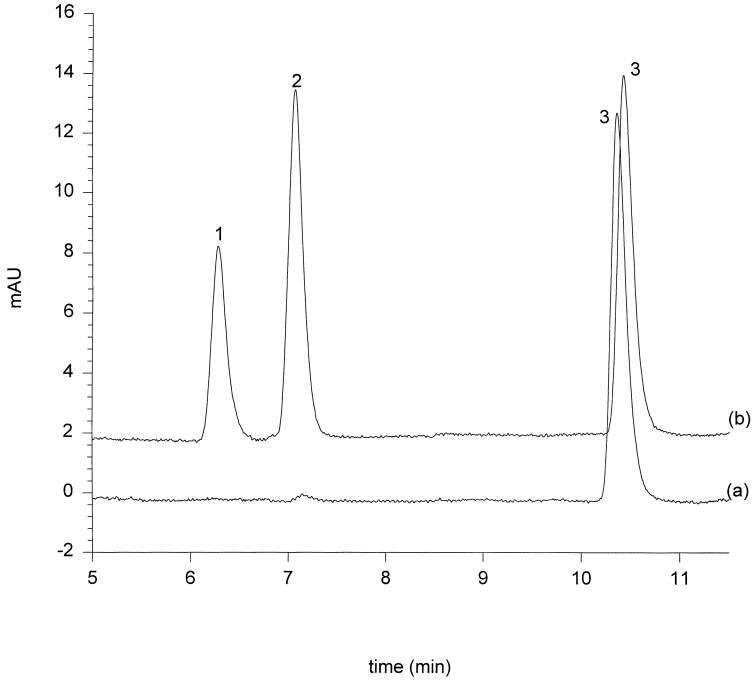

Fig. 6. These chromatograms were obtained from extracts of plasma from one patient who received a 1 h bolus infusion of 23.5 mg/m^2 of O6BG during the phase I clinical trial of O^6BG. Both plasma samples were processed as described in Section 2.8. (a) Chromatogram of an extract of a patient plasma sample drawn before the infusion of O^6BG. (b) Chromatogram of an extract of a plasma sample drawn from the same patient immediately after a 1 h infusion of O^6BG. Peaks 1, 2 and 3 represent O^6BG, 8-oxo-O^6BG and pCl-O^6BG, respectively. The chromatographic conditions were as described in Fig. 5.

8-oxo-O^6BG were determined for pharmacokinetic interpretation. Chromatograms of plasma extracts obtained from a patient before and after a 1 h infusion of 23.5 mg/m^2 O^6BG are represented in Fig. 6a and Fig. 6b. The plasma concentrations of O^6BG and 8-oxo-O^6BG immediately after the 1 h infusion were 436 mg/ml and 659 mg/ml, respectively. These representative chromatograms show no interference from endogenous plasma compounds.

In the Phase I pharmacokinetic trial, blood samples were drawn prior to the infusion, and then at intervals of 15 min until termination of the 1 h infusion. After the infusion, blood samples were drawn at 5 min intervals for 20 min, and then subsequently at 30, 45, 60, 90 and 120 min. Samples then were taken after each 2 h period until 8 h after infusion, and again at 12 and 24 h after infusion termination. Representative plots of plasma concentration vs. time for O^6BG and 8-oxo-O^6BG are shown in Fig. 7. The O^6BG concentration vs. time data set was described well by a two exponential term pharmacokinetic model which revealed two elimination processes. The apparent half life of the comparatively fast elimination process was 0.05 h, and that of the comparatively slow elimination process was 0.47 h The apparent plasma elimination half life of 8-oxo-O^6BG extracted from a single exponential model fit of the metabolite concentration vs. time data subset after disappearance of O^6BG was 3.8 h. Data sets obtained from study of seventeen patients who received 1 h bolus infusion doses of 10.0–23.5 mg/m^2 O^6BG all were characterized by the rapid rise to steady state plasma concentration evident in Fig. 7. In about half of the data sets, two elimination processes were disclosed by nonlinear fitting of a two exponential term pharmacokinetic

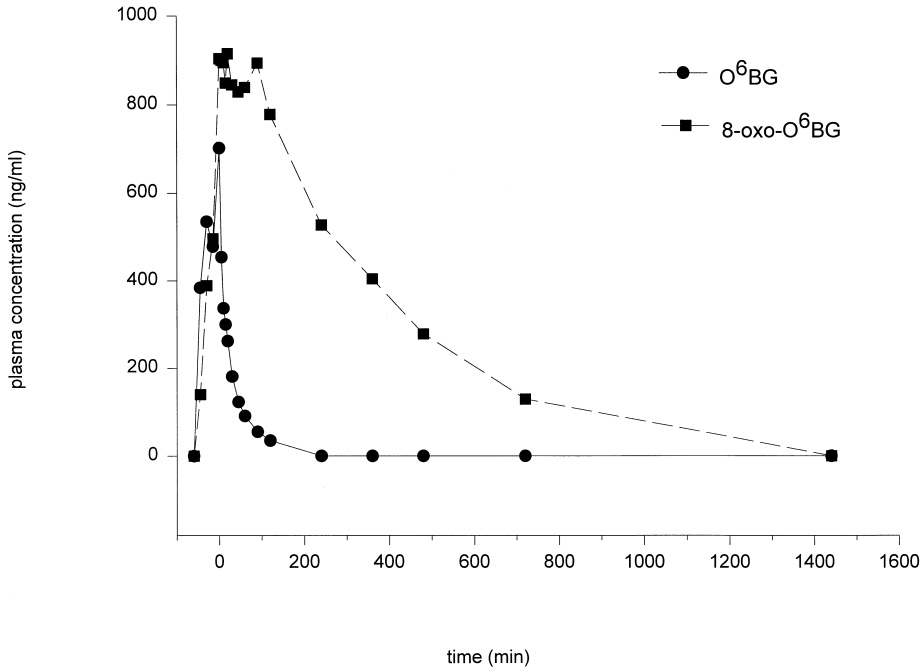

Fig. 7. Plots of plasma concentration of O^6BG and 8-oxo-O^6BG vs. time from a patient who received a 1 h bolus infusion of O^6BG (23.5 mg/m^2). Blood samples were drawn prior to the infusion, and then at intervals of 15 min for the duration of the infusion. After infusion termination, blood samples were drawn at 5 min intervals for 20 min, and then subsequently at 30, 45, 60, 90 and 120 min. Samples then were taken after each succeeding 2 h period until 8 h after infusion, and then again at 12 and 24 h. This O^6BG concentration vs. time data set was described well by a two exponential term pharmacokinetic model which disclosed two elimination processes. The most rapid of these proceeded with an apparent half life of 0.05 h, and the least rapid proceeded with and apparent half life of 0.47 h. A single exponential term equation fit of the 8-oxo-O^6BG data subset of values obtained after disappearance of O^6BG from plasma revealed a substantially longer plasma elimination half life of 3.8 h for the metabolite.

model. Full results of the pharmacokinetic study will be published elsewhere.

The acetylated animal metabolites of O^6BG [6] never were observed in human plasma samples analyzed by the antecedent method [5] or by this method. These metabolites also were not detected in plasma specimens subjected to HPLC–selected positive ion monitoring ES-MS.

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