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Determination of 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 O^6-benzylguanine utilizing liquid–liquid extraction and reversed-phase chromatography has been developed. Plasma samples were alkalinized, extracted into ethyl acetate, evaporated, and the residues were reconstituted and chromatographed. Separation was accomplished by gradient elution with a mobile phase of methanol, acetonitrile, and phosphate buffer, pH 3.2. Eluted compounds were detected spectrophotometrically at 280 nm. Sample quantitation was obtained from the regression line of six-point standard curves ranging from 25 to 400 ng/ml. O^6-Benzylguanine peak heights were compared to peak heights of O^6-(*p*-chlorobenzyl)guanine (internal standard). The average regression coefficient was 0.999 (n = 4). High concentration (305 ng/ml) and low concentration (38 ng/ml) quality control samples were determined with a day-to-day relative standard deviation of 7 and 8%, respectively (n = 18). The within-day relative standard deviations were 2.7 and 3.0% (n = 18) for the high and low concentration quality control specimens, respectively. Sample quantitation was reliable to 25 ng/ml with a signal-to-noise ratio of 8:1. This method was applied to plasma samples obtained from patients in a clinical trial of O^6-benzylguanine.

Keywords: O^6-Benzylguanine

1. Introduction

O^6-Benzylated guanine derivatives effectively inactivate the repair protein O^6-alkylguanine–DNA alkyltransferase by covalent transfer of the benzyl group to the cysteine residue in the protein active site [1–3]. Inhibition of this repair protein enhances the cytotoxic effects of chloroethylating agents [1,2]. Studies in human tumor xenograft-bearing athymic mice have shown marked potentiation of antitumor effect [4]. Recently, investigations have shown O^6-benzylguanine (O^6BG) to be more effective than other substituted purine derivatives [2]. O^6BG pharmacokinetic studies in rats and mice showed a number of metabolites, including acetylated and oxidized forms [5]. In addition, guanine is readily oxidized by reactive oxygen metabolites such as hydroxyl radical and singlet oxygen [6–8]. From this

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information we anticipated that 8-oxo-O^6BG and the acetylated metabolites found in the animal studies could appear in humans treated with O^6BG [5]. Phase I clinical trials have begun on O^6-benzylguanine. This necessitated the development of a method for specific determination of O^6BG in human plasma which resolves O^6BG from these possible metabolites.

2. Experimental

2.1. Materials

The reagents used in the mobile phase were purchased from Fisher Scientific (Pittsburg, PA, USA) and include monobasic sodium phosphate, phosphoric acid (85%, w/w), methanol (HPLC grade) and acetonitrile (HPLC grade). Ethyl acetate and sodium hydroxide pellets (Aldrich, Milwaukee, WI, USA) were used in the extraction procedure. A MilliQ water system (Millipore, Milford, MA, USA) was used to produce HPLC grade water. Compressed air was passed through a mechanical filter and a silica moisture trap before use for all sample evaporation operations. O^6BG, O^6-(*p*-chlorobenzyl)guanine (*p*Cl-O^6BG), and the rat and mouse metabolites 8-oxo-O^6-benzylguanine (8-oxo-O^6BG), N^2-acetyl-O^6BG and N^2-acetyl-8-oxo-O^6BG were supplied by Dr. Robert Moschel, NCI Frederick Cancer Research and Development Center (Frederick, MD, USA). Dr. Moschel also supplied several O^6BG analogs for possible use as an internal standard, including O^6-benzylhypoxanthine, O^6-benzyl-9-pivaloyloxymethylpurine, O^6-*p*-methylbenzylguanine, O^6-benzylthioguanine, O^6-benzyl-9-ethoxycarbonylmethylpurine and O^6-*p*-methylbenzylthioguanine.

2.2. Equipment

The chromatographic equipment consisted of a 1050 series quaternary pump, autosampler and multiple wavelength detector, purchased from Hewlett-Packard (Avondale, PA, USA). A Gateway 2000 P4D-66 microcomputer running under Microsoft Windows 3.11 (Microsoft, Redmond, WA, USA) and Hewlett-Packard LC ChemStation software were used for collection and processing of chromatographic data. The chromatographic separation was performed on a Hypersil BDS C_{18}, 100 × 4 mm, 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).

2.3. Chromatographic conditions

Two mobile phases were used for gradient elution of O^6BG and the internal standard. Mobile phase A consisted of methanol–acetonitrile–phosphate buffer, pH 3.2 (15:15:70, v/v). Mobile phase B consisted of acetonitrile–phosphate buffer, pH 3.2 (70:30, v/v). The phosphate buffer was prepared by dissolving 6.9 g (0.05 mol) of $NaH_2PO_4 \cdot H_2O$ in 1.0 l of HPLC grade water and adjusting to pH 3.2 with concentrated phosphoric acid. Mobile phase A was prepared by vacuum filtration of 150 ml of acetonitrile, 150 ml of methanol and 700 ml of the phosphate buffer through a 0.45-μm Nylon membrane. Mobile phase B was similarly prepared with 700 ml of acetonitrile and 300 ml of phosphate buffer.

Mobile phase A was pumped isocratically for 5.00 min after sample injection. This was followed by an 8.00-min linear mobile phase B concentration gradient which increased to a final concentration of 90% of mobile phase B. These final conditions were sustained for 3.00 min, then the chromatographic eluent was reset to the initial conditions and the column was allowed to equilibrate for 3.00 min. The total analysis time was 19.00 min.

2.4. Extraction

O^6BG was extracted from plasma using seven different solvents to identify a solvent that provided satisfactory extraction efficiency and selectivity for O^6BG. Samples containing 50 μl of O^6BG (0.10 mg/ml) in methanol were added to fourteen tubes and evaporated to dryness. Two additional tubes were prepared for use as calibration standards. To the fourteen residues, 250 μl of chromatographically screened non-interfering plasma (drug-free plasma)

and 50 μl of 0.1 *M* NaOH were added and vortex-mixed. This resulted in a final drug concentration in plasma of 20 μg/ml. The extraction was carried out with 3 ml of the following solvents, each in duplicate: ethyl acetate, diethyl ether, dichloromethane, trichloromethane, *n*-butylchloride, hexane–2-propanol (9:1, v/v) and toluene–2-propanol (9:1, v/v). Samples were vortex-mixed and centrifuged at 1760 *g*. An amount of organic phase ranging from 2.0–2.7 ml was transferred to 12 × 75 mm borosilicate glass tubes, evaporated to dryness, and reconstituted in 50 μl of phosphate buffer–acetonitrile (85:15, v/v) containing 0.1 mg/ml of *p*Cl-O^{6}BG. The amount removed was dependent upon the extent of emulsification that occurred with each solvent. Calibration standards were prepared by reconstituting the two additional O^{6}BG residues directly in the 0.10 mg/ml *p*Cl-O^{6}BG reconstitution solvent. A 10-μl aliquot was injected onto the chromatographic system. Chromatographic peak-area ratios were corrected for partial transfer of the organic phase after sample extraction. These corrected ratios were compared with the ratios obtained from calibration standards and the percent of O^{6}BG extracted was calculated.

A thorough extraction study for O^{6}BG and *p*Cl-O^{6}BG using ethyl acetate was done based on the results of the solvent selection study. The investigation was carried out as described for the solvent selection study with changes in the concentrations of O^{6}BG and *p*Cl-O^{6}BG and in the volume of plasma. Samples containing 500 μl of drug-free plasma were used due to the greater detection sensitivity required for the patients' samples during the pharmacokinetic studies. The extraction of O^{6}BG and *p*Cl-O^{6}BG was studied at 160 ng/ml, which is representative of the concentration range of actual patient samples. Reconstitution was carried out with 40 μl of methanol–acetonitrile–phosphate buffer (35:35:30, v/v, reconstitution solvent) containing 160 ng/ml *p*Cl-O^{6}BG and 15 μl was injected. The extraction efficiency was calculated as described above.

2.5. Sample preparation

Patient samples and quality control samples were stored at −70°C. Prior to extraction, the samples were thawed at room temperature. Once thawed, they were placed in an ice bath, mixed by inversion several times, a sample was removed, and the remainder was immediately returned to the freezer. Samples containing 500 μl of plasma were dispensed into 13 × 100 mm borosilicate glass tubes containing 50 μl of internal standard solution (2.0 μg/ml *p*Cl-O^{6}BG in HPLC grade H_2O). To each tube, 100 μl of 0.1 *M* NaOH and 3.0 ml of ethyl acetate were added. The samples were vortex-mixed and centrifuged (1760 *g*) to facilitate phase separation. The organic layers were transferred to 12 × 75 mm glass tubes and evaporated to dryness under dry, oil-free, compressed air. The resulting residues were reconstituted in 40 μl of reconstitution solvent.

2.6. Linearity

Calibration standards ranging from 25 to 400 ng/ml were prepared in drug-free plasma. Internal standard solution (50 μl) was added to each calibration specimen. Extraction was carried out, in duplicate, on 500-μl aliquots of each calibration standard as described in Section 2.5. Standard curves were constructed from peak-height ratios of detector response for O^{6}BG and *p*Cl-O^{6}BG, and the linear regression was calculated.

2.7. Day-to-day precision

Quality control samples were prepared by diluting a stock standard solution (1.0 mg/ml O^{6}BG) to final concentrations of 305 ng/ml and 38 ng/ml, each in 10 ml of drug-free plasma. Six samples at each concentration were extracted, chromatographed, and calculated on three separate days. Quantitation of samples was obtained by interpolation of peak-height ratios from the regression line.

2.8. Method application

A typical daily sample set consisted of a six-point standard curve, duplicate high (305 ng/ml) and low (38 ng/ml) concentration quality control samples, and patient samples. The samples and standards were prepared and chromatographed as described above for sample preparation. Quantitative results for patient and QC samples were obtained as described above for day-to-day precision.

3. Results and discussion

3.1. Extractions

An exploratory investigation for the purpose of selecting an extraction solvent that provided the greatest extraction efficiency and selectivity for O^6BG was carried out using seven different organic solvents (Table 1). Ethyl acetate and toluene–2-propanol (9:1, v/v) showed superior extraction efficiencies for O^6BG from plasma. Also, these solvents appeared to extract equal amounts of endogenous plasma substances from blank plasma specimens. Ethyl acetate was selected due to its slightly higher extraction efficiency for O^6BG and because it is less toxic than toluene. Separate detailed extraction studies for O^6BG and *p*Cl-O^6BG were performed using ethyl acetate, with each compound added in the expected concentration range of the patients' samples. The extraction efficiency was determined by external standardization. Results for the extraction of 160 ng/ml O^6BG in plasma were 99 ± 8% (n = 9) and 96 ± 5% (n = 9) for 160 ng/ml of *p*Cl-O^6BG.

3.2. Chromatographic conditions

Initially, the separation of O^6BG from the possible metabolites N^2-acetyl-O^6BG, N^2-acetyl-8-oxo-O^6BG, and 8-oxo-O^6BG was attempted on an end-capped C_{18} reversed-phase column. Using this column and a mobile phase of acetonitrile–sodium phosphate buffer, pH 6.9, the acetylated metabolites eluted first, followed by 8-oxo-O^6BG and O^6BG, which co-eluted. Because these conditions did not isolate O^6BG, a second separation attempt was made with the pH of the phosphate buffer mobile phase lowered to 3.2. This resulted in later elution of O^6BG with its complete separation from the possible metabolites, however severe peak tailing was a consequence. In separate experiments, triethylamine and sodium dodecyl sulfate were added to the mobile phase, but produced no improvement in peak shape.

Table 1
Extraction of O^6BG from plasma with various solvents

Solvent	Recovery of O^6BG (%)
Ethyl acetate	99
Diethyl ether	84
Dichloromethane	80
Trichloromethane	70
N-Butyl chloride	4
Hexane–2-propanol (9:1, v/v)	15
Toluene–2-propanol (9:1, v/v)	96

O^6BG (20 μg/ml) was added to drug-free plasma and extracted with these solvents as described in Section 2.4.

The acetonitrile–sodium phosphate buffers mentioned previously were tried with a base-deactivated C_{18} reversed-phase column. The peak tailing did not occur on this column. Additionally, the O^6BG still co-eluted with 8-oxo-O^6BG at pH 6.9, but it was retained less as the pH of the mobile phase was lowered. At a pH of 3.2, the acetonitrile–sodium phosphate mobile phase eluted O^6BG before the metabolites, with baseline resolution. Although these separation conditions were satisfactory, this eluent was not suitable for application to plasma samples due to an endogenous plasma compound that co-eluted with O^6BG. Methanol was added to this mobile phase in an attempt to achieve greater resolution, and with minor changes in the volume ratio of methanol and acetonitrile, this was successful. In order to minimize the total analysis time, an organic modifier gradient was necessary to rapidly elute the internal standard. Final eluent conditions were sustained for 3.00 min to elute other strongly retained plasma constituents. These chromatographic conditions achieved separation of O^6BG from endogenous plasma compounds as well as from the metabolites that may appear in human plasma samples (Fig. 1 and Fig. 2).

3.3. Possible internal standards

Several compounds were investigated as candidates for use as the internal standard (Table 2). *p*Cl-O^6BG was selected as the internal standard for our assay because it was resolved easily from O^6BG, 8-oxo-O^6BG, and from acetylated metabolites seen in the animal studies [5]. Methanolic solutions of *p*Cl-O^6BG were stored for 6 months at −70°C without evidence of deterioration.

3.4. Reconstitution

A relative standard deviation of more than 25% was observed for the recovery of *p*Cl-O^6BG when

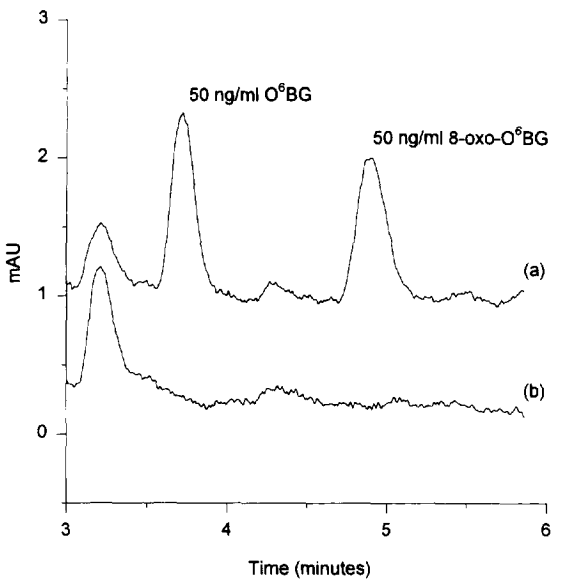

Fig. 1. Chromatogram of a plasma sample to which O⁶BG and 8-oxo-O⁶BG were added in the concentration range of actual patient samples (a) and a blank plasma sample (b). This demonstrates the absence of endogenous plasma compounds in the region of the chromatogram where O⁶BG elutes.

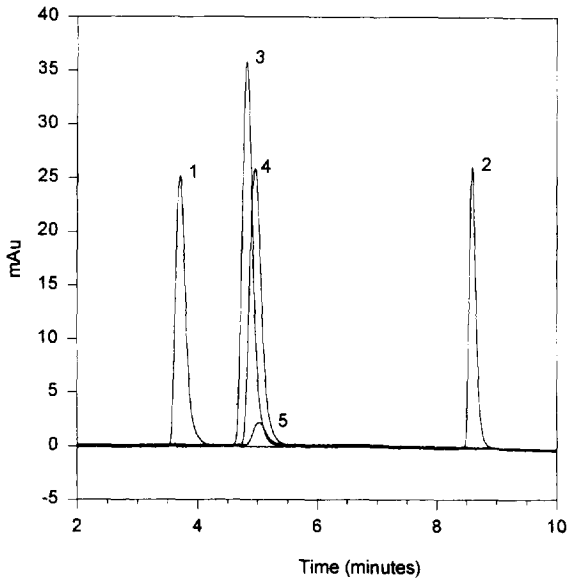

Fig. 2. This chromatogram shows O⁶BG (peak 1), the acetylated and 8-oxo metabolites (peaks 3–5) and the internal standard (peak 2). O⁶BG and the internal standard are baseline resolved from the possible metabolites. The standards were prepared in reconstitution solvent and chromatographed as described in Section 2.3.

Table 2
Possible internal standards

Compound	Selectivity factor[a]
O⁶-Benzylhypoxanthine	No response
O⁶-Benzyl-9-pivaloyloxymethylpurine	1.00
O⁶-Benzylguanine	1.00
O⁶-*p*-Methylbenzylguanine	1.73
O⁶-Benzylthioguanine	1.74
O⁶-*p*Cl-Benzylguanine	1.89
O⁶-Benzyl-9-ethoxycarbonylmethylpurine	2.21
O⁶-*p*-Methylbenzylthioguanine	2.24

Each compound was diluted to 1.0 μg/ml with phosphate buffer. Chromatographic conditions were as described in Section 2.3. Capacity factor k' was calculated according to the equation $k' = (t_{r\,\text{compound}} - t_0)/t_0$, where t_0 was defined as the time of initial baseline upset after injection.

[a]Selectivity factor = Capacity factor ratio ($k'_{\text{compound}}/k'_{\text{O6BG}}$).

sample residues were reconstituted in phosphate buffer, pH 3.2. This was corrected by reconstituting the residues in mobile phase A. However, when this solution was used to reconstitute the residues, an emulsion frequently formed. Residue emulsification was eliminated by increasing the ratio of organic solvent (methanol and acetonitrile proportionately) to phosphate buffer (30:30:35, v/v) in the solution used for residue reconstitution. The use of this reconstitution solvent resulted in a within-day relative standard deviation of internal standard peak heights of 7.0%.

3.5. Interfering drugs

Patients participating in the Phase I clinical trial were prescribed several therapeutic compounds which we investigated for possible chromatographic interference. The retention characteristics of these compounds were studied on our chromatographic system (Table 3). This was done by injecting standard solutions directly onto the chromatographic system. If a compound eluted in the chromatographic regions of O⁶BG, the possible metabolites, or the internal standard, its extent of extraction under these sample isolation conditions was tested. Among the drugs tested, only ondansetron interfered chromatographically with the O⁶BG quantitation and was extracted under our sample preparation conditions. Therefore, it is suggested that subjects receiving ondansetron not be evaluated using this procedure. Every set of plasma specimens obtained in the O⁶BG

Table 3
Drugs tested for interference with O^6BG quantitation

Compound	Concentration (μg/ml)	Retention time (min)	Extraction with ethyl acetate
Acetaminophen	100	n.d.	
Codeine	200	n.d.	
Erythromycin	500	n.d.	
Fentanyl	50	n.d.	
Flurazepam	100	n.d.	
Guanine	500	n.d.	
Morphine	400	3.090	n.d.
Ondansetron (Zofran)	200	3.95 and 4.23	Yes
Oxycodone	~100	4.023	n.d.
Prochlorperazine	500	n.d.	
Propoxyphene	~1000	n.d.	
Ranitidine	100	n.d.	
Trimethoprim/sulphamethoxazole	800/4000	2.76, 2.82 and 2.94	Yes
O^6BG	100	3.895	Yes
*p*Cl-O^6BG	100	8.678	Yes

The compounds were diluted in phosphate buffer, pH 3.2, to the concentrations listed above. A 15-μl aliquot was directly injected and chromatographed as in Section 2.3. Those compounds that co-eluted, or nearly co-eluted, with O^6BG or *p*Cl-O^6BG were extracted from an aqueous solution using the procedure described in Section 2.4 and re-chromatographed. Quantities of each drug in the μg/ml range were injected to ensure detection of those drugs that have therapeutic plasma concentrations in the μg range.
[a] n.d. = not detected.
[b] Yes = detected after extraction of aqueous standard solution.

clinical trial included one specimen taken before infusion of the drug. This measure discloses unexpected chromatographic interference.

3.6. Method validation

Linearity of the standard curve was validated from 25–400 ng/ml with an average slope of 0.975, an average regression coefficient of 0.999, and an average *y*-intercept of −0.023, (n = 4). At 25 ng/ml, samples were reliably quantitated with a signal-to-noise ratio of 8:1. Plasma concentrations of O^6BG in six patients studied extended from 300 ng/ml to the reliably quantitated level of 25 ng/ml. Six replicates of high and low concentration quality control samples were assayed each day for three days (n = 18) for day-to-day and within-day precision studies. The relative standard deviations for the high concentration quality control (305 ng/ml) and low concentration (38 ng/ml) quality control specimens from day-to-day were 8 and 7%, respectively. The within-day relative standard deviations were 2.7 and 3%, for the high and low concentration specimens, respectively. O^6BG apparently is stable in plasma specimens stored for 6 months at −70°C.

3.7. Method application

This assay procedure was used to evaluate the pharmacokinetic properties of O^6BG in patients in a Phase I clinical trial. The pharmacokinetic behavior of O^6BG in the first patient studied differed considerably from that expected on the basis of animal study data [5]. Therefore, the trial protocol blood-sampling intervals were shortened from the hourly intervals specified originally to provide better resolution of the O^6BG plasma elimination time course. Patients studied subsequently were given a 1-h bolus infusion of O^6BG (10 mg/m^2/h). Blood samples were drawn every 15 min throughout the infusion period and subsequently for 1 h, at 90 min and 2 h, and thereafter at hourly intervals until 8 h after termination of the infusion. Chromatograms of extracted plasma specimens obtained from one patient before and at the end of the infusion are shown in

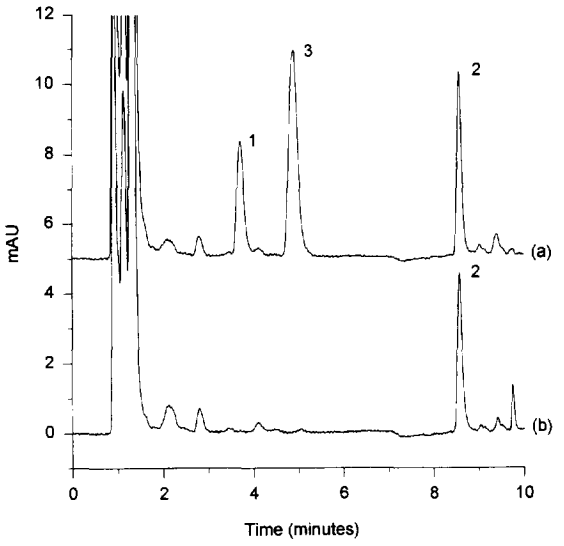

Fig. 3. Sample chromatograms from human plasma samples taken during the phase I clinical trial of O^6BG. (a) Sample chromatogram from a patient after a 1-h infusion with O^6BG. O^6BG and the tentatively identified 8-oxo-O^6BG metabolite appear as peaks 1 and 3, respectively. The internal standard, *p*Cl-O^6BG appears as peak 2. The extraction and chromatography were carried out as described in Section 2. (b) Representative chromatogram of a pre-infusion plasma sample from the same patient.

Fig. 3a and Fig. 3b. The concentration of O^6BG at the end of the infusion was 140 ng/ml. The chromatographic peak visible in Fig. 3a at 5.0 min, was identified tentatively as 8-oxo-O^6BG on the basis of retention behavior under other chromatographic conditions which resolved 8-oxo-O^6BG from acetylated metabolites, and by UV absorption spectra identical with authentic 8-oxo-O^6BG (data not shown).

As shown in Fig. 4, plasma steady-state concentration (Css) of O^6BG was achieved during the 1-h bolus infusion. The steady-state concentration was determined for each patient by averaging at least three infusion-period plasma concentrations. The O^6BG plasma elimination phase half-life was determined graphically from semi-log plots of O^6BG concentration vs. time. The average elimination phase half-life was remarkably short (29 min). The systemic clearance was calculated according to a non-compartmental pharmacokinetic model for each patient from the dose per 1-h infusion and the determined Css. These systemic clearance values are unusually large. Values obtained for these pharmacokinetic parameters are presented in Table 4. Details of the clinical Phase I study will be published elsewhere.

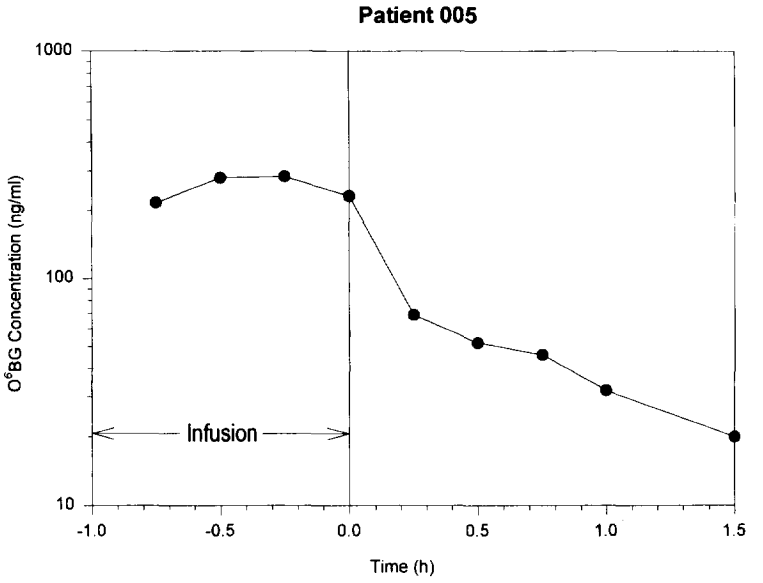

Fig. 4. Semi-log plot from a patient receiving a 1-h infusion bolus dose of O^6BG (10 mg/m^2/h). Plasma samples were drawn every 15 min from the beginning of the infusion until 1.0 h after the infusion. A steady state was established by the end of the infusion.

Table 4
Pharmacokinetic data

Patient	Dose (mg)	C_{ss} (ng/ml)	$t_{1/2}$ (min)	Clearance (l/min)
001	16.2	188	25	1.44
002	14.7	216	20	1.13
003	23.5	194	13	2.00
004	16.0	218	31	1.22
005	20.0	252	42	1.86
006	21.0	188	42	1.86
Mean		209	28.8	1.50
S.D.		24.8	11.8	0.36

Six patients received a 1-h infusion of O^6BG as described in Section 3.7 and the infusion period plasma concentrations of O^6BG were averaged to estimate the concentration at steady-state (C_{ss}). Semi-log plots (Fig. 4) were constructed, and the plasma elimination phase half-life was estimated from the elimination phase slopes of these plots. Clearance was calculated from the C_{ss} and the individual dose.

Acknowledgments

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