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Note

Quantitation of methanol formed in cell culture cytotoxicity assays and as a metabolite in microsome suspensions

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Many important cancer chemotherapeutic agents, mutagens and carcinogens are known to undergo a chemical or metabolic conversion to a methylating intermediate [1, 2]. Included in this category are methylnitrosamine, nitrosourea, hydrazine, azo, diazo, azoxy, diazotate, nitrosocarbamate, nitrosonitroguanidine and triazene containing compounds [3], which are precursors to the methyldiazonium ion. The pharmacologic activity of these agents is thought to result from the small fraction of methyldiazonium ion that reacts with cellular macromolecules. The fraction, over 95%, of methyldiazonium ion that does not covalently bind to large molecules reacts with water to form methanol [4]. Thus the amount of methanol formed in these reactions is a good measure of the amount of pharmacologically active species that has been produced. It is of interest to be able to relate the activity of an agent in a cytotoxicity or mutagenicity cell culture assay to the actual amount of active methyldiazonium ion formed during the assay procedure. Similarly, for agents that require metabolic activation, it is important to know the percent conversion of parent agent to active species that occurs in in vitro metabolic systems. This paper reports analytical methods for the measurement of methanol formed in in vitro rat liver microsome preparations and in cell culture assay incubations at concentrations as low as 10 nmol/ml.

There are a number of problems associated with the analysis of trace amounts of methanol in aqueous mixtures. The high volatility and polarity of methanol precludes extensive sample handling and extraction techniques. Derivatization methods for methanol become unreliable in dilute solutions [5, 6] and methanol itself is not readily detected using spectrophotometric or electrical

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detectors. Head-space analysis is unsatisfactory due to large errors in the results, 20—30% error has been reported with methanol [7, 8]. Gas chromatography with flame ionization detection can quantify methanol concentrations of 10^{-4} *M* [9]. Attempts to increase the sensitivity require large sample sizes, > 5 ml, and have lower precision [5].

This paper reports a gas chromatographic—mass spectrometric (GC—MS) assay that employs methane chemical ionization and selected ion monitoring to quantify 0.5 ng methanol per μl injected. Microsomal incubation preparations were found to contain significant amounts of methanol and ethanol as background impurities. Quantitation of methanol formed as a metabolite under these conditions is possible only if the parent agent contained a deuterium-labeled methyl group and ethanol-d_5 was used as an internal standard.

EXPERIMENTAL

Instrumentation

Mass spectra were obtained on a Finnigan 4023 mass spectrometer equipped with a dual chemical ionization (CI) electron impact source and operated in the CI mode. Methane was used as a carrier and reagent gas at an indicated ion chamber pressure of 0.25 torr. Samples were introduced through a gas chromatograph containing a 180 cm × 6.3 mm O.D. glass column packed with Porapak Q-S at a column oven temperature of 110°C. Eluting ions were detected using an Incos 2000 data system capable of monitoring selected ions.

Reagents and materials

1-Methyl-3-(4-methylphenyl)triazene, 1-methyl-3-phenyltriazene and 1-methyl-3-(4-carboxymethylphenyl)triazene were prepared by the method of White et al. [10]. 1-Hydroxymethyl-3-(4-carboxymethylphenyl)triazene was prepared by the method of Gescher et al. [11]. 1-Methyl-1-nitrosourea was from Sigma (St. Louis, MO, U.S.A.). Synthesis of methyl-d_3-benzylhydrazine hydrobromide will be described elsewhere [12]. Methanol-d_4 was from Aldrich (Milwaukee, WI, U.S.A.), ethyl-d_5 alcohol from MSD Isotopes (St. Louis, MO, U.S.A.). Dimethylsulfoxide (DMSO) (Mallinckrodt, St. Louis, MO, U.S.A.) was purified over 10-Å molecular sieves. Ethyl alcohol absolute was from U.S. Industrial Chemicals (New York, NY, U.S.A.), glucose-6-phosphate, NADP and glucose-6-phosphate dehydrogenase from Sigma. Fischer's medium from Bellco Biotechnology (Vineland, NJ, U.S.A.), 10% (v/v) donor horse serum from Gibco Labs. (Grand Island, NY, U.S.A.). Hypo-vials™ were from Pierce (Rockford, IL, U.S.A.).

Mass spectral analysis

Chemical reactions in aqueous solution or cell culture medium and metabolic reactions in microsome suspensions were conducted in 6-ml and 50-ml hypo-vials, respectively. The vials were sealed with PTFE-coated silicone septa. Larger vials were used in the microsome metabolisms to ensure an adequate supply of oxygen. The chemical or metabolic reactions were carried out at 37°C at a pH of 7.4 on 5 ml of reaction medium. The use of septum-sealed vials

prevented extensive loss of methanol which would have occured if the reaction medium were open to the atmosphere. In the chemical and cell culture reactions, a known amount of methanol-d_3 was added as an internal standard. The methanol-d_3 was added to the medium by injection through the septum prior to incubation. After incubation a known amount of ethanol was added as a second internal standard. In the metabolic reactions a known amount of ethanol-d_5 was added as the internal standard after the incubation. After the incubation reactions were complete, the vials were cooled to near 0°C and 1 μl of the solution was removed for injection into the GC—MS instrument. Chromatographic separation of the solvent water, methanol and ethanol was obtained by operating a Porapak Q-S column at 110°C. Elution times observed using methane as a carrier gas at a flow-rate of 35 ml/min were 1.7 min and 4.5 min for methanol and ethanol, respectively. Methanol, methanol-d_3, ethanol and ethanol-d_5 were detected by monitoring the respective protonated molecular ions. The amount of methanol present was quantified by measurement of the GC—MS peak height ratio with the ethanol standard with reference to a standard curve. The ratio of the methanol-d_3 to ethanol was monitored to determine if methanol was lost through evaporation or metabolism during the course of the incubation period and analysis.

Cell culture incubations

The cell incubation experiments were carried out with P388 lymphocytic leukemia cells suspended in Fisher's growth medium at pH 7.4. The medium was supplemented with 10% (v/v) donor horse serum, 100 μg/ml of streptomycin and 100 units per ml of penicillin G. Prior to each incubation, the cells were counted on a hemocytometer and the cell count was adjusted to a

TABLE I

METHANOL ANALYSIS IN P388 LEUKEMIA CELL SUSPENSION AFTER TREATMENT WITH METHYLNITROSOUREA AND METHYLTRIAZENES

Compound	Reaction time (min)	Initial concentration (μmol/5 ml)	Methanol calculated (pmol/μl)	Methanol observed (pmol/μl)	Percent difference*
Blank (DMSO)	15	0.0	0.0	40.6	
1-Methyl-1-nitrosourea	60	2.48	497	531	1.4
1-Methyl-3-phenyltriazene	12	3.62	650	709	2.8
1-Methyl-3-(4-methylphenyl)triazene	40	1.93	422	422	—9.5
1-Methyl-3-(4-carboxymethylphenyl)triazene	76	1.88	372	406	—1.9
1-Hydroxymethyl-3-(4-carboxymethylphenyl)-triazene	88	1.80	359	381	—5.3

*Percent difference = observed — blank value.

concentration of $1 \cdot 10^6$ cells per ml. The cell suspension was placed in a 6-ml hypo-vial and sealed. A 5-μl aqueous solution containing 10 μmol of methanol-d_3 and 86 μmol of ethanol was added by syringe through the septum followed by addition of a 25-μl dimethylsulfoxide—drug solution (see Table I for drug concentrations). Each drug was then incubated in a Dubnoff Metabolic Shaking Incubator at 37°C for a period of time equal to four half-lives (see Table I). At the conclusion of the incubation the mixture was cooled in an ice-bath and 1 μl of the solution was removed and analyzed by GC—MS.

Microsomal metabolism

The metabolism experiments were performed with rat liver microsomes isolated from phenobarbital-induced (6 mg per day for ten days) Fischer (F-344) male rats. The 100,000 *g* rat liver microsomal pellet suspensions were prepared according to the procedure of Fouts [13]. Metabolism reactions were carried out in 50-ml hypo-vials. The metabolism solutions contained 24 mg of microsomal protein (8 mg/ml), 9.4 m*M* glucose-6-phosphate, 3.7 units of glucose-6-phosphate dehydrogenase, 2.9 m*M* NADP, 6.1 m*M* $MgCl_2 \cdot 6H_2O$, 9.5 m*M* phosphate-buffered saline (PBS) "A" (pH 7.4) buffer and 0.11 m*M* methyl-d_3-benzylhydrazine hydrobromide. The solution was placed in the hypo-vial and the metabolism was initiated by addition of the glucose-6-phosphate dehydrogenase and the vial was then immediately sealed. The metabolism solution was incubated at 37°C for 20 min in a Dubnoff Metabolic Shaking incubator. After 20 min the metabolism vial was cooled to 0°C in an ice-bath and the internal standard, $C^2H_3C^2H_2OH$, was added by syringe through the septum. The samples were then analyzed by GC—MS using 1 μl of the metabolism solution.

RESULTS AND DISCUSSION

Reactions in aqueous solution, P388 cell culture medium or microsomal suspension were conducted in sealed vials to prevent loss of methanol by evaporation. Addition of standards and removal of samples from the reaction medium were made using a syringe inserted through a PTFE-coated silicone septum. No sample workup procedures were used because of the difficulties in handling trace amounts of volatile methanol. Instead, the reaction mixture was injected directly onto the GC column and the methanol and internal standards were detected with adequate sensitivity by methane CI-MS using selected ion monitoring. A standard curve for methanol in water gave a slope of 0.876 with a correlation coefficient of 0.966. The curve was linear over a range of 25 to 1100 pmol methanol injected.

The methanol MH^+ ion appears at *m/z* 33. At this low mass some sensitivity is lost due to the presence of an appreciable level of background ion current. An attempt was made to increase the observed mass of methanol by employing a CI reagent gas that formed higher mass association ions. Our most successful effort used ethylenediamine (E) as a reagent gas which was added to the methane carrier gas at a partial pressure that was great enough to give a reagent gas ion plasma consisting primarily of an ethylenediamine dimer $(E—H—E)^+$. When methanol was introduced into this plasma the only ion produced was $(CH_3OH—H—E)^+$. The stability of this ion was presumably due to the fact that

protonated methanol can form two hydrogen bonds to the ethylenediamine amino groups. Unfortunately, the efficiency of the ionization process was low and the net sensitivity was somewhat less than that of methane reagent gas.

We also employed tetramethylsilane as a reagent which has been reported to selectively form trimethylsilyl adduct ions with alcohols [14]. This report was confirmed by our results, but we experienced repeated serious losses of sensitivity related to contamination of the ion source by this reagent gas.

The cytotoxic activity of methylnitrosourea and 1-methyl-3-aryltriazenes can be measured by incubating these agents with suspended P388 leukemia cells in Fischer's medium containing 10% (v/v) donor horse serum at pH 7.4, 37°C for defined periods, usually 30 to 120 min. When the drug effect of treated vs. non-treated cells is related to the drug concentration decrease occurring during the cell exposure period, these agents are found to have equal activity [15]. If the concentration decrease is related to the amount of methyldiazonium ion formed, this provides further evidence that the toxic effect of presumably randomly generated methyldiazonium ion is independent of parent drug structure [16]. The relationship between parent drug concentration decrease, cytotoxicity and methanol formation may be defined more directly by measuring the amount of methanol formed during the assay incubation period.

The methanol assay procedure was tested in two experimental systems, an in vitro cell culture cytotoxicity assay and a microsomal metabolism preparation. The drugs listed in Table I were incubated with P388 cell suspension at 37°C by the method described above. Each drug was incubated for a period of time equal to four half-lives. After incubation the samples were analyzed by GC—MS. Table I shows the measured methanol concentration expected from disappearance of parent drug assuming 100% conversion to methanol. The small amount of methanol observed in the blank was an impurity in the dimethylsulfoxide. The impurity was reduced to the amount observed in the blank by treatment of the dimethylsulfoxide with 10-Å molecular sieves. Acetone has been found to contain less methanol impurity and may be used as an alternative solvent. The observed value less the blank corresponds closely to the expected value of methanol indicating that a single reaction pathway is operating with these agents. The average difference is −2.5% with a 4.4% standard deviation. Triazenes produce a 50% decrease in P388 cell growth rate at a dose that leads to the formation of 80 to 120 μM methanol. The concentrations reported in Table I are below the limits of detectable toxicity.

Methanol was also measured as a product of the phenobarbital-induced rat liver 100,000 *g* microsomal metabolism of 1-methyl-2-benzylhydrazine hydrochloride. Methylhydrazines are converted to azo, azoxy and azoxycarbinol metabolites by cytochrome P-450 enzymes [17]. The proposed azoxycarbinols react chemically to give methyldiazonium ion intermediates and methanol as a stable product [18]. Measurement of methanol formation would indicate the extent of conversion of parent drug to the ultimate active species, methyldiazonium ion. Analysis of the incubation mixture in the absence of substrate showed high levels of both methanol and ethanol. This material was present in the glucose-6-phosphate (methanol) and NADP (ethanol) and could not be

completely and reproducibly removed. Since the amount of methanol formed in this reaction was small, the presence of even low amounts of background methanol and ethanol prevented its measurement.

An accurate analysis of methanol metabolite formation could be made, however, by using a deuterium-labeled substrate, 1-methyl-d_3-2-benzylhydrazine hydrobromide and ethanol-d_5 as a standard. Fig. 1 shows a representative selected ion chromatogram of methanol-d_3, m/z 36, and standard ethanol-d_5 formed during the 20 min incubation of 1.02 μmol (142 μg)

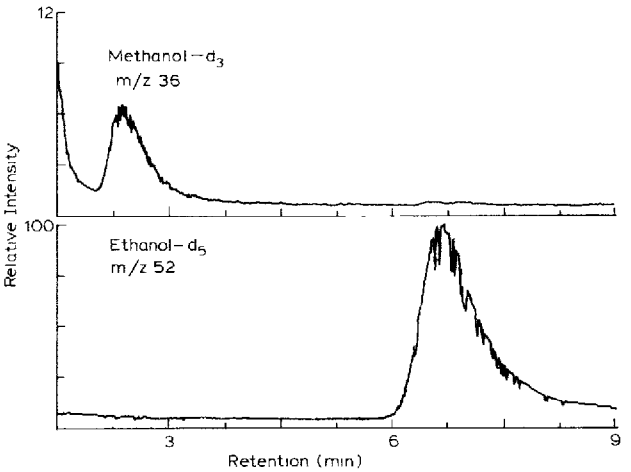

Fig. 1. Selected ion chromatogram of methanol-d_3, m/z 36, and internal standard ethanol-d_5, m/z 52, present in 1 μl of a 100,000 g microsomal metabolism mixture of 1-methyl-d_3-2-benzylhydrazine. The methanol peak at retention time 1.7 min represents 10.9 pmol injected onto a Porapak Q-S column at 110° C.

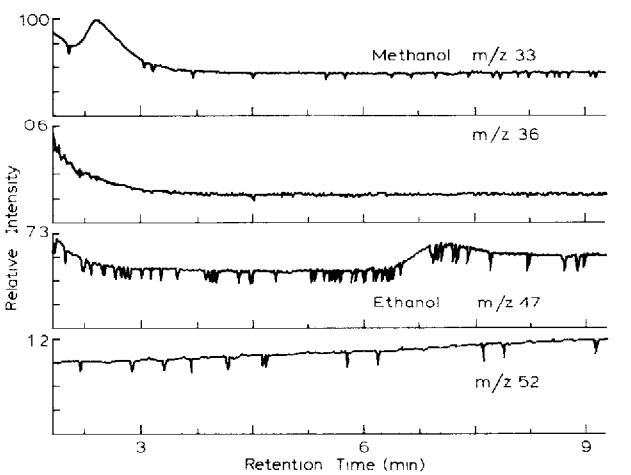

Fig. 2. Selected ion chromatograms from the injection of 1 μl of 100,000 g microsome suspension containing all cofactors and solvents normally present in a drug metabolism experiment. Methanol, m/z 33, and ethanol, m/z 47, are present as significant impurities that could not be completely removed.

deuterated 1-methyl-2-benzylhydrazine in 5 ml of rat liver 100,000 *g* microsome suspension containing 24 mg microsomal protein. The methanol peak of Fig. 1 represents 10.9 pmol injected onto the column or 54.5 nmol of methanol formed in the metabolism reaction, 5.3% of initial substrate. A 5.4% standard deviation was observed on three replicate incubations. Fig. 2 shows a selected ion chromatogram of a 100,000 *g* microsomal suspension that contains all cofactors and solvents but not substrate. Methanol, *m/z* 33, and ethanol, *m/z* 47, are present as impurities at a concentration comparable to the amount of methanol formed in these inefficient metabolism reactions. No interfering peaks are present at the masses of the deuterated variants.

These results demonstrate that methanol can be quantitated in the low pmol range from complex mixtures. This assay can provide a measure of the amount of active methyldiazonium ion formed from cytotoxic and carcinogenic methylating agents and will aid in the understanding of the mechanism of action of these compounds.

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