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Determination of a guanosine–malonaldehyde adduct in urine by high-performance liquid chromatography with a thiobarbituric acid reaction detector

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ABSTRACT

A new method for the determination of a guanosine–malonaldehyde adduct, β-D-ribofuranosylpyrimido[1,2-*a*]purin-10(3*H*)-one (GMA), in rat and human urine is described. The method involves rapid pretreatment using, in sequence, polyamide, ion-exchange and reversed-phase cartridges; determination is by means of high-performance liquid chromatography with a thiobarbituric acid reactor in series with a fluorescence detector. This device can quantitatively determine the adduct at the sub-picomole level. This rapid, selective and sensitive method is suitable for the determination of guanine–malonaldehyde adducts in biological samples, such as human and rat urine. A semi-preparative method for the extraction and purification of these adducts from rat urine and for their identification by mass spectrometry and high-performance liquid chromatography with ultraviolet detection is also reported.

INTRODUCTION

Malonaldehyde (MA), a product of lipid peroxidation and prostaglandin biosynthesis [1], is both mutagenic [2,3] and carcinogenic [4,5]. MA reacts with various functional substances, such as proteins [6–8] and nucleic acids [9,10], to form adducts. These adducts are metabolized and are subsequently excreted in the urine as TBA (2-thiobarbituric acid) reactants (TBAR) [11]. An adduct [pyrimidopurin-10(3*H*)-one] of guanine with MA was isolated from a large amount (1 l) of rat urine by Hadley and Draper [12]. Urinary excretion of guanine–MA adducts may serve as a marker for nucleic acid modification caused by lipid peroxidation *in vivo* [12]. Although these adducts have been identified in urine, no suitable method for their determination has yet been reported. Urinary excretion of guanine–MA adducts is expected to be very small because the reaction between guanine base and MA is slow under physiological conditions [13]. For this reason, a highly selective and sensitive detection method is required.

The adducts are reactive toward TBA [10,13], and a TBA–MA complex, a red fluorophor, is formed. For the quantitation of the adducts in biological samples,

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a system of post-column reaction with TBA was developed. This paper describes a simple and rapid sample purification method and a sensitive and selective TBA reaction detector to be used in high-performance liquid chromatographic (HPLC) system for the determination of β-D-ribofuranosylpyrimido[1,2-*a*]purin-10(3*H*)-one (GMA) in human and rat urine. GMA is an MA-modified nucleoside, which is a slightly different chemical species from the compound isolated from rat urine by Hadley and Draper [12]. The identification of GMA in urine is also reported, using a semi-preparative HPLC purification with in-line UV detection and mass spectrometry (MS).

EXPERIMENTAL

Reagents

GMA was prepared according to a previously described method [13]; it was synthesized by the reaction of guanosine with MA under acidic conditions at 37°C. The fluorescent compound (excitation maximum 365 nm, emission maximum 500 nm) was isolated by means of preparative reversed-phase HPLC. TBA was purchased from Merck (Darmstadt, Germany).

HPLC system

System A. Preparative HPLC was carried out with a Jasco 880PU pump (Tokyo, Japan), a Rheodyne 7125 injector (Berkeley, CA, USA) with 10-ml sample loop, a Merck LiChroprep RP-18, 300 mm × 22 mm I.D. column (15–25 μm particle diameter), a UV detector (Oyobunko Kiki Uvilog II, path-length 1 mm, detection wavelength 254 nm, Tokyo, Japan), and a Sekonic SS-250F recorder (Tokyo, Japan).

System B. The analytical HPLC system for identification consisted of a Jasco 880PU pump, a Rheodyne 7125 injector, a Macherey-Nagel (Düren, Germany) Nucleosil $5C_{18}$ column (250 mm × 4.6 mm I.D.), a Whatman (Clifton, NJ, USA) Partisil 10 SAX column (250 mm × 4.6 mm I.D.), and a Hewlett-Packard 1040M photo-diode array detector (Palo Alto, CA, USA), recording the UV spectra of the eluted compounds.

Systems C. Analytical HPLC for quantitation was carried out with a Jasco 880PU pump, a Rheodyne 7125 injector, a Nucleosil $7C_{18}$ column (250 mm × 4.6 mm I.D.), equipped with a post-column labeling device consisting of the same type of pump for the reagent [0.5% TBA in 10% (v/v) H_3PO_4–water], a reaction coil (0.5 mm I.D., 1-ml volume stainless-steel pipe) in a water-bath at 85 ± 0.2°C, a Hitachi (Tokyo, Japan) F-1000 fluorescence detector (excitation 520 nm, emission 550 nm) and a Sekonic SS-250F recorder. A diagram of the this system is shown in Fig. 1. The flow-rates of the mobile phase and reagent were 0.7 and 0.3 ml/min, respectively.

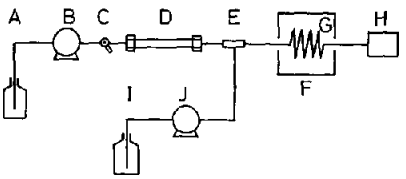

Fig. 1. Diagram of the system: A = eluent reservoir; B = pump 1; C = injector; D = column; E = joint (mixer); F = water-bath; G = reaction coil; H = detector; I = reagent reservoir; J = pump 2.

Mass spectrometry

MS analyses were carried out on a VG 70S mass spectrometer (Manchester, UK).

Collection of rat urine

Adult, specific pathogen-free Fisher 344 DuCrj rats (Nippon Charles River, Atsugi, Japan) were fed an ordinary diet. Each animal was put into a glass metabolism cage (Sugiyama-Gen Environmental Science, Tokyo, Japan). The urine was collected for 24 h and stored frozen. The animals were given water *ad libitum* but were not allowed to feed during the urine collection period. The rats were put into the cages in shifts of 24 h.

Identification of GMA in rat urine

A 1-l volume of rat urine was freeze-dried. The residue was dissolved in 100 ml of water, then 900 ml of acetonitrile were added. The mixture was shaken in a separation funnel for 5 min and allowed to stand. The aqueous layer was transferred to another separation funnel, and re-extraction was carried out by mixing with an additional 900 ml of acetonitrile. Water and acetonitrile are miscible in any proportions under normal conditions but in the present case separate phases were obtained because most of inorganic salts dissolved in water but not in acetonitrile. This desalting process gave a moderate recovery (52–57%) of GMA from rat urine.

The acetonitrile layers were combined and evaporated to dryness *in vacuo*. The residue was dissolved in 50 ml of water, and subjected to a polyamide column (an open glass column, 350 mm × 50 mm I.D., Wako Pure Chemicals C-200 gel, Osaka, Japan), conditioned with methanol and then water. The column was eluted with 350 ml of water. The eluate was reduced in volume to *ca.* 50 ml with a rotary evaporator, and this solution was subjected to purification on a second polyamide glass column (300 mm × 20 mm I.D.) with water as the eluent. The fractions from 20 to 100 ml were collected and concentrated to 5 ml by evaporation.

The solution was injected into a LiChroprep RP-18 preparative HPLC column and chromatographed with 7% (v/v) acetonitrile–water at a flow-rate of 5.0 ml/min, using system A. The fractions of GMA (50–70 min) were collected and

evaporated to dryness *in vacuo*. The residue was dissolved in 150 μl of water, and an aliquot (30 μl) of the solution was injected into a Nucleosil $5C_{18}$ column [mobile phase, 10% (v/v) acetonitrile–water; flow-rate, 1.0 ml/min], using system B. The fractions from 6 to 7 min were collected and evaporated to dryness *in vacuo*.

The residue was dissolved in 150 μl of water, and an aliquot (30 μl) was injected into a Partisil 10 SAX column [mobile phase, 0.05 *M* KH_2PO_4 (pH 3.5); flow-rate, 1.0 ml/min], using system B. UV spectra of the eluates were recorded during HPLC analysis. The GMA peak fraction at 4.4 min was collected and evaporated to dryness.

Trimethylsilylation of GMA in the residue was carried out as follows. The residue was added to 50 μl of acetonitrile and 50 μl of N,N-bis(trimethylsilyl)trifluoroacetamide (Pierce, Rockford, IL, USA). The mixture was kept in a sealed vial at 70°C for 3 h, then analysed with a VG 70S mass spectrometer (electron-impact mode; direct insertion; ionization, 70 eV, 200 μA; acceleration voltage, 7 kV).

Quantitation of GMA in urine

The sample preparation system consisted, in sequence, of a polyamide gel (Wako, C-200) cartridge (PC, 35 mm × 12 mm I.D.), a Bond Elut SAX anion-exchange cartridge (100 mg, Analytichem International, Harbor City, CA, USA) (AC) in the phosphate form, a Bond Elut SCX cation-exchange cartridge (100 mg) (CC) in the proton form and a Bond Elut C_{18} cartridge (1.5 g) (OC), coupled to a vacuum device.

The analytical procedure for GMA in urine was as follows. Either 10 ml (human) or 5 ml (rat) of urine were applied. The system was developed with 10 ml of water, and PC, AC and CC were removed. OC was washed with 2 ml of water. GMA was recovered from OC with 10 ml of 30% (v/v) acetonitrile–water. The eluate was evaporated to dryness, and the residue was dissolved in 200 μl of water. An aliquot (50 μl) of this solution was injected into the analytical HPLC system (system C). The GMA content of the sample was calculated from a standard curve prepared with authentic GMA.

RESULTS AND DISCUSSION

Identification of GMA in rat urine

The recovery of GMA from rat urine through the whole preparative process averaged 44% (41–47%). An example of the final chromatogram of a rat urine extract containing GMA is shown in Fig. 2. The retention time of authentic GMA was 4.4 min. Since the chromatogram indicated incomplete resolution of the peak at 4.4 min, the UV spectrum (Fig. 3B) was obtained by subtraction of the UV absorption spectrum at 4.0 min from the spectrum at 4.4 min. The difference spectrum thus obtained was similar to that of authentic GMA (Fig. 3A). The

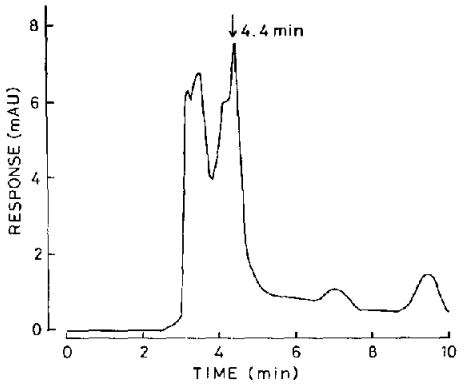

Fig. 2. Typical final chromatogram of a rat urine extract: authentic GMA was eluted at 4.4 min. Column, Partisil 10 SAX (250 mm × 4.6 mm I.D.); mobile phase, 0.05 *M* KH_2PO_4 (pH 3.5); flow-rate, 1.0 ml/min; detection, absorption detector with scanning. The ordinate axis represents absorption at 254 nm.

absorption maxima at 214, 253 and 350 nm, and the absorption minimum at 237 nm, were identical with those of authentic GMA. The mass spectrum was obtained after trimethylsilylation of GMA in the fraction at 4.4 min. The mass spectrum of the trimethylsilyl (TMS) derivative showed a molecular ion peak (M^+) at *m/z* 535 $[C_{13}H_{10}N_5O_5(TMS)_3]$ and its related ion peak, $[M-CH_3]^+$ at *m/z* 520 (Fig. 4). These results indicate that the peak at 4.4 min is due to GMA.

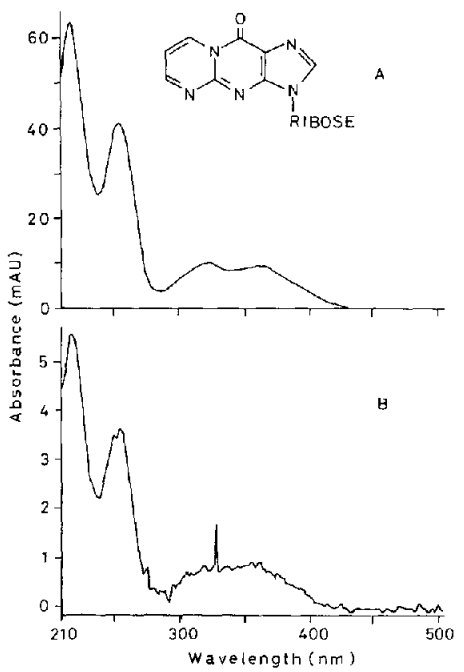

Fig. 3. UV absorption spectra of authentic GMA (A) and the compound (B) eluted at 4.4 min on the chromatogram in Fig. 2.

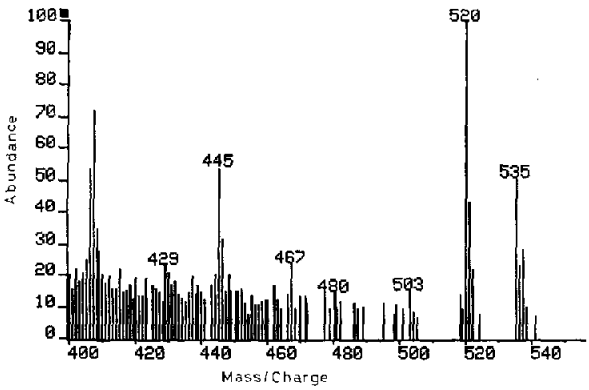

Fig. 4. Mass spectrum of the trimethylsilyl derivative of GMA isolated from rat urine.

Quantitation of GMA in urine

GMA is reactive toward TBA, and the TBA–MA complex is formed in the post-column derivatization system. The solution of the complex fluoresces strongly at 550 nm when excited at 520 nm. Thus the system is highly selective and sensitive for the detection of GMA.

The linearity of the response was excellent: correlation coefficients of greater than 0.9999 for injected amounts of 0.1–50 pmol of GMA were observed. The calibration curve was described by the equation $y = 16.0x + 1.65$. The relative standard deviation (R.S.D.) on five repeated injections of 4.3 pmol was 2.1%. The detection limit of GMA with this system was 0.09 pmol at a signal-to-noise ratio of 3. With a 50-μl injection volume, from a volume of 10 ml for urine, this resulted in a detection limit of *ca.* 0.04 pmol/ml. The recovery of GMA from human urine averaged 83.1 ± 2.9% ($n = 5$), when 43.2 pmol of GMA were added to 10 ml of urine. The recovery at the level closer to the sensitivity limit averaged 79.7 ± 6.8% ($n = 5$), when 2.2 pmol of GMA were added to 10 ml of urine. Analyses of six separate extractions on different days of 43.2 pmol per 10 ml of GMA-spiked human urine samples gave values of 3.58 ± 0.17 pmol/ml (mean ± S.D.; R.S.D. = 4.7%).

Typical chromatograms of GMA in rat urine (F344/DuCrj, male, sixteen weeks) and human urine are shown in Fig. 5B and C, respectively. The urinary GMA co-eluted with the authentic GMA. The peak in Fig. 5B represents 2.4 pmol of GMA in 1 ml of rat urine. When the sample extracted from rat urine was chromatographed on another column (Partisil 10 SAX or LC-HISEP, Supelco, Bellefonte, PA, USA), the retention time of the peak again coincided with that of authentic GMA (data not shown). Quantitation of the adduct also gave a satisfactory result.

The adduct was also detected in human urine (healthy adults age 21–60 years) and averaged 0.3 ± 0.3 pmol/ml ($n = 13$). The normal level of urinary TBAR (as MA) in humans is 3 nmol/ml (Seto, unpublished results). Our results thus showed

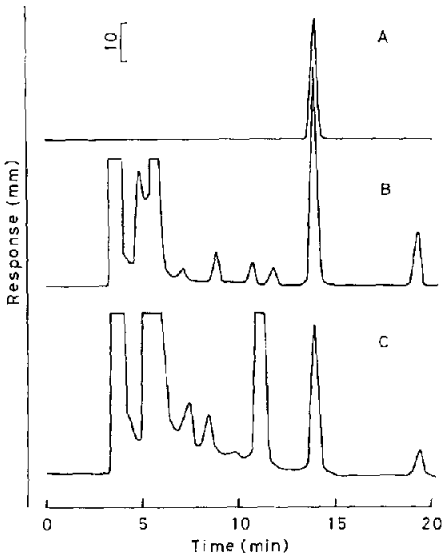

Fig. 5. Typical chromatogram after TBA post-column labeling of authentic GMA and urine extracts. (A) Authentic GMA (1.7 pmol); (B) rat urine; (C) human urine. Column, Nucleosil 7C_{18} (250 mm × 4.6 mm I.D.); mobile-phase, 7% (v/v) acetonitrile–water; flow-rate, 1.0 ml/min; reagent, 0.5% TBA in 10% (v/v) H_3PO_4–water; flow-rate, 0.3 ml/min; reaction temperature, 85°C; detection, fluorescence (excitation at 520 nm, emission at 550 nm). The ordinate axis represents fluorescence intensity (mm).

that the concentration of urinary GMA was only *ca.* 10^{-4} of that of total urinary TBAR. However, chemical modifications of genetic materials by MA *in vivo* may still represent a significant risk to humans.

Guanine is released from nucleic acids mainly in the form of nucleosides and partially as the free base [12], so that monitoring of modified nucleosides such as GMA is advantageous over that of modified bases produced by lipid peroxidation *in vivo*.

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