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## Note

# Determination of methylglyoxal bis(guanylhydrazone) in cells in culture using high-performance liquid chromatography

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Methylglyoxal bis(guanylhydrazone) (MGBG) is a potent antiproliferative drug, which was used in the early 1960s in the treatment of acute leukaemia [1, 2]. However, it proved to be extremely toxic and its use was discontinued. In the early 1970s interest in the drug was revived when it was shown to be an irreversible inhibitor of eukaryotic S-adenosylmethionine decarboxylase (EC 4.1.1.50), one of the enzymes involved in the biosynthesis of the polyamines [3]. Since then, MGBG has been used as an experimental tool in numerous systems to deplete cells of polyamines [4—7]. It has been assumed that the antiproliferative action of the drug is mediated via its effect on polyamine synthesis, since the addition of exogenous polyamines reverses the growth inhibition produced by drug treatment. However, MGBG utilises the same transport system as the polyamines, spermidine and spermine [8], and their antagonistic effect on MGBG action may be due to competition for uptake rather than direct reversal of the drug's effects.

MGBG is currently being used clinically in combination with another polyamine antimetabolite, α-difluoromethylornithine [9, 10], in the treatment of acute leukaemia and patients treated with a sequential regime of these drugs have shown significant therapeutic response [9]. The potential of this type of combination therapy remains to be tested, but if MGBG is to be used effectively there must be a suitable assay system to measure cellular concentrations of the drug in order to keep them within the therapeutic range. In this

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study, we have developed a simple and rapid assay for MGBG, and have measured MGBG concentrations in two cell types in tissue culture; a rapidly growing cell, BHK-21/C13, and a non-proliferating cell, adult rat hepatocytes.

## EXPERIMENTAL

### *Materials*

MGBG dihydrochloride monohydrate was obtained from Aldrich (Gillingham, U.K.) and [$^{14}$C]MGBG was from Amersham International (Amersham, U.K.). Polyamine hydrochlorides, insulin and hydrocortisone 21-acetate were bought from Sigma (Poole, U.K.). Waymouth's medium, Dulbecco's medium and horse serum were purchased from Gibco Europe (Paisley, U.K.).

### *Sample preparation*

Stock solutions (1 m*M*) of MGBG were prepared by dissolving the compound in 0.9% (w/v) sodium chloride and diluting before use with either 0.9% (w/v) sodium chloride or 0.2 *M* perchloric acid. Samples were injected onto the high-performance liquid chromatography (HPLC) column in 0.2 *M* perchloric acid.

Polyamines and MGBG were extracted from cells in culture as described previously [11] and the polyamines analysed by a modification of the method of Seiler and Knödgen [12]. The efficiency of extraction of MGBG from these cells by 0.2 *M* perchloric acid was tested using [$^{14}$C]MGBG and was found to vary between 76 and 84% (not shown).

### *HPLC analysis*

Final separation of MGBG was achieved on a reversed-phase $C_{18}$ $\mu$Bondapak column (30 cm × 3.9 mm, Waters Assoc., Milford, MA, U.S.A.). The elution was isocratic in 0.1 *M* sodium phosphate buffer, pH 7.4–acetonitrile (9:1) at a flow-rate of 1.0 ml/min. Buffers were filtered through a Millipore filter (0.22 $\mu$m pore size) and dissolved gases were removed by bubbling the solutions with helium prior to use. MGBG was detected at 286 nm on a Cecil UV detector using a deuterium lamp. Samples were chromatographed at room temperature (approx. 20°C).

### *Cell culture*

Hepatocytes were isolated from male Sprague–Dawley rats (250–350 kg) by the method of Seglen [13]. All perfusion buffers were filter-sterilized before use and all instruments and glassware were sterilized by autoclaving. Cells were plated at a density of $9.4 \cdot 10^4$ cells per cm$^2$ and were grown for 24 h at 37°C in an atmosphere of air–carbon dioxide (95:5) in Waymouth's medium supplemented with 1% (v/v) horse serum, 1 $\mu M$ insulin and 10 $\mu M$ hydrocortisone 21-acetate. At the time of plating 10 $\mu M$ MGBG was added to the cultures.

BHK-21/C13 cells were grown in Dulbecco's medium supplemented with 10% (v/v) horse serum as described previously [11].

Protein content was measured by a modification of the method of Lowry et al. [14].

## RESULTS

The wavelength at which maximum absorbance of MGBG occurs varies with pH. Under our assay conditions $\lambda_{max}$ was found to be 286 nm.

A number of different mobile phases were tested. These included 0.1 *M* sodium acetate, pH 4.5—methanol in ratios of 6:4 to 9:1 in 10% increments and 0.1 *M* sodium acetate, pH 4.5—acetonitrile (8:2 and 9:1). Each of these solvent systems partially separated MGBG from the solvent front. However, the resolution was not sufficient for the purpose of this assay. The pH of the sodium acetate solution was increased in 0.5 pH units to pH 6.0. The buffer was then changed from sodium acetate to 0.1 *M* sodium phosphate and the pH again increased in 0.5 pH units to pH 7.5. At each pH value acetonitrile was added in amounts ranging from 5 to 15% (v/v).

Optimum conditions for separation were found to be an isocratic elution with 0.1 *M* sodium phosphate—acetonitrile (9:1) as the mobile phase at a flow-rate of 1.0 ml/min. MGBG was eluted from the column at 5.3 min. Fig. 1 shows a typical chromatogram of MGBG standard solution. MGBG (10 nmol) was injected onto the column and eluted as described in the figure legend. The sensitivity of the detector for this particular chromatogram was 1.00 OD units full scale deflection. The retention time and the half-height peak width

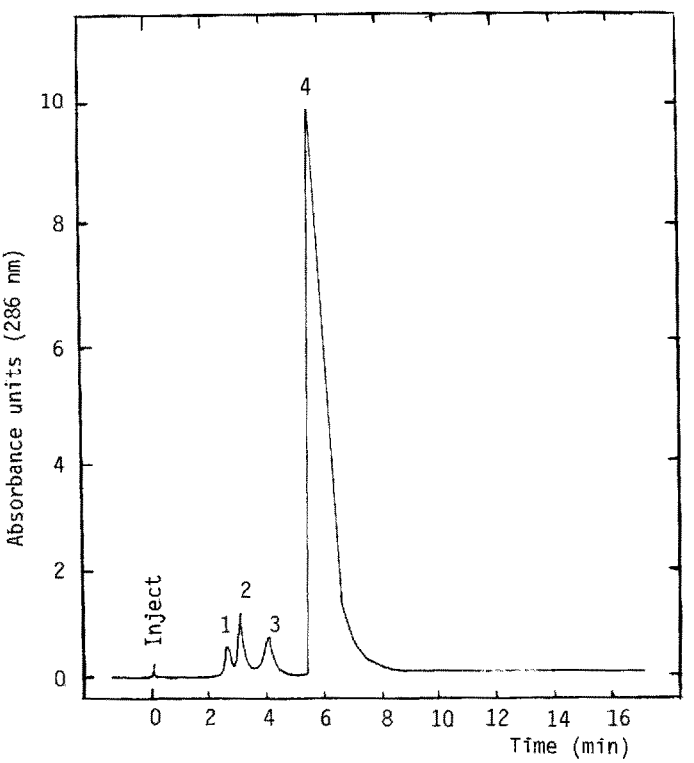


Fig. 1. Chromatogram of MGBG. MGBG (10 nmol) was chromatographed on a Waters $C_{18}$ μBondapak reversed-phase HPLC column. Separation was achieved by elution with 0.1 *M* sodium phosphate buffer, pH 7.4—acetonitrile (9:1) at a flow-rate of 1.0 ml/min. MGBG eluted at 5.3 min. Peaks: 1, 2, 3 = solvent front followed by perchloric acid peak; 4 = MGBG.

(3 mm) remained constant irrespective of the amount of MGBG injected. The standard curves of peak height and absorbance were linear with amount of drug up to 10 nmol with a correlation coefficient of 0.999 for both curves (not shown).

MGBG was extracted from cells in 0.2 *M* perchloric acid. In order to check for interference by other acid-soluble compounds, an acid extract from freshly isolated adult rat hepatocytes was mixed with an equal volume of drug and chromatographed using this method. There was no interference from the cell extract either to the retention time or to the half-height peak width. The amounts of MGBG determined from the peak height and standard curve compared well with the known amounts of drug injected on to the column. The largest variation observed was 7%. Samples were stored at −20° C for seven days and then rechromatographed. Again the variation was less than 10% (not shown).

The intracellular concentrations of MGBG and polyamines were measured in two cell types, BHK-21/C13 cells, and primary rat hepatocytes, after continuous exposure to the drug for 24 h. In each case, MGBG treatment increased putrescine and decreased spermidine concentrations (Table I). The spermine content of the BHK cells was also decreased markedly but the drug had little effect on the spermine content of hepatocytes. High concentrations of MGBG were observed, greater in fact than the total amount of polyamines in the cells (Table I). The concentrations of MGBG attained in rat hepatocytes and BHK-21/C13 cells were 1.2 and 4.6 m*M*, respectively, as calculated from the values measured (Table I), the volume of $10^6$ cells (3.76 and 1.6 $\mu$l) and the amount of protein per $10^6$ cells (1 and 0.33 mg).

## DISCUSSION

There are a number of ways of determining the intracellular concentrations

TABLE I

MGBG AND POLYAMINE CONCENTRATIONS IN MAMMALIAN CELLS AFTER 24 h IN CULTURE

Cells were grown for 24 h as described in the Experimental section. Control cultures were treated with 0.9% (w/v) sodium chloride and sample cultures with 10 $\mu M$ MGBG added to the extracellular medium. Results are the average of two experiments and are the values obtained at 24 h.

| Sample | Concentration (nmol/mg of protein) | | | | | Protein content (mg) |
|---|---|---|---|---|---|---|
| | MGBG | Putrescine | Spermidine | Spermine | Total polyamines | |
| *Hepatocytes* | | | | | | |
| Control | 0 | 0.30 | 1.41 | 1.59 | 3.30 | 0.93 |
| MGBG | 3.96 | 0.53 | 0.96 | 1.61 | 3.10 | 0.98 |
| *BHK-21/C13 cells* | | | | | | |
| Control | 0 | 0.03 | 2.17 | 2.22 | 4.42 | 0.37 |
| MGBG | 22.33 | 0.13 | 0.47 | 0.22 | 1.00 | 0.25 |

of MGBG. The most recent method is an enzymic inhibition assay which uses a dual enzyme system of diamine oxidase (non-competitive inhibitor) and S-adenosylmethionine decarboxylase (competitive inhibitor) [15]. This method has high sensitivity but requires partial purification of both enzymes prior to assay and is therefore technically more difficult and time-consuming than the HPLC method described in this paper.

If a drug is to be effective therapeutically it must be taken up by the cells and concentrated at its site of action. MGBG is known to be concentrated by cells in culture and concentrations of 4—6 m*M* can occur under conditions of rapid cell growth [15]. In this study we found that both cell types tested assimilated MGBG. The drug decreased the viability of the BHK cells by about 20% after 24-h treatment but had little effect on the viability of the hepatocytes. Primary cultures of hepatocytes attained an intracellular concentration of 1.2 m*M*. This value is lower than that obtained for the BHK cells (4.6 m*M*, Table I). The uptake of the drug does, however, depend on the growth rate of the cell [4] and therefore lower intracellular concentrations would be expected in non-proliferating primary hepatocytes compared to the rapidly growing BHK cells. The drug did prevent spermidine formation in both cell types but had no effect on the concentration of spermine within hepatocytes. This result would suggest that hepatocytes do synthesise spermidine, but not spermine in culture.

MGBG inhibited cell growth in the BHK cells as measured by the change in protein content but the degree of inhibition did not correlate with the intracellular concentration of drug. The drug did not affect the protein content of hepatocytes, probably because of their non-proliferating nature.

The present work describes an HPLC method for determining the cellular content of MGBG. The method is rapid and reproducible and is sensitive to 0.1 nmol. The method can be fully automated using a Waters Intelligence Sample Processor (WISP) to control the run time and injection cycle. Also since it was not affected by other acid-soluble compounds such as polyamines no further extraction of the drug is required.

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REFERENCES

1 E. Mihich, Cancer Res., 23 (1963) 1375.
2 R.H. Levin, E. Henderson, M. Karen and E.J. Freireich, Clin. Pharmacol. Ther., 6 (1964) 31.
3 H.G. Williams-Ashman and A. Schenone, Biochem. Biophys. Res. Commun., 46 (1972) 288.
4 P. Seppanen, L. Alhonen-Hongisto and J. Janne, Biochim. Biophys. Acta, 67 (1981) 169.
5 P. Seppanen, R. Fagerstrom, L. Alhonen-Hongisto, H. Elo, P. Lumme and J. Janne, Biochem. J., 221 (1984) 483.
6 J. Janne and D.R. Morris, Biochem. J., 218 (1984) 947.

7 L. Alhonen-Hongisto, V.A. Levin and L.J. Marton, Cancer Res., 45 (1985) 509.
8 E. Mihich, C. Dave and H.G. Williams-Ashman, Prog. Chemother. (Antibact., Antiviral, Antineoplast.), Proc. Int. Congr. Chemother., 8th, 1973, 3 (1974) 845.
9 M. Siimes, P. Seppanen, L. Alhonen-Hongisto and J. Janne, Int. J. Cancer, 28 (1981) 567.
10 R.P. Warrell, C.J. Coonley and J.H. Burchenal, Cancer Chemother. Pharmacol., 11 (1983) 134.
11 H.M. Wallace and H.M. Keir, Biochem. J., 202 (1982) 785.
12 N. Seiler and B. Knödgen, J. Chromatogr., 221 (1980) 227.
13 P.O. Seglen, Methods Cell Biol., 13 (1976) 29.
14 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
15 P. Seppanen, L. Alhonen-Hongisto, H. Poso and J. Janne, FEBS Lett., 111 (1980) 99.