

Stability and Intra-Individual Variation of Urinary Malondialdehyde and 2-Naphthol

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Objectives : Malondialdehyde (MDA), a lipid peroxidation by-product, has been widely used as an indicator of oxidative stress. Urinary 2-naphthol, a urinary PAH metabolite, is used as a marker of ambient particulate exposure and is associated with lung cancer and chronic obstructive pulmonary disease. However, the stability and intra-individual variation associated with urinary MDA and 2-naphthol have not been thoroughly addressed. The objective of this study was to assess the stability and intra-individual variation associated with urinary MDA and 2-naphthol.

Methods : Urine samples were collected from 10 healthy volunteers (mean age 34, range 27~42 years old). Each sample was divided into three aliquots and stored under three different conditions. The levels of urinary MDA and 2-naphthol were analyzed 1) just after sampling, 2) after storage at room temperature (21°C) for 16 hours, and 3) after storage in a -20°C freezer for 16 hours. In addition, an epidemiological study was conducted in 44 Chinese subjects over a period of 3 weeks. The urinary MDA and 2-naphthol were measured by HPLC three times.

Results : There was no difference in the levels of urinary MDA and 2-naphthol between the triplicate measurements (n=10, p=0.84 and p=0.83, respectively). The intra-class correlation coefficients (ICC) for urinary MDA and 2-naphthol were 0.74 and 0.42, respectively. However, the levels of PM2.5 in the air were well correlated with the levels of both MDA and 2-naphthol in the epidemiological study.

Conclusions : These results suggest that urinary MDA and 2-naphthol remain stable under variable storage conditions, even at room temperature for 16 hours, and indicate that these markers can be used in epidemiological studies involving various sample storage conditions. The intra-CC of urinary 2-naphthol and MDA were acceptable for application to epidemiological studies.

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Key words : Malondialdehyde, Oxidative stress, Biomarkers, Stability

INTRODUCTION

Generations of epidemiologists, physicians, and scientists have used biomarkers to study a variety of conditions. Especially, biomarkers of internal dose and early biological effects as a biomarker of oxidative stress have been used in epidemiological studies [1]. Although a number of cohort studies have collected and preserved various biospecimens for use in molecular and genetic epidemiological studies [2,3], it is surprising that there has been little documentation concerning the intra-person variance and inter-person variance reliability associated with biomarkers [4]. Therefore, investigators have considered using biological sample storage and freeze-thaw cycles [5]. In

urine, this is particularly useful for biomarker studies due to non-invasive and easy sampling [6]. Urinary determination of polycyclic aromatic hydrocarbon(PAH) metabolites, such as 1-hydroxy pyrene and 1-hydroxypyrene glucuronide, and oxidative stress biomarkers are already used routinely [7]. Urinary 2-naphthol, a urinary PAH metabolite that reflects ambient particulate exposure [7], has been widely used as a biomarker of PAH exposure in many epidemiological studies [8,9]. Urinary 2-naphthol was measured in occupational workers [10-12] and as a marker of exposure to environmental PAHs [8,13].

PAHs and other chemicals, or their metabolites, produce reactive oxygen species and oxidative stress, mediated by these reactive oxygen species, cause lipid peroxidation,

protein modification, membrane disruption, and mitochondrial damage [14]. Especially, urinary malondialdehyde (MDA) can be monitored to serve as an indicator for oxidative stress and lipid peroxidation [15,16]. Urinary MDA was evaluated as a useful marker in rats over a period of 14 days to predict the development of intra-renal oxidative stress in cisplatin-induced acute renal failure(ARF) [17]. In addition, urinary MDA was used as a biomarker of lipid peroxidation to prove that 1 hour of exercise may cause oxidative damage in a study of 18 selected men [18]. However, the plasma MDA was not stable when stored after the alkaline hydrolysis step and was stable for 3 days when stored after n-butanol extraction [19]. Most previous studies have not found in detail at the stability of the MDA over a longer time period [20].

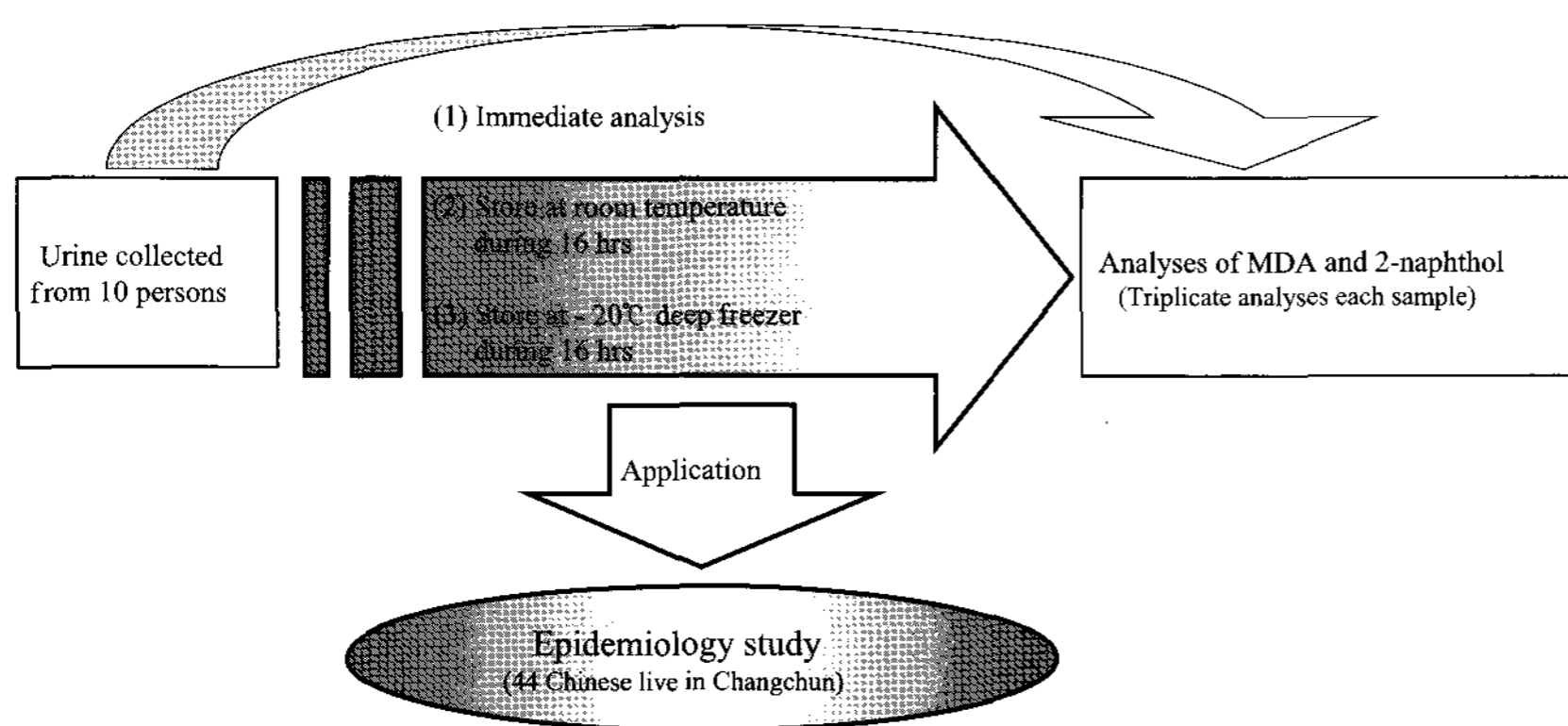


Figure 1. Scheme of study design.

The usefulness of urinary MDA and 2-naphthol determined in those epidemiological studies has not been validated, i.e., the stability and intra-individual variation of urinary MDA and 2-naphthol have not been thoroughly addressed. In order to take full advantage of the opportunities afforded by the use of biomarkers in epidemiological studies, careful attention to biospecimen processing, the stability of the biomarker and the precautions to be taken during the transportation and storage of samples need to be taken into consideration [3].

The objective of this study was to assess the stability and intra-individual variation associated with urinary MDA and 2-naphthol and to apply these biomarkers to epidemiological studies.

MATERIALS AND METHODS

I. Materials

The 2-naphthol standard was obtained from Sigma-Aldrich Korea (Yongin, Kyunggi, Korea). Methanol and acetonitrile were purchased from HAYMAN (Witham, Essex, U.K.) with a purity of 99.85%. Two-thiobarbituric acid (TBA) and malondialdehyde (MDA) standard were also purchased from Sigma-Aldrich Korea. All other chemicals were obtained in the greatest purity available from commercial suppliers.

II. Study Subjects and Design

Urine samples were collected from 10 healthy volunteers (mean age 34, range 27–42

years old). Each sample was divided into 3 aliquots and maintained under 3 different storage conditions. The levels of urinary MDA and 2-naphthol were analyzed 1) just after sampling from volunteers, 2) after storage at room temperature (21°C) for 16 hours in a laboratory and 3) after storage in a -20°C freezer for 16 hours (Figure 1).

As an application of epidemiological study findings, 97 Chinese subjects (49 children and 48 their mothers) participated in the Asian dust event study from April 1 to April 30, 2004. However, 44 selected subjects participated in this study over the course of 3 weeks in April 2004. An epidemiological study was conducted in 44 Chinese volunteers, which included 22 children and their mothers (average 23.4 years old, range 10–46) over a period of 3 weeks, and urine samples were collected from participants once a week (3 times). The spot urine samples were collected in the morning. All samples were coded, transported to the laboratory, processed and kept in aliquots at -20°C.

III. Analysis of Urinary 2-naphthol

Urinary 2-naphthol was determined using reverse-phase high-performance liquid chromatography (HPLC) [21]. Briefly, 0.5-ml urine samples were buffered with 50 μ l of 2.0 M sodium acetate buffer (pH 5.0) and hydrolyzed with 10 μ l of β -glucuronidase/sulfatase (Sigma, St. Louis). The urine mixture was incubated at 37°C for 16 hours in a

shaking water bath. After hydrolysis, 0.5 ml of acetonitrile was added to the mixture.

We centrifuged the mixture and collected a 100 μ l sample of the supernatant for HPLC analysis. The HPLC system was comprised of a Waters 515 HPLC Pump, Waters Automated Gradient Controller, Waters 717plus Autosampler, and Waters™ 474 Scanning Fluorescence Detector. The HPLC parameters were as follows: column, Xtera C18 (4.5 mm x 250 mm); mobile phase, 50% acetonitrile in water; flow rate, 0.8 ml/min. The excitation/emission wavelength used in the detection of 2-naphthol was 227/355 nm. The limit of detection was 0.5 ng/ml and the coefficient of variation was less than 15%.

IV. Analysis of Urinary Malondialdehyde (MDA)

MDA was measured according to a previously described method [22], with slight modification. The most common method of measuring MDA is based on the reaction with 2-thiobarbituric acid (TBA). A 10 mmol/L stock standard of MDA was prepared by dissolving 123.5 μ l of 1,1,3,3-tetraethoxypropane in 50 mL of ethanol (40% ethanol by volume). TBA-MDA adducts were prepared in glass tubes with a polypropylene stopper. In each tube, 300 μ l of phosphoric acid (0.5 M) was mixed with 50 μ l of urine and 150 μ l of TBA reagent. The mixtures were incubated at 95°C for 1 h, and methanol (500 μ l) was added to each tube. After 5 min of centrifugation (5,000 x g), the samples were analyzed using HPLC on a 4 x 150 mm Xtera C18 column with a fluorescent detector (Waters 707) at a wavelength of 532/553 nm (excitation/emission). The mobile phase was potassium phosphate (0.05 mol/L; pH 6.8) and methanol (58:42, v/v). The flow rate was 0.8 mL/min. MDA (Sigma-Aldrich, St. Louis, MO, T-8998) was used as an external standard. MDA standards (0.1, 0.5, 1, 2, and 4 μ mol/L) were prepared with 1,1,3,3-tetramethoxypropane. The LOD was 0.05 μ mol/L and the

Table 1. Stability of urinary malondialdehyde (MDA) level on 3 difference status (room temperature on 0 hour, 21 °C over 16 hours and in -20 °C freezer over 16 hours)

	N	Immediate analysis	Store at room temp(16hr)	Store at -20°C(16hr)	p-value [†]	ICC [‡]
		Mean ± SE*	Mean ± SE	Mean ± SE		
MDA (μmol/L)	10	2.43 ± 0.19	2.28 ± 0.19	2.27 ± 0.22	0.84	0.99
2-naphthol (ng/ml)	10	11.00 ± 3.80	13.68 ± 4.14	14.57 ± 4.82	0.83	0.97

* Arithmetical means and standard error

† By ANOVA test

‡ intraclass correlation coefficients

Table 2. Urinary levels of MDA and 2-naphthol and intra-class correlation coefficients (ICC) in Chinese samples for April 2004

	N	1st week (April 2nd)	2nd week (April 9th)	3th week (April 16th)	p-value [†]	ICC [‡]
		Mean ± SE*	Mean ± SE	Mean ± SE		
MDA (μmol/L)	44	1.52 ± 0.20	1.38 ± 0.19	1.98 ± 1.20	0.08	0.74
2-naphthol (ng/ml)	44	19.23 ± 2.77	13.56 ± 2.52	17.44 ± 2.22	0.27	0.42

* Arithmetical means and standard error

† By ANOVA test

‡ intraclass correlation coefficients

correlation for the linearity of the standard curve was 0.99. The coefficient of variation was less than 10%.

V. Ambient Air Quality Data

Data on fine particulates, which are defined as being 2.5 μm in diameter or less (PM_{2.5}), were collected using a GT-331 (Sibata Science Technology LTD, Tokyo, Japan) portable particle mass monitor. This device recorded the average levels of PM_{2.5} over five-minute periods. From April 1 to 30, 2004, we obtained ambient air quality data for average PM_{2.5} mass from several areas in Changchun, China. We then selected air monitoring data from a 3-day period (1st, 8th and 15th April), with a 1-day lag time in collecting urine samples.

VI. Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS 12.0, SPSS Inc., Chicago, IL, USA). An ANOVA test was used to compare the mean levels of MDA and 2-naphthol between the 3 sample groups. In order to calculate objectivity and stability, Intraclass correlation coefficients (ICC) were calculated first using a one-way ANOVA model analysis. Intra-class correlation coefficients (ICC) were calculated using the

SPSS statistical package. The definition of ICC is inter-group variance divided by the sum of the inter-person and intra-person variances. ICC was calculated using the following formula: $ICC = (MS (group) - MS (within group)) / (MS (group) + MS (within group))$. Where $MS (group)$ is mean squares between subjects and $MS (within group)$ is mean squares within subjects [23]. This coefficient can range from 0 to 1, where 1 is perfect agreement. The exact 95% confidence interval (CI) was calculated from the ANOVA table based on the F distribution. Statistical significance was defined as $p < 0.05$.

RESULTS

Table 1 represents the arithmetical mean, standard error (SE) and p-value by ANOVA for the levels of urinary 2-naphthol and urinary MDA determined in an experimental test that was performed three times under three different conditions: the urine samples were analyzed 1) immediately after they were collected from volunteers, 2) after being kept at room temperature for 16 hours and 3) after being kept in a -20 °C freezer for 16 hours.

The mean (± standard error) urinary MDA levels for each condition were 2.43 (0.19), 2.28 (0.19) and 2.27 (0.22) μmol/L, respectively,

under the conditions described above. The levels of urinary 2-naphthol were 11.00 (3.80), 13.68 (4.14) and 14.57 (4.82) ng/ml, respectively, under the conditions described above. There was no difference in the levels of urinary MDA and 2-naphthol under the three different conditions ($n=10$, $p=0.84$ and $p=0.83$ by ANOVA, respectively) (Table 1). The intraclass correlation coefficients (ICC) of urinary MDA and 2-naphthol were 0.99 and 0.97, respectively (Table 1).

The levels of PM_{2.5} and biomarkers, such as urinary MDA and 2-naphthol, were measured in Changchun, China during April 2004. The concentrations of PM_{2.5} were 77.40, 28.74 and 85.77 μg/m³ on April 1, April 8 and April 15, respectively (Figure 2). There was no significant difference in the levels of urinary MDA and urinary 2-naphthol among the participants during the study period ($n=44$, $p=0.08$ and $p=0.27$ by ANOVA, respectively) (Table 2). There was a similar pattern between the levels of PM_{2.5} (one day lag) in the Changchun area and the mean urinary MDA concentration (Figure 2A) and urinary 2-naphthol levels (Figure 2B) of all participants ($n=44$) living in that area.

DISCUSSIONS

We found that levels of biomarkers, such as 2-naphthol and malondialdehyde (MDA), in collected urine samples remained stable when analyzed under the following three conditions: 1) immediately after sampling, 2) after storage at room temperature for 16 hours, and 3) after storage at -20 °C in a deep freezer for 16 hours. In a previous study, Gika et al. [24] evaluated the stability of the principal components for quality control in collected urine samples. They found that human urine samples were stable for up to 1 month at -20 °C. Feldman et al. [25] compared the effects of different storage variables, such as temperature (25, 4, and -20 °C) and storage time (1, 2, 4, and 8 months), on the stability of arsenic species in urine and found that the

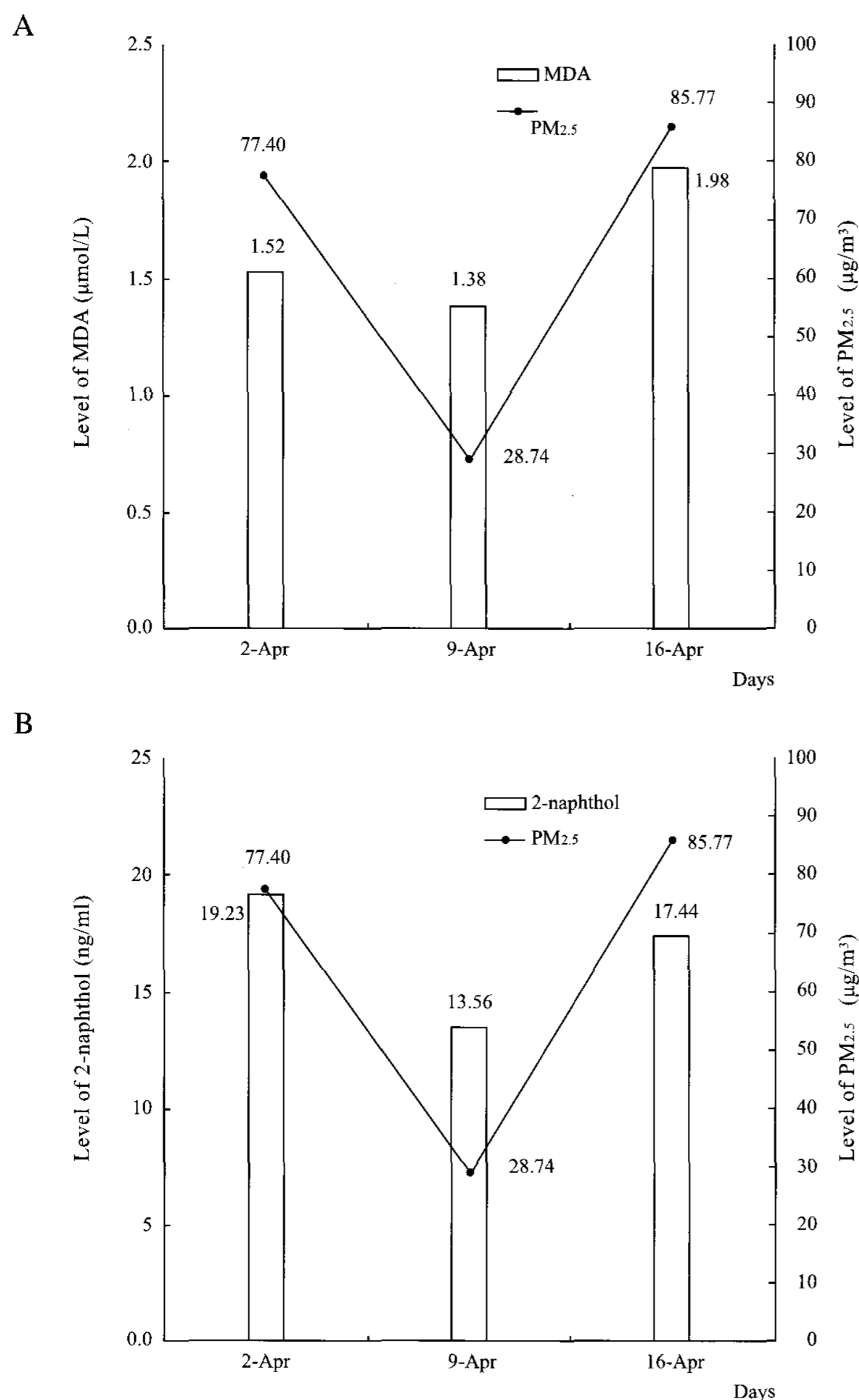


Figure 2. Levels of urinary malondialdehyde (A) and levels of urinary 2-naphthol (B) compared to air pollutant levels such as PM_{2.5} in Changchun, China.

urinary arsenic species remained stable for at least 2 months when stored at 4 and -20°C [25]. In the stability study of human urine in UK Biobank sample collection, either processed by freezing at -80°C or stored at 4°C for 0 or 24 hours before being frozen, was compared in a study of samples. No differences in the metabolic profiling variance were observed between technical replicates and the variance between samples stored at -80°C or 4°C for 0 or 24 h were detected for human urine [26].

We estimated the stability of urinary MDA, however Dr. Wu and colleagues have shown

that the intra-class correlation coefficient (ICC) for plasma MDA was 0.45 for up to 24 hours [27]. This result suggested that MDA is much more stable in urine than in plasma. Few studies have used 2-naphthol in plasma as an indicator of PAHs, and most studies of biomarkers use urinary 2-naphthol in epidemiological-type studies [8,28]. The results of the present study also indicate that urinary 2-naphthol is stable enough to serve as a biomarker in epidemiological studies. When we applied our hypothesis to an epidemiological study conducted in 44 Chinese subjects over a period of 3 weeks, there was a good

correlation between the levels of biomarkers and PM_{2.5} as a measure of air particulate pollutants. In a previous study, PM_{2.5} was significantly associated with biomarkers of oxidative stress [29]. Urinary 2-naphthol is an especially good marker of particle air pollutant concentration [28].

The use of spot urine sample collection instead of 24 hour collection was a limitation of this study. However, according to previous study, 24 hours urine was not necessary for measurement of biomarkers and there was no statistically significant difference between spot and 24 hours urines [30]. The small number of study participants (n=10) was also a limitation of this study. Despite these limitations, our study findings suggest that urinary MDA and 2-naphthol are stable under variable storage conditions, even at room temperature for 16 hours, and indicate that these markers can be used in epidemiological studies involving various sample storage conditions. The ICCs of urinary 2-naphthol and MDA determined in this study were not high, but were acceptable for application to epidemiological studies. Our future study is focused on observing the effects of long or short-term and difference storage-degree and developing an ideal method for urine storage.

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