Determination of 8-oxoguanine in human plasma and urine by high-performance liquid chromatography with electrochemical detection

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Abstract

A highly sensitive and selective method for determining 8-oxoguanine in plasma and urine was developed by high-performance liquid chromatography with electrochemical detection. The compound was separated by gradient elution on a C_{18} reversed-phase column with a mobile phase of acetonitrile and 0.1 M sodium acetate, pH 5.2. 8-Hydroxy-2′-deoxyguanosine was used as internal standard. 8-Oxoguanine was detected electrochemically by setting the potential to +300 mV vs. Pd reference. The sensitivity of the assay was 22 ng/ml with a signal-to-noise ratio of 7:1. The within-day relative standard deviations for 8-oxoguanine quality control samples with concentrations of 3340, 1340 and 84 ng/ml were 3.6, 4.3 and 5.7% for plasma, and 4.1, 4.6 and 6.2% for urine, respectively. The day-to-day relative standard deviations for the same samples were 3.8, 6.8 and 7.1% for plasma, and 3.9, 7.0 and 7.9% for urine, respectively. The method is designed to study the pharmacokinetics and metabolic fate of O^6-benzylguanine in a phase I clinical trial. Previously, O^6-benzyl-8-oxoguanine was identified as the primary metabolite of O^6-benzylguanine in humans. We now demonstrate that 8-oxoguanine is a further metabolite of O^6-benzylguanine.   © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

O^6-Benzylguanine (BG#) is a potent inactivator of the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT), that enhances sensitivity of tumor cell lines and tumor bearing animals to nitrosoureas [1]. Inactivation of the alkyltransferase protein by non-toxic doses of BG renders a variety of human tumor cell lines more sensitive to the cytotox-

ic effects of alkylating agents including 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), chlorozotocin, clomesone, streptozotocin and temozolomide [2–4]. BG treatment prior to BCNU results in significant growth inhibition of nude mice carrying human brain [5–7] and colon tumor xenografts [8,9] compared to animals treated with BCNU alone. BG in combination with BCNU is presently in phase I/II clinical trials at several institutions [10–13].

In studying the metabolic fate of BG in rats, mice, nonhuman primates and humans, it was determined that BG is oxidized to an equally potent derivative,

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O^6-benzyl-8-oxoguanine (8-oxoBG). BG and 8-oxoBG in patient plasma and urine were previously identified using HPLC with UV detection [10,14,15]. Rat studies suggested oxidation of BG and de-benzylation of 8-oxoBG [16], however, there have been no studies to date reporting debenzylation in humans. Debenzylated products most likely eluted at the solvent front and therefore were not detected by using the traditional HPLC–UV detection system. In order to identify whether 8-oxoguanine was a meta-bolic product of BG in humans (Fig. 1), an HPLC with electrochemical detection (ED) assay was de-veloped to separate and detect this compound.

HPLC–ED based techniques to measure 8-oxo-guanine from DNA extracts have been reported previously [17–22]. Under these conditions, 8-oxo-guanine was separated from other artifacts of DNA extracts using anion-exchange columns [17], im-munoaffinity columns [18,19], and in vitro enzymatic preparation [20]. Reports of the determination of 8-oxoguanine in urine [23–27] have utilized an immunoaffinity column. In the present paper we describe a conventional HPLC system coupled with ED for biological samples from patients treated with BG that separates 8-oxoguanine from other metabo-lites and endogenous peaks in plasma and urine.

2. Experimental

2.1. Materials

Sodium acetate, glacial acetic acid, HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Itasca, IL, USA). Double-distilled deionized water was obtained by passing water through a Milli-Q reagent water system (Millipore, Bedford, MA, USA). The standard compounds of 8-oxoguanine, 8-hydroxy-2′-deoxyguanine (8OH2dG), 1-methylguanine, 7-methylguanine and uric acid were purchased from Sigma-Aldrich, (St. Louis, MO, USA). BG and 8-oxoBG were kindly supplied by Dr. Robert C. Moschel, NCI Frederick Cancer Research and Development Center (Fre-derick, MD, USA). O^6-Methylguanine was obtained from the Drug Synthesis and Chemistry Branch at the National Cancer Institute (Rockville, MD, USA).

2.2. Instrumentation

A model AS300 autosampler in line with a P4000 pump were purchased from Spectra-Physics (Riviera Beach, FL, USA). A CoulArray ED connected to a model 6210 electrochemical cell, and 5600 software used for collection and processing of chromatograph-ic data were from ESA (Chemsford, MA, USA). The chromatographic separation was achieved on a TosoHaas ODS-80TM column (5 μm particle size, 4.6×250 mm) purchased from TosoHaas (Mont-gomeryville, PA, USA).

2.3. Chromatographic conditions

Two mobile phases were required for gradient elution of BG, 8-oxoBG, 8-oxoguanine and internal standard 8OH2dG. Mobile phase A consisted of 0.1 M sodium acetate adjusted to pH 5.2 with glacial acetic acid. The buffer was then filtered through a 0.22 μm nylon membrane by vacuum filtration.
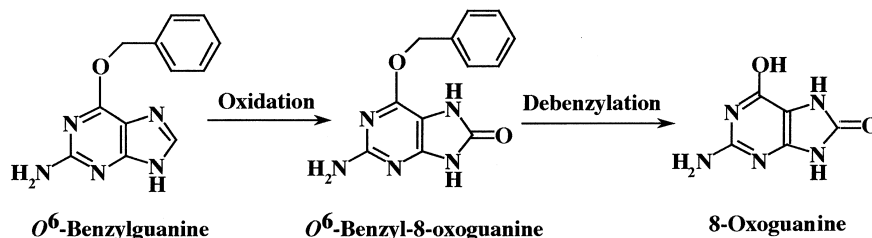

Fig. 1. Oxidation of O^6-benzylguanine and debenzylation of O^6-benzyl-8-oxoguanine.

Mobile phase B consisted of 100% acetonitrile (HPLC grade). The pump was set to deliver 2% of mobile phase B and 98% of mobile phase A at a flow-rate of 1.0 ml/min for 10 min after injection. The concentration of mobile phase B was then increased linearly to 40% over 10 min. This condition was sustained for 5 min to allow washing out of additional peaks. Mobile phase B was brought back to 2% within 0.1 min and the column was re-equilibrated for another 9.9 min. The column temperature was 31°C. CoulArray potentials were set as follows: channel 1 at +280 mV and channel 2 at +300 mV for detection of 8-OH2dG and 8-oxoguanine, and channel 3 at +400 mV for 8-oxoBG.

2.4. Sample preparation

The plasma samples were initially extracted using ethyl acetate. The results showed that only 17% of 8-oxoguanine was recovered. Methanol was then chosen to precipitate protein from plasma and 93% of 8-oxoguanine was recovered.

Patient samples and quality control samples were stored at −70°C. The plasma samples were thawed on ice and gently vortex-mixed. Aliquots of 500 μl of plasma were extracted by the addition of 5 ml of methanol and 10 μl of 8OH2dG (0.25 m*M* in MeOH, internal standard) in borosilicate glass tubes (16×100 mm). The mixture was vortex-mixed for 15 s and centrifuged at 1320 *g* for 20 min to achieve separation. The supernatant was dried under a stream of nitrogen gas. The resulting residues were reconstituted in 200 μl of a solution containing acetonitrile–0.1 *M* sodium acetate buffer, pH 5.2 (2:98, v/v) (reconstitution solvent). The reconstituted sample was centrifuged again for 10 min at 2800 *g*. Aliquots of 50 μl supernatant were injected onto the HPLC system.

Urine samples were centrifuged in plastic microfilter tubes (Nylon, size 0.22 μm, Corning Costar Co., Cambridge, MA, USA) at 20 800 *g* for 5 min. Filtered urine (130 μl) was added to 20 μl of reconstitution solvent and 50 μl aliquots were injected onto the HPLC column.

A typical daily sample set consisted of eight calibration standards, a plasma or urine blank, duplicate high, medium and low concentration quality control (QC) samples and several patient samples.

3. Results and discussion

3.1. Separation and identification of 8-oxoguanine

The oxidation of BG to 8-oxoBG and subsequent debenzylation to 8-oxoguanine in rats was reported previously by our laboratory [16]. In humans, BG rapidly converts to an equally potent AGT inactivator, 8-oxoBG, following intravenous infusion of BG, and only 0.2 and 3.1% of BG and 8-oxoBG is accounted for in the urine [10]. The further metabolism of BG in humans has not been reported. Using a C_{18} reversed-phase column with UV detection, 8-oxoguanine eluted with the solvent front and thus was difficult to quantitate. Consequently, this work was undertaken to determine if 8-oxoguanine was a product of BG metabolism in plasma and urine in humans.

HPLC–ED has been used for the detection of easily oxidizable compounds such as 8-oxoguanine [17–27] and 8-hydroxy-2′-deoxyguanosine [28–30]. In our CoulArray system, multiple channels were set at different potentials to give a two dimensional analysis. A peak is identified not only by retention time but by dominant channel (e.g. 8-oxoguanine at 300 mV) and peak ratios across the channels (as compared to authentic standard). This method is especially useful when evaluating components in a complex mixture such as urine [31,32]. Fig. 2 illustrates a chromatogram obtained after injection of an extract of plasma obtained from a patient who received a dose of 120 mg/m^2 BG i.v. over 1 h. Figs. 3 and 4 illustrate chromatograms obtained after injection of urine collected from two individual patients after BG infusion. The chromatographic peak at 11.2 min on channel 2 (+300 mV) corresponds to the elution of 8-oxoguanine. Other chromatographic peaks at 6.8 min and 18.5 min of channel 1 (+280 mV) and 24.3 min of channel 3 (+400 mV) correspond to elution of uric acid 8-hydroxy-2′-deoxyguanosine and 8-oxoBG, respectively. We have set channel 4 for detection of BG at 770 mV (not shown), but many oxidative artifacts
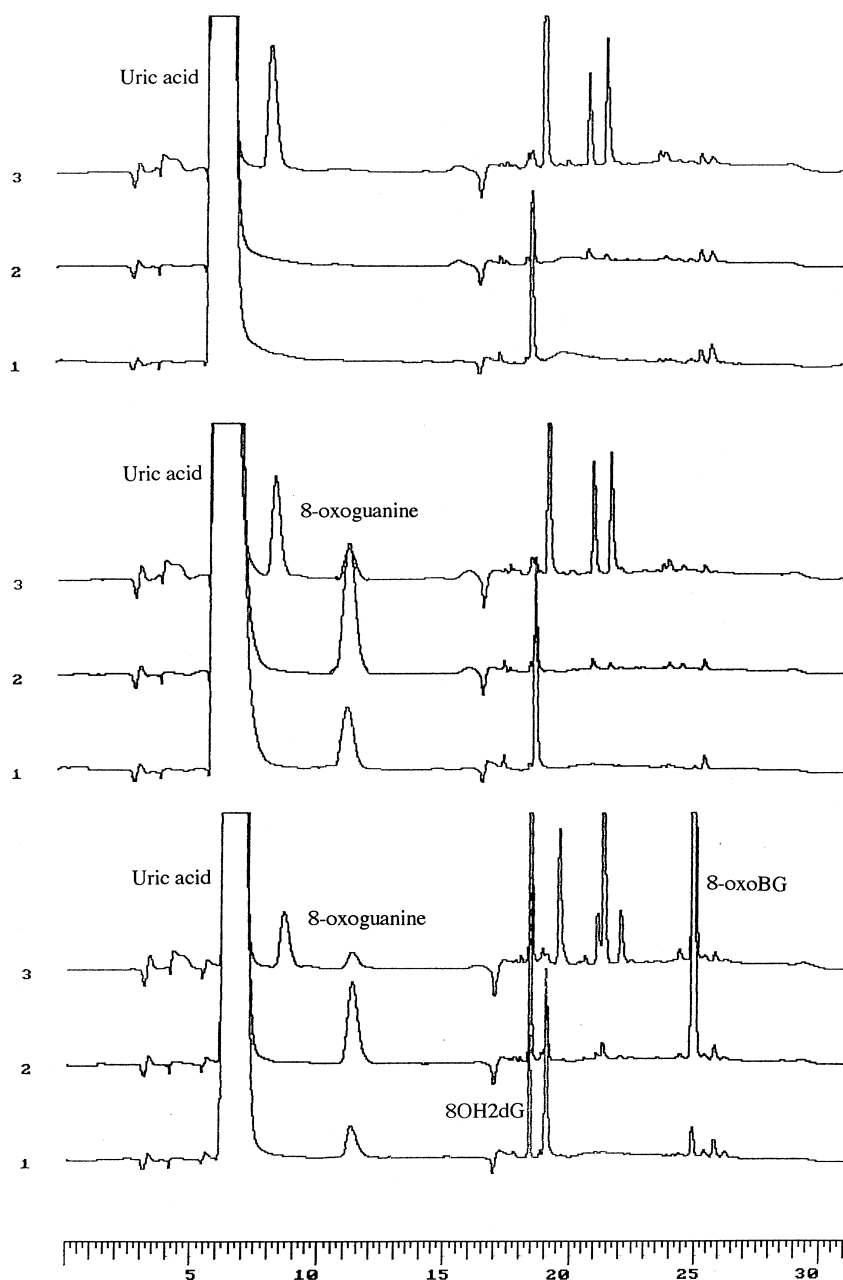
Fig. 2. Chromatographic profiles of plasma from patient #654. Channel 1=+280 mV; 2=+300 mV; 3=+400 mV (gain 5 μA). Graph A – control plasma; B – control plasma spiked with standard 8-oxoguanine; C – plasma drawn 8 h after BG infusion.

were also observed at this high voltage. BG and 8-oxoBG were quantified by HPLC–UV detection [10]. All peaks were identified by comparison of oxidation behavior potential and retention time with authentic compounds.

3.2. Chromatographic conditions

Separation of uric acid and 8-oxoguanine was attempted using several different columns and HPLC conditions including reversed-phase and anion-ex-
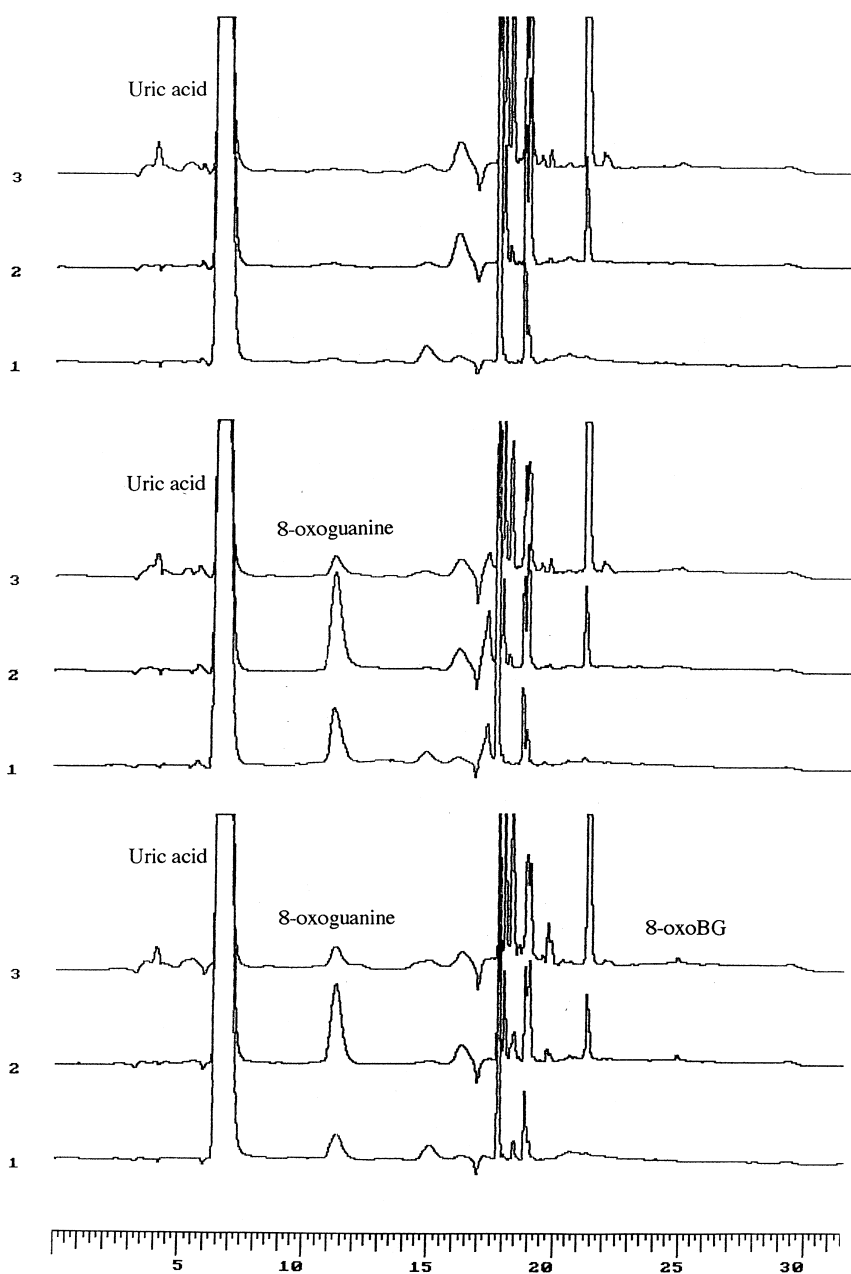
Fig. 3. Chromatographic profiles of urine from patient #619. Channel 1=+280 mV; 2=+300 mV; 3=+400 mV (gain 5 μA). Graph A – control urine; B – control urine spiked with standard 8-oxoguanine; C – urine collected 8 h after BG infusion.

change chromatography. The separation of 8-oxo-guanine from uric acid and other endogenous peaks in urine and plasma was achieved on an ESA Meta-250 (C_{18} reversed-phase) column with a mobile phase of 0.1 M sodium acetate buffer (pH 5.2) with methanol increasing from 0% to 50% over 45 min. However, after several runs, the 8-oxoguanine peak broadened and peak tailing was observed.
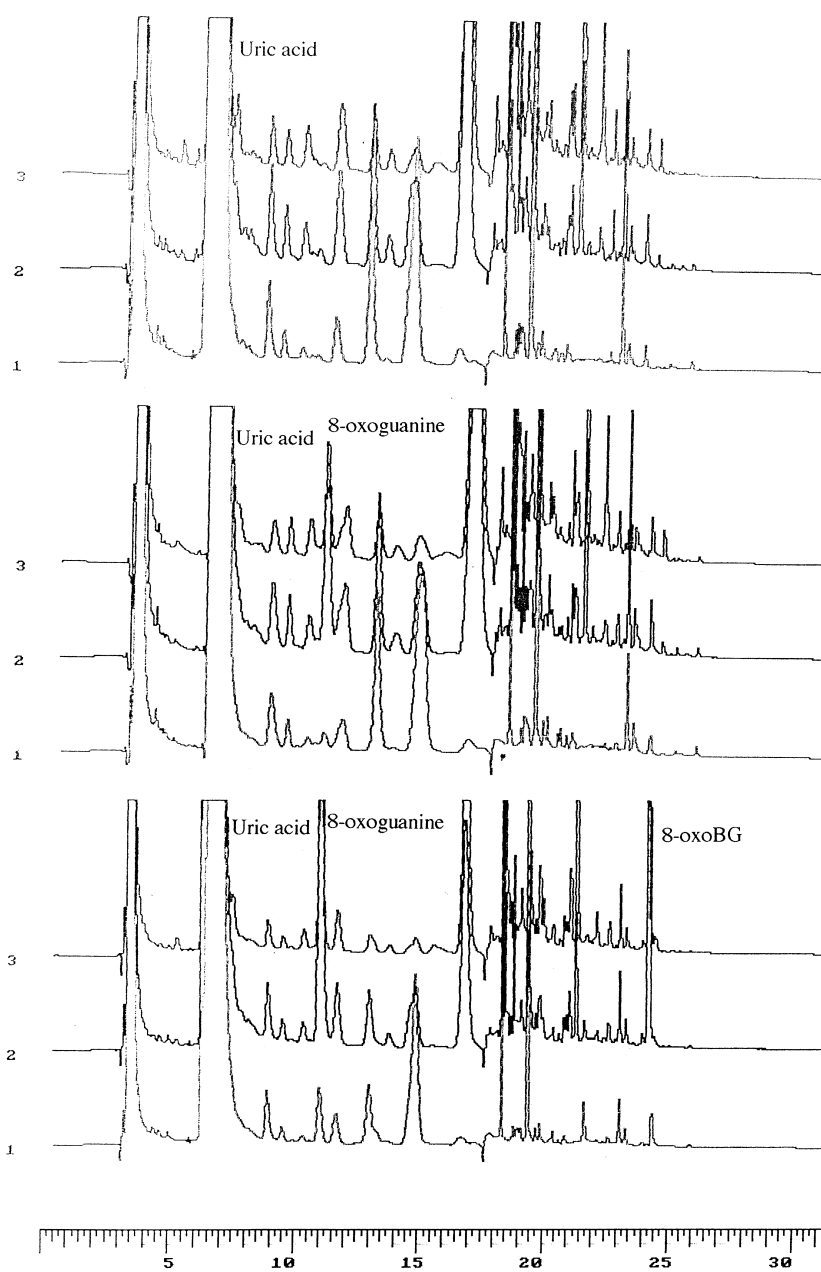
Fig. 4. Chromatographic profiles of urine from patient #658. Channel 1=+280 mV; 2=+300 mV; 3=+400 mV (gain 5 μA). Graph A – control urine; B – control urine spiked with standard 8-oxoguanine; C – urine collected 8 hour after BG infusion.

Using an acetonitrile–sodium acetate buffer on a TosoHaas ODS-80TM (C_{18} reversed-phase column), uric acid and 8-oxoguanine eluted at 6.8 and 11.2 min, respectively (Figs. 2–4).

3.3. Internal standard

Several compounds, including 8OH2dG, 1-methyl-guanine, O^6-methylguanine, 7-methylguanine and

Table 1
Possible internal standards

Compounds	Retention time (min)	Detection potential (vs. Pd, mV)	Recovery[a] rate (%)
8-Hydroxy-2′-deoxyguanosine	18.5	+300	98
1-Methylguanine	17.8	+650	96
O^6-Methylguanine	19.5	+660	89
7-Methylguanine	18.2	+800	84
Guanine	10.3	+590	94

[a] Recovered using 100% of methanol to precipitate protein.

guanine, were investigated as candidates for use as the internal standard for plasma (Table 1). 8OH2dG was selected as the internal standard for the assay because it was well separated from 8-oxoguanine, exhibited excellent recovery rate (98%) and was detected at a potential similar to that of 8-oxoguanine.

8-Oxoguanine in urine was quantitatively determined using an external standard since all the candidate internal standard compounds co-eluted with endogenous peaks in control urine. The control urine was spiked with increasing concentrations of 8-oxoguanine. It was observed that a small amount of 8-oxoguanine existed in control urine samples of four out of 60 patients. In these cases, the amount of 8-oxoguanine in control urine was subtracted from that collected after BG administration.

3.4. Method validation

Linearity of the standard curve for 8-oxoguanine in human plasma was validated from 22–3340 ng/ml with an average slope of 0.0039 ($n=8$), an average weighted linear regression coefficient of 0.998 ($n=8$) and an average y-intercept of −0.0697 ($n=8$). 8-Oxoguanine concentrations of patients studied extended from 1300 ng/ml to a reliably quantitated level of 33 ng/ml for plasma. The lowest limit of quantitation was 33 ng/ml with a signal-to-noise ratio of 7:1.

Linearity of the standard curve for 8-oxoguanine in human urine was validated from 22–66 800 ng/ml with an average slope of 0.1141 ($n=8$), an average weighted linear regression coefficient of 0.998 ($n=8$) and an average y-intercept of −0.1417 ($n=8$), when standard 8-oxoguanine was spiked into control urine.

8-Oxoguanine concentrations in 60 patients studied extended from 65 000 ng/ml to the reliably quantitated level of 22 ng/ml with a signal-to-noise ratio of 7:1 for urine.

Five replicates of high, medium, and low concentration samples (quality control) were assayed each day for three days ($n=15$) for within-day and day to day precision studies. The within-day relative standard deviations for 8-oxoguanine quality control samples with concentrations of 3340, 1340 and 84 ng/ml were 3.6, 4.3 and 5.7% for plasma, and 4.1, 4.6 and 6.2% for urine, respectively. The day-to-day relative standard deviations for the same samples were 3.8, 6.8 and 7.1% for plasma, and 3.9, 7.0 and 7.9% for urine, respectively.

3.5. Method application

The chromatographic procedure described in this paper was developed for identification and quantification of a BG metabolite that was observed in patient plasma and urine samples. When 8-oxoBG was incubated with human liver microsomes, 8-oxoguanine was detected using the described HPLC–ED system. The separation and identification of 8-oxoguanine in human plasma and urine following BG administration suggests debenzylation of 8-oxoBG in humans. This could result from hydroxylation on the phenyl ring or at the benzyl carbon and subsequent degradation.

In our phase I clinical trial, blood samples were drawn prior to the infusion, at 30 min during infusion and at the end of the 1 h infusion. After the infusion, blood samples were drawn at 10, 20, 30, and 45 min, and then at 1, 2, 4, 6, 8, 24 and and 48 h. Fig. 5 shows a semi-log plot of 8-oxoguanine
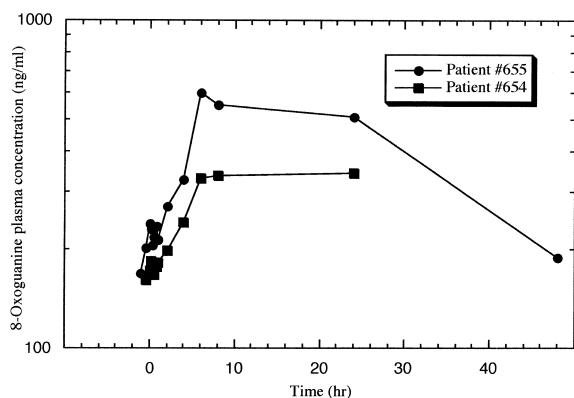
Fig. 5. Semi-log plot of plasma concentration of 8-oxoguanine from two patients receiving a 1 h intravenous infusion of BG. Plasma samples were drawn 30 and 60 min during infusion, and 10, 20, 30, 45 min, 1, 2, 4, 6, 8, 24 and 48 h after infusion. Time 0 indicates the end of infusion.

plasma concentration from two patients. 8-Oxo-guanine was detected immediately following the start of the infusion and the maximum concentration was reached at 8 h following the infusion. Urine samples were collected prior and 1, 4, 8, 24 and 48 h after the infusion. As shown in Table 2, 8-oxo-guanine was detected in urine samples collected at all time points after infusion. The maximum urine concentration was found in the urine collection at approximately 8 hour.

The elimination of 8-oxoguanine from plasma was slow. The enzyme responsible for debenzylation of 8-oxoBG is still unknown, however, preliminary data from our laboratory suggest that this is most likely a

Table 2
Summary of O^6-benzylguanine, O^6-benzyl-8-oxoguanine, and 8-oxoguanine detected in patient #619 urine sample[a]. (BG and 8-oxoBG were detected using a UV detector, $\lambda = 280$ nm)

Sampling point	[BG] (ng/ml)	[8-oxoBG] (ng/ml)	[8-oxoG] (ng/ml)
pre	0	0	0
1 h post	146	761	2500
4 h post	13.4	997	7360
8 h post	14.5	875	9250
24 h post	0	73.8	5170
48 h post	0	0	1090

[a] Total dose administered to the patient was 77.2 mg. The percentages of O^6-benzylguanine, O^6-benzyl-8-oxoguanine and 8-oxoguanine recovered in urine unchanged were 0.11, 1.34 and 28%, respectively.

microsomal reaction. Further studies to identify the enzyme(s) involved in this reaction are ongoing.

4. Abbreviations

BG	O^6-benzylguanine
8-oxoBG	O^6-benzyl-8-oxoguanine
8OH2dG	8-hydroxy-2′-deoxyguanosine
HPLC	high-performance liquid chromatogram
UV	ultraviolet
ED	electrochemical detector
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
CCNU	1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
AGT	O^6-alkylguanine-DNA alkyltransferase

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