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GAS CHROMATOGRAPHIC ASSAY FOR THE NEW ANTITUMOR AGENT PYRAZINE-2-DIAZOHYDROXIDE (DIAZOHYDROXIDE) AND ITS STABILITY IN BUFFER, BLOOD AND PLASMA

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SUMMARY

Diazohydroxide is a new antitumor agent being considered for clinical trial. A sensitive and specific assay for diazohydroxide in physiological media, plasma and blood has been developed based on conversion of diazohydroxide to 2-chloropyrazine in the presence of strong hydrochloric acid. The 2-chloropyrazine is extracted into the ethyl acetate and separated by capillary gas chromatography with nitrogen—phosphorus detection. Using 0.2 ml plasma the assay was linear up to 100 μg/ml diazohydroxide and had a lower limit of detectability for diazohydroxide of 50 ng/ml. The coefficient of variation of the assay at 1 μg/ml was 6.7%. Breakdown of diazohydroxide was rapid under mild acid conditions but slower under alkaline conditions. The half-life of diazohydroxide in 0.1 *M* sodium phosphate buffer, pH 6.0, at room temperature was 5 min and at pH 8.0, 480 min. Breakdown of diazohydroxide in plasma was biphasic. In fresh mouse plasma diazohydroxide had a terminal half-life at 37°C of 72 min while in fresh human plasma the terminal half-life was 23 min and in fresh blood 21 min. Diazohydroxide accumulated in red blood cells at 37°C to a concentration 68% above the concentration in plasma. Diazohydroxide was 49% bound to human plasma proteins at room temperature.

INTRODUCTION

Pyrazine-2-diazohydroxide (diazohydroxide), whose structure is shown in Fig. 1, has exhibited antitumor activity when administered intraperitoneally in a number of animal tumor models including L1210 and P388 leukemias, B-16 melanoma, M5076 sarcoma and in human MX-1 mammary xenograft [1]. The compound is currently being considered by the National Cancer Institute, U.S.A., for eventual clinical trial. We report a sensitive gas chromato-

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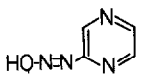

Fig. 1. Structure of diazohydroxide.

graphic (GC) assay for diazohydroxide in biological fluids based on its conversion to 2-chloropyrazine in the presence of strong hydrochloric acid. The assay has been used to show rapid breakdown of diazohydroxide in buffers under neutral and mild acid conditions and to study the stability of diazohydroxide in mouse plasma and in human blood and plasma.

EXPERIMENTAL

Drugs

Diazohydroxide (NSC-361456) was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, U.S.A.). Diazohydroxide was formulated immediately before use as a 10 mg/ml solution in 0.1 *M* sodium bicarbonate. 2-Chloropyrazine and 2,5-dimethylpyrazine were purchased from Aldrich (Milwaukee, WI, U.S.A.). All other chemicals were reagent grade.

Preparation of samples

A 0.2-ml volume of buffer, plasma or blood containing diazohydroxide was thoroughly mixed at room temperature with 50 μl of concentrated hydrochloric acid. The hydrochloric acid caused the breakdown of diazohydroxide to 2-chloropyrazine. The solution was then made basic by addition of 75 μl of 10 *M* sodium hydroxide. The mixture was shaken vigorously for 20 min with 1 ml ethyl acetate containing 1 μg 2,5-dimethylpyrazine as internal standard and then centrifuged at room temperature for 10 min at 1000 *g*. The upper ethyl acetate layer was removed and taken for GC analysis. To correct for any 2-chloropyrazine originally present in the sample before reaction with acid, a pre-mixed solution of 75 μl of 10 *M* sodium hydroxide and 50 μl of concentrated hydrochloric acid was added to a separate 0.2-ml volume of sample and extracted as previously described. Because this solution was basic, diazohydroxide was not converted to 2-chloropyrazine and any 2-chloropyrazine detected was due to prior chemical or metabolic breakdown of the drug. Diazohydroxide was measured as the difference in the amount of 2-chloropyrazine present in the acid-treated sample and in the base-treated sample. It should be noted that freshly prepared solutions of diazohydroxide contained negligible amounts of 2-chloropyrazine.

Gas chromatography

2-Chloropyrazine formed from diazohydroxide was assayed on a Hewlett-Packard 5880A gas chromatograph with a 7672A automatic sample injector and a nitrogen–phosphorus detector. The output from the detector was fed into a Hewlett-Packard 5880A gas chromatograph terminal and peak areas were integrated. Ethyl acetate, 3 μl, containing 2-chloropyrazine and 2,5-dimethylpyrazine internal standard was injected in the splitless mode onto a

25 m × 0.31 mm I.D. cross-linked 5% phenyl methyl silicone capillary column (Hewlett-Packard, Avondale, PA, U.S.A.) with helium as the carrier gas. The oven temperature was maintained at 50°C, injector temperature 200°C and detector temperature 300°C. Mass spectral analysis employed a Kratos MS50 gas chromatograph—mass spectrometer and 70-eV electron-impact ionization.

Stability studies

Studies on the stability of diazohydroxide at 100 μg/ml were conducted at room temperature in 5% dextrose, 0.9% sodium chloride, 0.1 *M* sodium phosphate buffer, pH 6.0, 7.0, 8.0 and 9.0, and 0.9% sodium chloride adjusted to pH 10.0 with 1 *M* sodium hydroxide. The stability of diazohydroxide in blood and plasma was studied by adding drug to fresh citrate-buffered human blood or plasma, or to fresh heparinized CDF_1 mouse plasma, at initial concentrations of 25, 50 and 100 μg/ml and incubating at 4, 20 and 37°C for up to 4 h. Stability studies in mouse plasma were conducted at 37°C only. Samples, 0.2 ml, of blood and plasma prepared from blood at the end of the incubation period were taken for analysis of diazohydroxide. Diazohydroxide and 2-chloropyrazine concentration data were subjected to non-linear least-squares regression analysis using the NONLIN computer program [2] and results expressed as half-life. Plasma protein binding studies were conducted with human plasma at 4°C and at room temperature with diazohydroxide at concentrations of 100, 50 and 25 μg/ml using Amicon C550A ultrafiltration cones (Amicon, Danvers, MA, U.S.A.).

RESULTS

The GC assay developed was based on conversion of diazohydroxide to 2-chloropyrazine by addition of strong hydrochloric acid. Positive identification of 2-chloropyrazine as the product of diazohydroxide breakdown was provided by comparison with a standard sample of 2-chloropyrazine and by mass spectral analysis of the chromatographic peak (Fig. 2). High-resolution mass determination gave a measured molecular weight of 113.9920 (calculated for $C_4H_3N_2{}^{35}Cl$ 113.9985) and for the major fragmentation peak 79.0252 (cal-

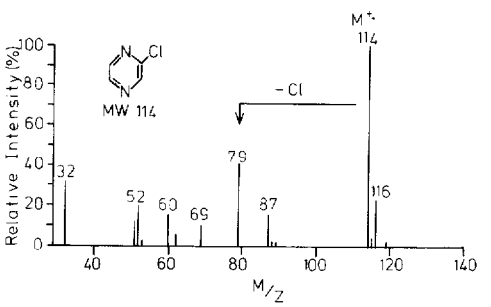

Fig. 2. 70-eV Electron-impact mass spectrum of the gas chromatographic peak of 2-chloropyrazine (inset) formed by addition of strong hydrochloric acid to diazohydroxide. The predominant molecular ion $M^{+\cdot}$ has a mass of 114 representing the ^{35}Cl form of 2-chloropyrazine with a smaller amount of the ^{37}Cl form at a mass of 116. The major fragment ion has a mass of 79 and shows no associated chlorine isotope peak due to the loss of Cl from the molecular ion.

culated for $C_4H_3N_2$ 79.0296). The formation of 2-chloropyrazine is similar to the Griess reaction where heating an acid or neutral aqueous solution of a diazonium salt in the presence of halide ion results in nucleophilic displacement of the diazonium group by halide [3]. The reaction of diazohydroxide with hydrochloric acid proceeded at room temperature and gave a yield of 2-chloropyrazine of 20.3%. 2-Chloropyrazine was not formed if hydrochloric acid was replaced by an equal amount of sulfuric acid in the reaction, providing confirmation that the reaction was proceeding by the halide-dependent Griess reaction. There was no detectable 2-chloropyrazine in control samples of freshly prepared diazohydroxide that had not been exposed to acid. In the related Sandmeyer reaction a metal, often a cuprous halide, is used to catalyze the displacement of the diazonium group by halide. Attempts to use cuprous chloride to increase the yield of 2-chloropyrazine from diazohydroxide were unsuccessful. Extraction of copper complexes into the ethyl acetate resulted in rapid deactivation of the GC column.

Extraction of 2-chloropyrazine into ethyl acetate was 67% at pH values between 1 and 12. Extraction of 2,5-dimethylpyrazine internal standard into ethyl acetate was more efficient at alkaline pH than at acid or neutral pH, and was 100% at pH 12. For this reason all samples were extracted after addition of excess sodium hydroxide.

A typical gas chromatogram for diazohydroxide added to human plasma is shown in Fig. 3. The GC assay was linear up to at least 100 μg/ml diazohydroxide and had a lower limit of detectability (peak > 3 × background) for diazohydroxide from 0.2 ml plasma of 50 ng/ml. The coefficient of variation of twelve repeated assays was 4.3% at 10 μg/ml, 6.7% at 1 μg/ml and 17.1% at 0.1 μg/ml.

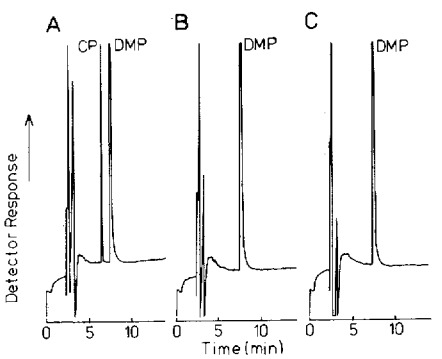

Fig. 3. Chromatogram of diazohydroxide added to human plasma at 1 μg/ml. (A) Plasma mixed with concentrated hydrochloric acid and extracted into ethyl acetate at pH 12. (B) Plasma extracted directly into ethyl acetate at pH 12. (C) Blank plasma mixed with concentrated hydrochloric acid and extracted into ethyl acetate at pH 12. Peaks: CP = 2-chloropyrazine; DMP = 2,5-dimethylpyrazine, internal standard, 5 μg/ml.

Having developed an assay procedure for diazohydroxide the stability of the drug was studied under a variety of conditions. Breakdown of diazohydroxide in 0.1 *M* sodium phosphate buffer was first-order and was much more rapid under acid than under basic conditions (Table I). When diazohydroxide was added to 5% dextrose or 0.9% sodium chloride the half-life was

variable, probably because of variations in the pH of the unbuffered solutions. Diazohydroxide was relatively stable when prepared in 0.9% sodium chloride adjusted to pH 10 with 1 *M* sodium hydroxide, having a half-life of 126 h at 4°C, 27.2 h at room temperature and 14.1 h at 37°C. A small amount of 2-chloropyrazine was formed spontaneously during breakdown of 2-diazohydroxide in medium containing chloride but was typically less than 3% of the total diazohydroxide breakdown.

TABLE I

STABILITY OF DIAZOHYDROXIDE IN VARIOUS MEDIA AT ROOM TEMPERATURE

The initial diazohydroxide concentration was 100 μg/ml.

Medium	Half-life (min)
Sodium phosphate buffer (0.1 *M*)	
pH 6.0	5
pH 7.0	44
pH 8.0	480
pH 9.0	1182
Sodium chloride (0.9%)	351, 672*
Dextrose (5%)	262, 330*
Sodium chloride (0.9%, pH 10)	1470, 1800*

*Values from two separate determinations.

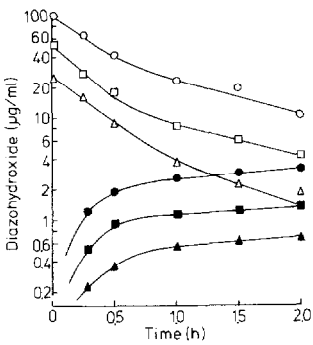

Fig. 4. Breakdown of diazohydroxide in fresh heparinized mouse plasma at 37°C. Diazohydroxide was added at initial concentrations of 100 μg/ml (○, ●), 50 μg/ml (□, ■) and 25 μg/ml (△, ▲). Open symbols are diazohydroxide, closed symbols are 2-chloropyrazine. Continuous lines are computer-generated fits to the data.

When diazohydroxide was added to fresh heparinized mouse plasma at 37°C biphasic decay curves were observed as shown in Fig. 4. The mean initial half-life for decay was 14.4 min and the terminal half-life 71.8 min. Small amounts of 2-chloropyrazine were formed by spontaneous breakdown of diazohydroxide in plasma and the rise in 2-chloropyrazine followed a single exponential with a half-life of 21.5 min. The amount of 2-chloropyrazine formed by 2 h was 3.2% of total amount of diazohydroxide initially present. When diazohydroxide was added to fresh citrate-buffered human blood and plasma the degradation was also biphasic. The half-lives are shown in Table II. Also

TABLE II

PROPERTIES OF DIAZOHYDROXIDE ADDED TO HUMAN BLOOD AND PLASMA

Temperature (°C)	Half-life* (min)				Red cell/plasma ratio**
	Plasma		Blood		
	α	β	α	β	
4	91	847	74	761	0.24
23	38	90	18	69	0.54
37	1	23	8	21	1.68

*Values are mean initial (α) and terminal (β) half-lives obtained at three concentrations of diazohydroxide (100, 50 and 25 μg/ml).

**Calculated from the extrapolated zero-time blood and plasma diazohydroxide concentrations when the same amount of diazohydroxide was added to plasma or to whole blood.

shown in Table II is the mean ratio of red blood cell diazohydroxide concentration to plasma diazohydroxide concentration calculated from the ratio of the extrapolated zero-time concentration of plasma diazohydroxide when the same amounts of diazohydroxide was added to plasma or to blood. The hematocrit of the blood used for the studies was 50%. The results show that accumulation of diazohydroxide by red blood cells increased as the temperature increased and reached a maximum of 168% of the plasma concentration at 37° C. Binding of diazohydroxide to proteins in human plasma determined by ultrafiltration was 62.6% at 4° C and 49.2% at room temperature.

DISCUSSION

Preliminary attempts to develop a sensitive high-performance liquid chromatography (HPLC) assay for diazohydroxide were unsuccessful. Diazohydroxide was not retained by a variety of normal- or reversed-phase HPLC columns. Reversed-phase ion-pair HPLC gave adequate retention but the limit of detectability for diazohydroxide measured by its absorbance at 280 nm was only 10 μg/ml. This lower limit of detectability was considered unlikely to be adequate for proposed studies in animals and clinical trials of the drug. Derivatization of diazohydroxide with diethyldithiocarbamate [4] was also unsuccessful because of a large interfering peak in plasma that could not be separated from the diazohydroxide-diethylthiocarbamate derivative peak.

Diazohydroxide can be converted to 2-chloropyrazine with an efficiency of approximately 20% in the presence of strong hydrochloric acid. The 2-chloropyrazine is readily extracted into ethyl acetate and can be analyzed by capillary GC giving a sensitive and reproducible assay for diazohydroxide. The breakdown of 2-diazohydroxide is very acid sensitive. Analogy to breakdown of pyridine-2-diazohydroxide [5] suggests that the rate-limiting step in the breakdown of diazohydroxide is conversion to the 2-diazonium ion which then reacts rapidly with either hydroxyl ion to form 2-hydroxypyrazine, or with other nucleophilic groups. The formation of small amounts of 2-chloropyrazine during breakdown of diazohydroxide in chloride-containing media is consistent with such a mechanism.

Generation of a reactive 2-diazonium ion might be related to the antitumor activity of diazohydroxide. Tumors frequently have a more acid local environment than normal tissue. Studies with experimental tumors have reported an interstitial fluid pH of 5.8—7.2 compared to a pH of 7.1—7.4 for normal tissue [6–8]. The acidic environment of a tumor might lead to increased formation of 2-diazonium ion and, possibly, enhanced cell tumor responsiveness to diazohydroxide, compared to normal tissue.

The present studies show that diazohydroxide is relatively stable in 0.9% sodium chloride adjusted to pH 10 with 1 *M* sodium hydroxide and this might form a suitable vehicle for formulating the drug for clinical use. The relative instability of diazohydroxide in plasma will necessitate that for in vivo pharmacokinetic studies of the drug the plasma will have to be adjusted to alkaline pH immediately after collection. It should then be possible to store the plasma frozen until assay of diazohydroxide.

In summary, a sensitive GC assay for diazohydroxide has been developed based on conversion of diazohydroxide to 2-chloropyrazine under acid conditions. The assay has been used to study the stability of diazohydroxide in buffers and in blood and plasma.

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