Recent Advances in Research on Bioactive Ingredients in Cigarette Smoke

Recent Progress in Analytical Methods for Determination of Urinary 3-Hydroxypropylmercapturic Acid, a Major Metabolite of Acrolein

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3-Hydroxypropylmercapturic acid (3-HPMA), a major metabolite of acrolein in urine, has been recognized as a noninvasive biomarker of exposure to cigarette smoke. Since acrolein is formed endogenously from polyamines and is also formed during oxidative stress and aggravates tissue damage by changing protein activity through its conjugation in pathological lesions, it is thought that the urinary 3-HPMA level is useful as a biomarker to monitor the severity of several diseases related to acrolein. To study the correlation between 3-HPMA and disease severity, it is important to understand the properties of analytical methods for determination of 3-HPMA. In this article, we summarize the analytical methods for determination of urinary 3-HPMA and discuss the utility of 3-HPMA as one of the biomarkers for the diagnosis of brain infarction.

Key words acrolein; 3-hydroxypropylmercapturic acid (3-HPMA); brain infarction; LC/MS/MS; GC/MS

1. INTRODUCTION

Acrolein (CH₂=CHCHO), the strongest electrophile among the 2-alkenals, is produced during the combustion of fossil fuels, including engine exhaust, wood, and tobacco and during the heating of cooking oils in the environment, indicating that monitoring acrolein is of significant importance to delineate the pathogenesis of various diseases related to exposure to acrolein.1) Acrolein is also endogenously generated through polyamines (especially spermine) oxidation by amine oxidase,2,3) myeloperoxidase-catalyzed threonine oxidation4) and, at least in part, lipid peroxidation5,6) in pathological lesions in which DNA, protein and lipids are attacked by acrolein directly and these functions are changed or abolished through the conjugation with acrolein. Protein conjugated acrolein (PC-Acro) containing N-(3-formyl-3,4-dehydropiperidino)lysine (FDP-Lys) and N-(3-methylpyridinium)lysine (MP-Lys) in particular is increased in pathological lesions and plasma in patients with various diseases.7–19) PC-Acro has been increasing in popularity as a reliable biomarker for the quantification of acrolein (Table 1). In general, determination of PC-Acro can be achieved using a commercially available enzyme-linked immunosorbent assay (ELISA) kit with a murine monoclonal antibody 5F6 (mAb5F6) which recognizes FDP-lys and MP-Lys, respectively.23,24)

On the other hand, acrolein is generally scavenged by glutathione (GSH) and metabolized to produce 3-hydroxypropylmercapturic acid (3-HPMA) as one of the major and stable metabolites in urine (Fig. 1A). In the 1970s, urinary 3-HPMA was found in rats as a metabolite of acrolein together with allyl alcohol, allyl formate, allyl propionate, allyl nitrate and cyclopropamidase using paper chromatography and gas chromatography.25,26) Parent et al. reported that the major pathway of [2,3-14C] acrolein excretion was via the kidney into the urine (66–69%) in intravenously dosed animals whereas fecal excretion was very low (less than 2%).27) There were no differences in the excretory patterns between males and females. Furthermore, compositions of urinary metabolites were analyzed; N-acetyl-S-2-carboxy-2-hydroxyethylcysteine (5.4–10.4%), N-acetyl-S-2-carboxyethylcysteine (2.6–18.3%), N-acetyl-S-3-hydroxypropyleysteine (3-HPMA: 52.5–73.8%) and 3-hydroxypropionic acid (5.2–33.2%) were found in urine from rats at 4–24 h after intravenous administration of [2,3,14C] acrolein.28) These compounds were quantified by gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS). When [2,3-14C] acrolein was taken orally, 3-HPMA (22.5–41.2%) and significant amounts of oxalic acid (16.8–34.9%), N-acetyl-S-2-carboxy-2-hydroxyethylcysteine (7.8–18.1%), N-acetyl-S-2-carboxyethylcysteine (3.1–11.7%), and 3-hydroxypropionic acid (5.6–30.8%) were produced in rat urine. Based on the above results, 3-HPMA has been recognized as a major urinary metabolite of acrolein.

3-HPMA data has become increasing popular as a biomarker to maintain human health because the noninvasive nature of measuring urinary 3-HPMA allows long-term monitoring of cigarette smoke containing acrolein. Investigation of the severity of acrolein exposure has been performed by liquid chromatography/mass spectrometry tandem mass spectrometer (LC/MS/MS) and it was concluded that the urinary 3-HPMA level in smokers was higher than that in nonsmokers.29–33) However, there is no report of acrolein metabolism in urine within 12 h after the onset of cigarette smoking. Watzek et al. determined acrolein content in commercially available potato crisps/chips (26.5±2.1 µg/kg, mean±standard deviation (S.D.) and monitored urinary 3-HPMA levels in volunteers after their consumption.34) As a result, urinary 3-HPMA levels were increased exponentially within the first 4 h and a gradual decrease was observed at 8 h after consuming the potato crisps. In light of this, 3-HPMA produced by the liver and

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kidney is rapidly excreted into urine,\textsuperscript{25,28} suggesting that the urinary 3-HPMA level quickly reflects the degree of acrolein exposure and consumption of GSH in the body. However, few reports have monitored the urinary 3-HPMA level in patients with several diseases. To further investigate the relationship between 3-HPMA and disease, it is important to understand the analytical methods established thus far. It is also thought that development of a less costly and more reliable analytical method will further facilitate the study of diseases related to acrolein.

In this review article, we introduce several analytical methods including paper chromatography, gas chromatography/mass spectrometry and liquid chromatography/mass spectrometry for 3-HPMA determination. Furthermore, the utility of urinary 3-HPMA analysis for the diagnosis of brain infarction is also discussed.

2. ANALYTICAL METHODS OF URINARY 3-HPMA

2.1. Paper Chromatography

Paper chromatography is an analytical technique for separating dissolved materials by taking advantage of their different rates of migration across sheets of paper. In the 1970s, the detection of 3-HPMA in urine was performed by paper chromatography. At first, a sample pretreatment procedure that included liquid–liquid extraction, fractionation with Amberlite CG 400 column (formate form) and desalting with a Zeo-Karb 225 column (H\textsuperscript{+} form) was performed before paper chromatography.\textsuperscript{25,26} After

\begin{table}
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\begin{tabular}{|c|c|c|c|c|}
\hline
Diseases & Sample & Substrate & Method & Compared with control subjects & References \\
\hline
Atherosclerosis & Arterial tissue & Protein conjugated acrolein (FDP-Lys) & Immunostaining & Increase & 7, 8) \\
\hline
Chronic renal failure & Plasma & & & & 9) \\
Brain infarction & & & & & 10) \\
Silent brain infarction & & & & & 11, 12) \\
Alzheimer’s disease & & & ELISA & Increase & 13, 14) \\
End-stage renal disease & & & & & 15) \\
Diabetic retinopathy & Haemoglobin & & Western blotting & Increase & 16) \\
Sjogren’s syndrome & Saliva & Protein conjugated acrolein (FDP-Lys) & & & 17) \\
Type1 and Type2 diabetes & Urine & Protein conjugated acrolein (FDP-Lys) & ELISA & Increase & 18, 19) \\
\hline
Brain infarction & Urine & 3-HPMA & LC/MS/MS & Decrease & 20) \\
Alzheimer’s disease & & & & & 21) \\
Cardiovascular disease risk & Urine & 3-HPMA & GC/MS & Increase & 22) \\
\hline
\end{tabular}
\caption{Correlation between Acrolein and Several Diseases}
\end{table}
extraction of 3-HPMA from urine, chromatograms are developed overnight on 3MM paper by the descending method at room temperature with one of the following solvent mixtures: A, butanol–water–acetic acid (12: 5: 3, v/v/v); B, butanol–pyridine–3 mol/L NH₃ solution (4: 3: 2, v/v/v); C, propanol–water–NH₃ solution (sp. gr. 0.88) (80: 19: 1, v/v/v). The Rf values of the reference compounds in these three solvent mixtures are 0.76–0.78, 0.49–0.52 and 0.47–0.48, respectively.25,26 After development, the chromatograms are dried and the 3-HPMA spot is visualized by dipping the papers in a platinum reagent (H₂PtCl₆). The detection range of sulfur-containing compounds using platinum reagent was reported to be 12–24 µg.25 The paper chromatography method is relatively easy, however, a large amount of urine was required due to the consumption of urinary sample during the extraction step and low sensitivity of the detection system. For this reason, there was a tendency to change the analytical method to LC/MS/MS.

Consequently, a conventional pretreatment procedure of urine samples with a column of solid phase extraction (SPE) was established for the analysis of LC/MS/MS or GC/MS.29,31 SPE is a technique designed for rapid, selective sample preparation and purification prior to chromatographic analysis. According to the method of Eckert et al., the recovery rate of 3-HPMA using Isolute ENV (Biotage, Grenzach-Wyhlen, Germany) was approximately 80%.29 It was also reported that mercapturic acids including phenylmercapturic acid (PMA) and benzylmercapturic acid (BMA) were sensitive to porcine acylase I, and o-phthalaldehyde derivatization of deacetylated compounds was achievable.26 The fluorescence detection limits of PMA and BMA were 0.5 µg/L. If 3-HPMA is sensitive to porcine acylase I, it is possible to establish an easier and more sensitive paper chromatography method by combining SPE and fluorescence detection.

2.2. GC/MS In 2009, Conklin et al. established a quantitative analysis method for 3-HPMA using GC/MS.38 In this system, a urine sample was initially treated with SPE (Oasis MAX; Waters, Milford, MA, U.S.A.). After extraction, samples were separated by HPLC with UV detection and 3-HPMA fractions were collected. 3-HPMA can be detected by UV detection (210 nm).39 In order to achieve an efficient ionization of the 3-HPMA, hydroxyl and carboxyl groups were derivatized with bistrimethylsilyltrifluoroacetamide for 1 h at 70°C. The resulting product (m/z 366) data were acquired in positive chemical ionization mode using a gas chromatograph equipped with an HP-5 capillary column (50 m×0.2 mm i.d.×0.5-µm phase thickness). DeJarnett et al. measured urinary 3-HPMA using GC/MS and reported that the urine 3-HPMA level was correlated with cardiovascular disease (CVD) risk.25 The median values of µg 3-HPMA/g Creatinine (Cre) in the low CVD risk category (n=49) and high risk category (n=168) were 181.5±30 µg/g Cre (0.821±0.135 µmol/g Cre) and 453.7±46.7 µg/g Cre (2.05±0.211 µmol/g Cre), respectively. In the case of nonsmokers, the median values of µg 3-HPMA/g Cre in the low CVD risk category (n=28) and high risk category (n=99) were 105.2±23 µg/g Cre (0.476±0.104 µmol/g Cre) and 220.9±26.7 µg/g Cre (1.00±0.121 µmol/g Cre), respectively.

2.3. LC/MS Most 3-HPMA analysis has been carried out by LC/MS/MS using a triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface. The 3-HPMA data were generally acquired in multiple reaction monitoring (MRM) mode through monitoring the following transitions: m/z 220 [M–H] to 91 [HO(CH₃)₂]⁻. The MRM mode has been proven to be one of the most sensitive and specific methods used in tandem mass spectrometry in which an ion of particular mass and an ion product of a fragmentation reaction of the precursor ion are selected, respectively.

In 2001, Mascher et al. were the first to develop an LC/MS/MS system with a positive ESI mode.29 In this report, positive ion m/z 222→163 was monitored and the limit of detection (LOD) was found at 50 µg/L. Sample preparation was performed by SPE using an ENV+ cartridge (Separtis GmbH, Grenzach-Wyhlen, Germany). In 2007, Carmella et al. developed the LC/MS/MS system with a negative atmospheric pressure chemical ionization (APCI) mode.30 In this system, monitoring of negative ion m/z 220→91 was performed and LOD was found at 0.9 µg/L. Sample preparation was performed by SPE using an Oasis MAX (Waters). In 2008, an LC/MS/MS system with a negative ESI mode was subsequently developed and LOD was 5 µg/L.31 SPE was performed with an ENV+ cartridge (Separtis GmbH, Grenzach-Wyhlen, Germany). In 2010, direct analysis of urinary samples using the LC/MS/MS system without the SPE step during sample preparation was developed (LOD: 22 µg/L).25 In 2014, Zhang et al. reported high sensitive analysis of 3-HPMA (LOD: 0.049 µg/L) using an API5500 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, U.S.A.) without the SPE step during sample preparation.25 Detection ranges of µg 3-HPMA/g Cre in urine from nonsmokers and smokers were 37–730 and 132–5345 µg/g Cre, respectively.

3. CORRELATION BETWEEN URINARY 3-HPMA LEVEL AND SEVERITY OF STROKE

One of the main endogenous sources of acrolein is amine oxidase-mediated degradation of both spermine and spermidine in pathological lesions.2,3 Tomitori et al. reported that FDP-Lys content and spermine oxidase (SMO) and acetylpolyamine oxidase (AcPDAO) activities in blood plasma were good markers for human stroke.30 If the urinary 3-HPMA level is correlated with the severity of brain infarction, it must be possible to develop a noninvasive biomarker of acrolein for the diagnosis of brain infarction. For this reason, we have measured urinary 3-HPMA from patients with brain infarction using the LC/MS/MS system using negative ESI mode.29

To begin with, the urinary 3-HPMA levels of smokers (n=16) and nonsmokers (n=74) were measured to judge whether our analytical condition was correct or not. The median values of µg 3-HPMA/g Cre in smokers (n=16) and nonsmokers (n=74) were 1635±517 µg/g Cre (7.40±2.34 µmol/g Cre) and 493±232 µg/g Cre (2.23±1.05 µmol/g Cre), respectively. These median values of urinary 3-HPMA obtained from smokers and nonsmokers are very similar to results reported previously.

We next determined the urinary 3-HPMA level from patients with brain infarction. Unexpectedly, we found that the 3-HPMA level in urine from stroke patients was lower than that in control subjects, unlike the increased level of FDP-Lys content in plasma from stroke patients (Fig. 1B). The median values of µmol 3-HPMA/g Cre in 90 control subjects (age 40 to 79) and 78 stroke patients (age 42 to 96) were 2.83 µmol/g
Cre (625.4 µg/g Cre) and 1.56 µmol/g Cre (344.8 µg/g Cre), respectively. This result was observed in terms of both the concentration of urinary 3-HPMA (µmol/L) and µmol/g Cre despite a lower urinary Cre level in stroke patients than in control subjects. Since the median age of the 90 control subjects (age 40 to 79) and 78 stroke patients (age 42 to 96) was 58 and 70 years, respectively, 3-HPMA levels were analyzed with the similar age group of control subjects and stroke patients (Fig. 1C). Significant decreases in 3-HPMA (µmol/L) and µmol 3-HPMA/g Cre were observed in stroke patients compared with control subjects. Furthermore, the size of the brain infarction was also inversely correlated with the urinary 3-HPMA level when stroke patients were subdivided into two groups, one with lesions ≥1 cm in diameter and the other with lesions <1 cm in diameter (data not shown). These results suggest that the monitoring of urinary 3-HPMA is useful for improving the diagnosis of brain infarction.

Why was the level of 3-HPMA in urine reduced in stroke patients? Khan et al. reported that the level of GSH at the locus of an infarction in stroke model rats was significantly lower than that in the brain of control rats.40 Thus, it is most likely that the decrease in 3-HPMA reflects the consumption of GSH at the region of the brain infarction. We have previously suggested that acrolein preferentially reacts with the thiol group of GSH rather than the NH₂-group of lysine residues in proteins, if cellular levels of GSH are not limiting.41,42 It is expected that an increase in PC-Acro at the locus of a cerebral infarction is a consequence of a decrease in GSH in brain tissues.

We have also measured PC-Acro levels in plasma and 3-HPMA levels in urine from control and mild cognitive impairment (MCI) plus Alzheimer’s disease (AD) subjects13,14,21 (Table 1). As expected, the urinary 3-HPMA level in MCI plus AD subjects was lower than that of control subjects. The median values of µmol 3-HPMA/g Cre in 74 control subjects (age 40 to 79) and 54 MCI plus AD subjects (age 57 to 92) were 2.23 µmol/g Cre (492.8 µg/g Cre) and 1.54 µmol/g Cre (340.3 µg/g Cre), respectively. Moreover, it was found that the monitoring of 3-HPMA/Cre in urine was a useful biomarker for distinguishing MCI subjects from AD subjects because urinary Cre in AD subjects was significantly higher than in MCI subjects.

4. PERSPECTIVE

In this review, we have focused on recent progress in the analytical methods of 3-HPMA. As reviewed here, sensitive detection of urinary 3-HPMA can be achieved by LC/MS/MS or GC/MS. However, since the maintenance of LC/MS/MS or GC/MS is generally expensive, more cost effective and easy analytical methods without mass spectrometry are required to expand the correlation studies between 3-HPMA and diseases. For this reason, continuous development to create a more sensitive detection reagent or system is required.

Acknowledgments This study was supported by the Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sport, Science and Technology of Japan and Smoking Research Foundation.

Conflict of Interest The authors declare no conflict of interest.

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