

Simultaneous Determination of Formaldehyde and Methylglyoxal in Urine: Involvement of Semicarbazide-Sensitive Amine Oxidase-Mediated Deamination in Diabetic Complications

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Abstract

The deamination of methylamine and aminoacetone by semicarbazide-sensitive amine oxidase (SSAO) produces formaldehyde and methylglyoxal, respectively, which have been presumed to be involved in diabetic complications. A high-performance liquid chromatography procedure using 2,4-dinitrophenylhydrazine (DNPH) as a derivatizing agent is developed to determine endogenous formaldehyde, methylglyoxal, malondialdehyde, and acetaldehyde. The devised DNPH method is sensitive enough to analyze aldehyde levels in urine. An increase in the excretion of formaldehyde, methylglyoxal, and malondialdehyde is confirmed in streptozotocin-induced diabetic rats. Following the chronic administration of methylamine, the urinary levels of both formaldehyde and malondialdehyde (a product from lipid peroxidation) are found to be substantially increased. A potent selective SSAO inhibitor, (E)-2-(4-fluorophenethyl)-3-fluoroallylamine hydrochloride (MDL-72974A), reduced the formation of formaldehyde, methylglyoxal, and malondialdehyde. The increase of the cytotoxic aldehyde levels as a result of increased SSAO-mediated deamination may occur in some pathological conditions.

Introduction

Semicarbazide-sensitive amine oxidase (SSAO, International Union of Biochemistry EC 1.4.3.6) is an enzyme or group of enzymes that are sensitive to semicarbazide and related hydrazine compounds and are distributed in different tissues (1), particularly in vascular smooth muscles and cartilage tissue (2). Methylamine and aminoacetone are considered to be physiological substrates for SSAO enzymes (3,4,5). Formaldehyde and

methylglyoxal are products deaminated from methylamine and aminoacetone, respectively.



Methylglyoxal has been found to be increased in diabetes and considered to be involved in protein glycation and associated diabetic vascular disorders (6). Interestingly, serum SSAO activity has been reported to be increased in diabetic patients (7–9) and also in the blood and kidney of streptozotocin (STZ)-treated diabetic rats (10). More recently, plasma SSAO was also found to be elevated in patients with congestive heart failure (11). It has been proposed that the increased SSAO-mediated deamination from methylamine and aminoacetone may be related to diabetic complications (12,13).

There are other sources for forming endogenous aldehydes besides the SSAO-mediated deamination. Formaldehyde, acetaldehyde, and malondialdehyde are common products of lipid peroxidation that reflect the interaction between molecular oxygen and polyunsaturated fatty acids (14,15). Methylglyoxal can be formed nonenzymatically or enzymatically by sugar fragmentation reactions in glycolysis (16,17). Formaldehyde and methylglyoxal can rapidly interact with cellular constituents or quickly metabolize. By tracing residual radioactivity after the administration of ^{14}C -methylamine in the presence or absence of specific SSAO inhibitors, the formation of formaldehyde and its irreversible interaction with tissue components was demonstrated in vivo (18). Several chromatographic procedures for the determination of aldehydes were described, such as high-performance liquid chromatography (HPLC) with fluorescence detection of 1,3-cyclohexanedione (19) or 4-(*N,N*-dimethylamino-sulphonyl)-7-hydrazino-2,1,3-benzoxadiazole derivatives (20), HPLC with ultraviolet (UV) detection of 2,4-dinitrophenylhy-

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drazine (DNPH) adducts (21–23), and gas chromatography (24). Methylglyoxal was determined following derivatization with *o*-phenylenediamine (*o*-PD) (25), or 1,2-diamino-4,5-dimethoxybenzene (26). None of these methods could simultaneously measure formaldehyde and methylglyoxal in a single run. DNPH derivatives of both formaldehyde and methylglyoxal were found to be coeluted under previously described conditions, whereas the *o*-PD derivative of formaldehyde could not be monitored at wavelength 315 nm, which was recommended for the *o*-PD–methylglyoxal derivative. In this paper, an HPLC procedure for the simultaneous determination of formaldehyde and methylglyoxal is established. In fact, this method can also be applied for the determination of acetaldehyde and malondialdehyde. The modified method was applied to the assessment of urinary levels of formaldehyde and methylglyoxal in experimental animals.

Experimental

Materials

Methylglyoxal, DNPH, methylamine hydrochloride, and 1,1,3,3-tetraethoxypropane were purchased from Sigma (St. Louis, MO). Aminoacetone was donated by Dr. Boomsma

(University Hospital Dijkzigt/Erasmus University, Rotterdam, The Netherlands). Malondialdehyde was prepared by the hydrolysis of 1,1,3,3-tetraethoxypropane according to Esterbauer et al. (27). (E)-2-(4-fluorophenethyl)-3-fluoroallylamine hydrochloride (MDL-72974A) was a gift previously provided by Marion-Merrell-Dow, Inc. (Cincinnati, OH). Formaldehyde was obtained from BDA (Ontario, Canada), and acetaldehyde and propionaldehyde were from Aldrich (Milwaukee, WI). All other chemicals were analytical grade.

Animal treatment and urine collection

Male Wistar rats (200–250 g) were used in the experiments. The animal treatments were in strict accordance with the guidelines established by the Canadian Council on Animal Care and were approved by the University of Saskatchewan Animal Care Committee. The rats were housed in hanging wire cages with free access to food and water on a 12-h light/dark cycle (lights on at 6:00 a.m.) at a temperature of 19–20°C. Diabetes was induced in animals by a single administration of STZ (60 mg/kg, ip). Serum glucose levels were assessed in order to ensure that diabetes was induced. The blood glucose levels of the control and STZ-induced diabetic rats were 1.61 ± 0.04 and 4.74 ± 0.35 mg/mL, respectively. Methylamine was chronically administered via drinking water (4 mg/mL). MDL-72974A (0.5 mg/kg) was

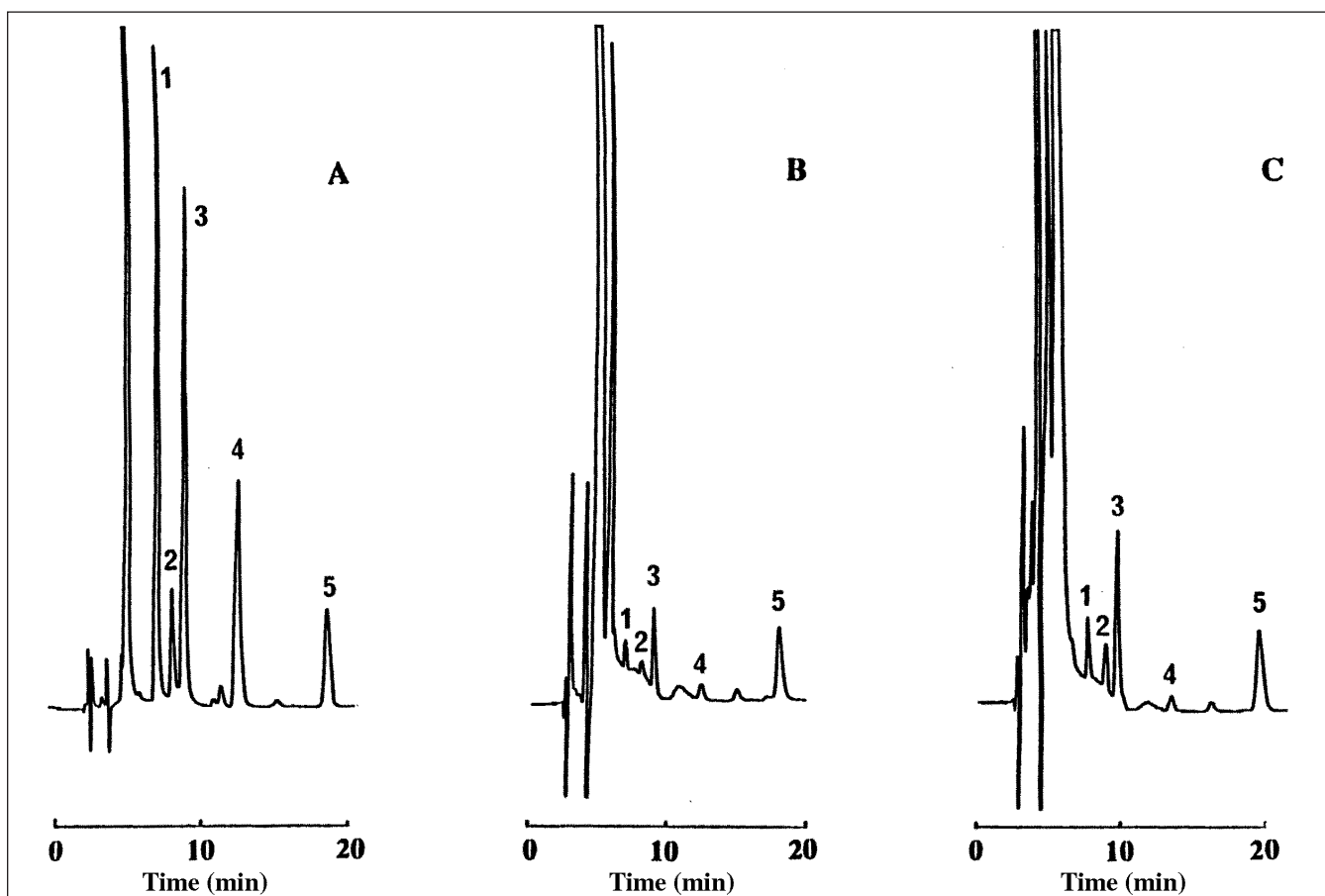


Figure 1. Chromatographic separations of DNPH derivatives of rat urinary aldehydes: standards (A), urine sample from the control animals (B), urine sample from the animals treated by STZ and methylamine (C). Separation conditions: column, reversed-phase Ultrasphere IP analytical column (250 × 4.6-mm i.d.); mobile phase, 20mM phosphate buffer (pH 4.6) containing 32% acetonitrile and 8% 2-methyl-1-propanol. Peaks: 1, malondialdehyde; 2, methylglyoxal; 3, formaldehyde; 4, acetaldehyde; 5, propionaldehyde (as internal standard).

injected intraperitoneally every second day. The control rats were supplied with tap water.

Twelve-hour urine was collected by using metabolic cages (Nalgene, Rochester, NY) at 2 weeks after the initial chronic treatment. Rats were allowed free access to tap water but received no food. The urine-collecting vessels were positioned over Styrofoam containers filled with dry ice that permitted the collection of urine in the frozen state over 12 h.

Derivatization of aldehydes

DNPH was used for the derivatization of the aldehydes. The reagent was prepared at the concentration of 10mM in 2M HCl solution. Propionaldehyde was selected as the internal standard. The volume of collected urine was first measured. In 25-mL screw-capped tubes, 200 μ L of distilled water, 500- μ L aliquots of urine, 100 μ L of internal standard solution, and 200 μ L of the DNPH reagent were mixed. The tubes were incubated at 37°C for 10 min. The derivatized urine samples were then extracted by the addition of 10 mL of pentane and shaken for 10 min. The pentane layers were removed and placed in clean glass tubes. The extraction of urine samples was repeated. The pentane extracts were combined and evaporated to dryness at 45°C in a water bath. The dried samples were reconstituted in 300 μ L of distilled water and subjected to HPLC.

Chromatographic assay

The HPLC system was composed of an SSI (Milford, MA) 222B HPLC pump, a WISP 712B autoinjector (Waters/Millipore, Milford, MA), and a Spectra-Physics (San Jose, CA) recorder. The separation was performed using a reversed-phase Ultrasphere (Beckman, Toronto, Canada) I.P. analytical column (5- μ m C₁₈, 250 \times 4.6-mm i.d.). Elution was isocratic with 20mM phosphate buffer (pH 4.6) containing 32% acetonitrile and 8% 2-methyl-1-propanol at a flow rate of 1.0 mL/min. Detection was performed by a Lambda-Max model 481 liquid chromatography spectrophotometer (Waters/Millipore) at 330 nm. Data represent the average of 4 or more analyses.

Statistics

The results were assessed using analysis of variance (ANOVA) followed by Newman-Keuls multiple comparisons. In general, the null hypothesis used for all analyses was that the factor has no influence on the measured variable, and significance was accepted at the > 95% confidence level.

Results and Discussion

Separation of DNPH derivatives of aldehydes

Figure 1 shows a typical chromatographic separation pattern of the standard DNPH derivatives of formaldehyde, methylglyoxal, acetaldehyde, malondialdehyde, and propionaldehyde using a mobile phase consisting of 20mM phosphate buffer (pH 4.6), 32% acetonitrile, and 8% 2-methyl-1-propanol. Propionaldehyde was selected as an internal standard. The HPLC procedure provides baseline separation for all of these DNPH aldehyde compounds.

Calibration curve, precision, and accuracy

The quantitative linearity of this assay was assessed using the aldehyde standards. Table I gives the results obtained by linear regression, which shows the dependence of the relative responses of analytes on their concentrations. A good linear relationship between concentrations and relative responses was obtained over the measured ranges from 10 to 200 pmol per injection. The correlation coefficients for formaldehyde, acetaldehyde, methylglyoxal, and malondialdehyde were from 0.95 to 0.98.

The precision and accuracy of this method with respect to these compounds were validated. A rat urine sample was spiked with 0.25 nmol of formaldehyde, methylglyoxal, and malondialdehyde; 1 nmol of acetaldehyde; and 2 nmol of propionaldehyde in 1 mL of the reaction mixture and subjected to extraction and concentration. The intraday precision of this assay is shown in Table II, and the coefficients of variation were found to be 9.8, 10.1, 5.8, and 11.9% for formaldehyde, acetaldehyde, methylglyoxal, and malondialdehyde, respectively. Table III summarizes the recoveries of the method for 4 compounds.

Determination of urinary aldehydes in diabetic and methylamine-treated rats

Two weeks after methylamine was administered via drinking water (4 mg/mL in water), the 12-h urine was collected and analyzed. Representative chromatograms are shown in Figures 1B and 1C. Methylamine and aminoacetone are endogenous SSAO substrates, and formaldehyde and methylglyoxal, respectively, are produced. Malondialdehyde, the end product of lipid peroxi-

Table I. Relationship Between the Relative Responses of Analytes and their Injection Amounts*

Compounds	Equations [†]	γ [‡]
Malondialdehyde	$y = -1.89 \pm 1.84x$	0.982
Methylglyoxal	$y = -1.86 \pm 1.22x$	0.966
Formaldehyde	$y = -1.55 \pm 3.04x$	0.990
Acetaldehyde	$y = -0.38 \pm 0.55x$	0.988

* The peak area of the analyte was expressed as relative response (i.e., the ratio of analyte to propionaldehyde) (0.2 nmol/injection).

[†] y, relative response; x, amount of injection.

[‡] γ , correlation coefficient

Table II. Intraday Precision (within 24 h) of the Assay for Malondialdehyde, Methylglyoxal, Formaldehyde, and Acetaldehyde

Compounds	Amount* (ng, mean \pm SD, n = 6)	Coefficient of variation
Malondialdehyde	30.31 \pm 3.61	11.9%
Methylglyoxal	20.69 \pm 1.19	5.8%
Formaldehyde	11.01 \pm 1.08	9.8%
Acetaldehyde	49.19 \pm 4.96	10.1%

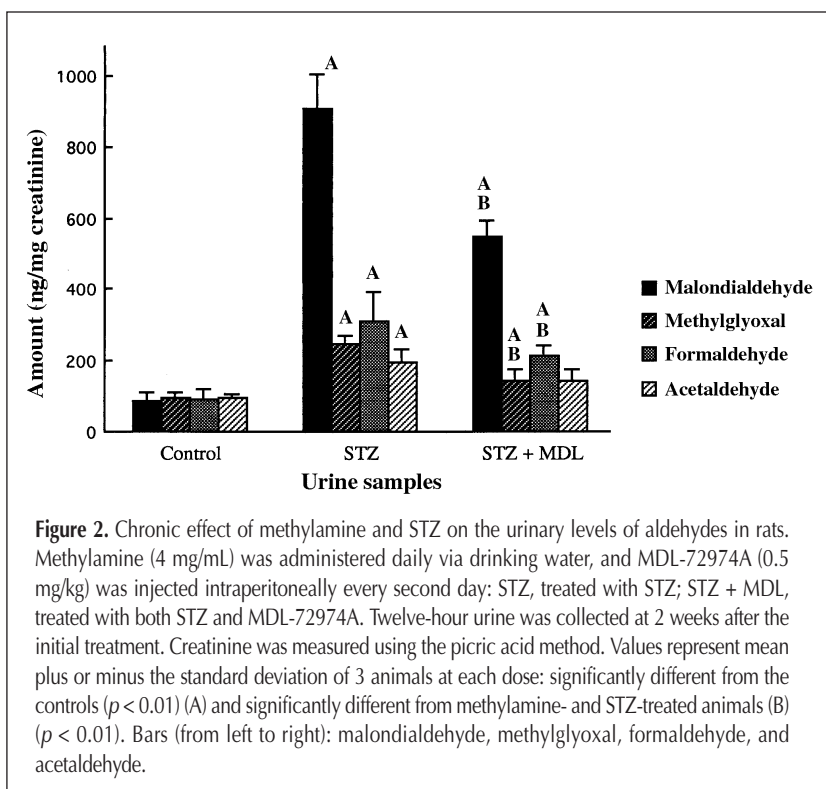
* The urine sample from control animals was spiked with 0.25 nmol of malondialdehyde, methylglyoxal, and formaldehyde and 1 nmol of acetaldehyde.

dation, is the most widely used for the assessment of oxidative stress. As can be seen in Figure 2, formaldehyde, methylglyoxal, and malondialdehyde were elevated significantly in the STZ-induced diabetic rats. MDL-72974A (0.5 mg/kg), a potent selective SSAO inhibitor, was found to reduce both formaldehyde and malondialdehyde levels and also reduced other aldehyde excretion.

After the administration of methylamine or aminoacetone, the increase in deaminated products (formaldehyde or methylglyoxal) was detected in the rat urine (28). The presence of both methylglyoxal and formaldehyde was also demonstrated in the urine of C57BL/6 mice, a strain very susceptible to atherosclerosis (29). Earlier reports on formaldehyde using the previous DNPH method may overestimate the levels due to the presence of methylglyoxal, because both aldehydes were not separated under the conditions previously described (21–23).

Compounds	Spiked amount* (ng)	Endogenous amount in urine (ng)	Amount found (ng, mean \pm SD, $n = 6$)	Recovery† (%)
Malondialdehyde	18.25	13.95	30.31 \pm 3.61	89.6%
Methylglyoxal	18.25	3.97	20.69 \pm 1.19	91.6%
Formaldehyde	7.5	4.56	11.01 \pm 1.08	86.0%
Acetaldehyde	44.0	8.94	49.19 \pm 4.96	91.4%

* The urine sample (500 μ L) from control animals was spiked with 0.25 nmol of malondialdehyde, methylglyoxal, and formaldehyde and 1 nmol of acetaldehyde.
 † Recoveries were calculated as follows: the value of amount found subtracted by the endogenous amount in the urine sample and then compared with the spiked amount.



The previous DNPH method uses a water–acetonitrile solvent system. It was initially hoped to follow the solvent system with the alteration of acetonitrile concentration from 0 to 80%, but no resolution of DNPH-derivatives of methylglyoxal and formaldehyde was achieved. 2-Methyl-1-propanol was then used as a secondary modifier to improve separation in this study. DNPH derivatives of formaldehyde and methylglyoxal are relatively hydrophilic compounds, thus they are not sufficiently retained on a reversed-phase column, although retention is essential for their separation. These results show that the application of 2-methyl-1-propanol as a secondary modifier significantly increases the retention of most DNPH aldehyde derivatives (except methylglyoxal and malondialdehyde). The addition of 2-methyl-1-propanol reduces the solvent polarity and thus possibly assists in expulsion of the DNPH derivatives out of the mobile phase to the C_{18} stationary phase. The precise mechanism remains to be established. The procedure could achieve the separation of DNPH formaldehyde and DNPH methylglyoxal derivatives conveniently and effectively. The composition of mobile phase has been optimized. Under the selected conditions, a complete baseline separation was obtained for the DNPH derivatives of formaldehyde, methylglyoxal, malondialdehyde, acetaldehyde, and propionaldehyde. The capacity ratios for these DNPH derivatives were between 1 and 10, the range recommended for optimum resolution. The reproducibility and recovery for the analysis of these biological aldehydes in spiked urine samples were found to satisfy recommended guidelines (15%) for assay precision and accuracy.

The SSAO-mediated deamination reaction of methylamine and aminoacetone to formaldehyde and methylglyoxal, respectively, were demonstrated in vitro and in vivo by several research groups (3,4,5). Methylamine was considered to be derived endogenously from adrenaline, creatinine, sarcosine, lecithin, nicotine, etc. (30,31) and probably inhaled from cigarette smoke, and ingested from food and drinks (32). Aminoacetone is a product of mitochondrial metabolism of glycine and threonine (33,34). Both methylamine and aminoacetone were demonstrated to be present in rat and human urine (33,35,36), blood (37), and tissues (38). The increased levels of formaldehyde and malondialdehyde were demonstrated in rat urine after chronic administration of methylamine (28). Interestingly, the urinary levels of formaldehyde, methylglyoxal, and malondialdehyde were elevated in the STZ-induced diabetic rats. Even though there are several sources for forming the endogenous formaldehyde and methylglyoxal, MDL-72974A, a potent selective SSAO inhibitor, reduced the formation of formaldehyde, methylglyoxal, and malondialdehyde, suggesting that the SSAO-mediated deamination of methylamine and aminoacetone indeed occurs in vivo. The results are consistent with the notion that increased deamination of methylamine or aminoacetone by SSAO could cause aldehyde stress, which may be a risk factor of health (18,39).

In addition to formaldehyde and methylglyoxal, H₂O₂, a reactive oxygen species, is also generated from the SSAO-mediated deamination. The increase of malondialdehyde in methylamine-treated and diabetic rats suggests that increased deamination of methylamine may also enhance oxidative stress. Increases in SSAO-mediated deamination, and subsequent increases in aldehyde and oxidative stress, may be related to some pathological conditions (i.e., atherosclerosis, nephropathy and diabetic complications, etc.)

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