# Quantification of Blood Betel Quid Alkaloids and Urinary 8-Hydroxydeoxyguanosine in Humans and their Association with Betel Chewing Habits

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## Abstract

**Chewing betel quid is a common habit in Taiwan and associated with the risk of oral cancer. Betel quid contains arecoline and arecaidine, which may serve as the exposure biomarkers of a chewing habit. We developed a liquid chromatography–tandem mass spectrometry method for the quantitative analysis of blood arecoline and arecaidine. Because 8-hydroxydeoxyguanosine (8-OH-dG) level in urine is only one early health marker of carcinogenesis, we also examined its association with chewing habit. We found a significant positive correlation between the quantities of betel quid used before the day of drawing blood and arecoline [(Spearman correlation coefficient ($r$) = 0.81; $p$ value < 0.01) or arecaidine levels ($r$ = 0.86; $p$ value < 0.01)]. Habitual use quantity (quids/day) showed moderate correlation with both arecoline ($r$ = 0.52; $p$ value < 0.05) and arecaidine concentrations ($r$ = 0.51; $p$ value < 0.05). However, there were no significant correlations between total chewing years and concentrations of arecoline and arecaidine in serum or 8-OH-dG in urine. In conclusion, serum arecoline and arecaidine levels are measurable and good indicators for recent betel quid use.**

## Introduction

After smoking, alcohol drinking, and caffeine use, consumption of betel quid is the world's fourth most common psychoactive and addictive habit (1). Betel quid (dietary food) is used by an estimated 600 million people worldwide (2); and this practice is widespread in Taiwan with approximately two million habitual users (10% of the population) (3). Our previous study reported that a high proportion of adolescent chewers were also smokers and alcohol drinkers (4). The increasing incidence in oropharyngeal cancer in Taiwan is probably heavily influenced by the rising consumption of alcohol and use of betel quid (5).

Taiwanese betel quid refers to a combination of areca nut, lime, and influorescence of *Piper Betle Linn.* or *Piper Betle* leaf (6). Habitual betel chewing is known to be deleterious to human health with problems including esophageal cancer, liver cancer, obesity, metabolic syndrome, heart disease, cerebrovascular mortality, schizophrenia, and adverse pregnancy outcome (7–14) and has been especially associated with increased risk of the development of oral/pharyngeal cancer (15–17). Although betel quid has varied components and practices in different parts of the world, areca nut is a major common ingredient.

The International Agency for Research on Cancer (IARC) has labeled areca nut without tobacco a Group 1 carcinogen to humans (18). In clinical toxicology, the major constituents of areca nut are alkaloids and polyphenols related to oral cancer (18). The main alkaloids in areca nut are arecoline, arecaidine, guvacine, and guvacoline (19). Arecoline is the major areca alkaloid and constitutes 0.15–0.67% dry weight of betel quid (19), followed by arecaidine. In in vitro studies, the genotoxicity and mutagenicity of areca-nut alkaloids (arecoline/arecaidine) have been detected from many short-term assays (20). Such alkaloids have widespread effects on the body, including the brain, cardiovascular system, and gut, because of their effect on parasympathetic, GABAnergic,

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and sympathetic functions (1,21). Ingredients in betel quid produce complex interactions during chewing. For example, when betel quid is chewed with lime, arecoline and guvacoline are metabolized into arecaidine and guvacine, respectively, and block the GABAnergic effects (21). In addition, arecoline might be rapidly metabolized in both the liver and kidney (18).

Reactive oxygen species (ROS) cause oxidative damage to nucleic acids, proteins, and lipids and are known to be associated with aging, carcinogenesis, and other diseases (22). ROS, produced from polyphenolic betel-quid ingredients with lime, induce chromosomal damage and are involved in pathological changes of oral mucosa (6,23,24). 8-Hydroxydeoxyguanosine (8-OH-dG), a good indicator of oxidative stress, can increase after environmental or occupational exposure to carcinogens. DNA templates containing 8-OH-dG are easily misread (25). In vitro studies have shown that treatment of DNA with betel quid extract under alkaline conditions leads to the formation of 8-OH-dG (24). Cytogenetic effects of ROS were found after applying betel nut extract with lime on the cheek pouch of Syrian golden hamsters (24). Therefore, the formation of 8-OH-dG can be considered a marker of mutation or carcinogensis (20).

Taken together, these biomarkers could be used to detect the exposure (arecoline and arecaidine) and adverse effect (8-OH-dG) of a betel quid chewer. In contrast to the situation with 8-OH-dG, little effort has been made to develop analytical methods for betel alkaloids. Moreover, to our knowledge, no study has revealed the correlation between those three chemical levels and the cumulated amount of betel quid consumed. This study has two main aims: to develop a sensitive and specific analysis of arecoline/arecaidine (Figure 1) in humans and to examine the correlation between the levels of plasma alkaloids or urinary 8-OH-dG and chewing habits (obtained by using a questionnaire).

## Materials and Methods

### Chemicals

Solvents and reagents were analytical grade. Reagents were purchased from the indicated sources: [$^2H_3$]-arecoline and [$^2H_3$]-arecaidine as internal standard (Ryss Lab., Taipei, Taiwan); arecoline and arecaidine (Sigma, St. Louis, MO); ethanol and formic acid (FA) (ECHO, Miaoli, Taiwan); and acetonitrile (ACN) (R.D.H, Seelze, Germany).

### Standard and sample preparation

Standard stock solutions were prepared by adding 1 mg of arecoline, arecaidine, and [$^2H_3$]-arecoline or [$^2H_3$]-arecaidine powder in 1 mL of distilled water (1000 ppm). Standard and quality control samples were obtained by adding known amounts of standard solutions (60 µL) to 1 mL of human plasma. The solutions were serially diluted by adding 5% ACN and 0.1% FA (prepared by adding 5 mL ACN and 0.1 mL FA in 95 mL distilled water) to prepare the internal standard and build a calibration curve.

### Study subjects, blood/urine sampling, and questionnaire

In total, 18 healthy workers of a rapid transit system in Kaohsiung, Taiwan volunteered to participate in this study. Only one of them was female; and the mean age was 39.7 ± 10.6 years with ages ranging from 24 to 57 years. Blood samples were collected from the peripheral veins of upper limbs from each subject. They were stored at –20°C and analyzed for arecoline and arecaidine levels within three days. Urine samples were kept at 4°C during sampling and then stored at –20°C before analysis. All of the subjects were interviewed by trained interviewers using a standardized questionnaire, slightly modified from one used in our previous study (13), to collect demographic characteristics and information about substance use (betel quid, tobacco, and alcohol). Subjects who had weekly chewed betel quid for at least six months during any period were defined as chewers. In the questionnaire, we also recorded what year the subject started and, if applicable, quit the habit, the duration of consumption, and the daily amount consumed. This study was approved by the internal review board of the Human Experimental and Ethics Committee of the Kaohsiung Medical University (KMUH-IRB-94046). All participants gave their written informed consent.

### Liquid chromatography–tandem mass spectrometry (LC–MS–MS) analysis of plasma arecoline/arecaidine (a new analytical method)

*Blood sample preparation.* Heparinized whole blood collected from study subjects was centrifuged at 2000 × *g* for 10 min to isolate plasma supernatant. Plasma specimens were stored at –20°C until analysis. One milliliter of plasma was added to 60 µL of internal standard mixture ([$^2H_3$]-arecoline/[$^2H_3$]-arecaidine, 100 ppb) and 1 mL of 99.9% ethanol. We also tried other solvents such as methanol, but 99.9% ethanol had much better solubility for the mixture. The sample solution was vortex mixed and dried under vacuum for 2 h. The residues was then redissolved in 1 mL of ethanol, mixed, and centrifuged for 10 min at 2000 × *g* at 20°C. One milliliter of the supernatant was pipetted out and collected. The residues was extracted again with 1 mL of ethanol, mixed, and centrifuged for 10 min at 2000 × *g* at 20°C. A total 2 mL of supernatant collected was then dried under vacuum and redissolved in 240 µL of solvent containing 5% ACN and 0.1% FA. It was followed by centrifugation and LC–MS–MS analysis.

*Calibration curve.* Calibration standards containing 180 µL of arecoloine and arecaidine mixture at serial concentrations of 0.2, 1.0, 5.0, 50.0, 100.0, 1000 ppb plus 60 µL, 100 ppb of internal standard mixture ([$^2H_3$]-arecoline, [$^2H_3$]-arecaidine)

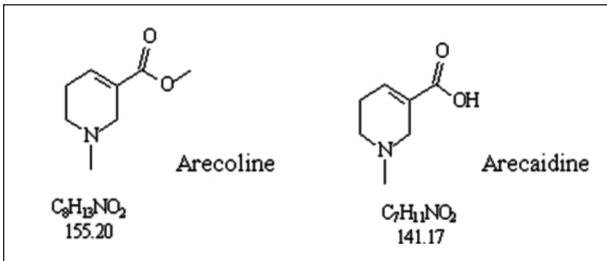


**Figure 1.** Structure and components of arecoline and arecaidine.

were prepared daily for each analytical batch by adding suitable amounts of ethanolic working solutions to 1 mL of plasma.

*LC.* The LC system consisted of two series 200 micro-pumps, a series 200 autosampler (Perkin Elmer, Boston, MA), and an analytical ACE5 $C_{18}$ column (250- × 4.6-mm i.d., 5 µm). Chromatographic separation was conducted at room temperature with a mobile phase consisting of 5% ACN with 0.1% FA (solvent A) and delivered at a flow rate of 600 µL/min for 3 min. It then was varied to 95% ACN with 0.1% FA (solvent B) within 5 min and rapidly back to solvent A with a linear gradient for 1 min. It was then maintained in equilibrium for 3 min.

*Electrospray ionization (ESI)-MS–MS.* The sample eluted from the HPLC system was infused into a TurboIonspray source installed on an API 3000 triple-quadrupole MS (Applied Biosystems, Foster City, CA). Standard solution (1 µg/mL) of arecoline, [$^2H_3$]-arecoline, arecaidine, and [$^2H_3$]-arecaidine was infused into the MS system, and the full-scan spectra of the four molecules were conducted using the positive mode. For all of the samples, the $(M+H)^+$ ion was selected by the first mass filter, and the collision energy was adjusted to optimize the signal for the most abundant product ions. Nebulizer and curtain gas flow rates were set to 15 (arbitrary units). Collision-assisted dissociation (CAD) gas and turbo gas were set at 12 and 8 (arbitrary), respectively. The collision energy was set at 35 eV with nitrogen as the collision gas. Peak widths were set at 0.7 Th (FWHM) for both Q1 and Q3. The protonated molecule of arecoline at *m/z* 156 fragmented in MS–MS to give a major product ion at *m/z* 44.2 (used for qualification). The second abundant ion generated was at *m/z* 80.9 (used for quantification). The mass transition pairs for qualification and quantification were 145/47.2 and 145/99.0 for arecoline-$d_3$, 142/44.2 and 142/42.1 for arecaidine, and 159/47.2 and 159/113.0 for arecaidine-$d_3$. Data acquisition and quantitative processing were accomplished using Analyst software (version 1.1, Applied Biosystems).

### LC–MS–MS analysis of urinary 8-OH-dG

*Urine sample preparation and analysis.* Urinary 8-OH-dG level was measured according to the method developed by Hu et al. (26). In brief, the urine samples were thawed, thoroughly mixed and underwent centrifugation at 5000 × *g* for 5 min. Twenty microliters of urine was diluted 10-fold with 5% methanol containing 0.1% FA. Forty microliters of $^{15}N_5$-8-OH-dG solution (20 µg/L in 5% methanol/0.1% FA) was added to the diluted urine as internal standard, and then the urine was vortex mixed for 5 s. The 8-OH-dG stock solution was prepared by dissolving 8-OH-dG in 5% methanol/0.1% FA; it was then serially diluted 1:1 with 5% methanol/0.1% FA to yield aqueous solutions for establishing the calibration curve.

The online solid-phase extraction LC–MS–MS analysis was used to measure of urinary 8-OH-dG according to methods described in a previous study (26). The column-switching system consisted of a switching valve and an Inertsil ODS-3 column. The switching valve function was controlled by PE-SCIEX control software (Analyst). When the switching valve was at position A, 100 µL of prepared urine sample was loaded on the cartridge by an autosampler, and a quaternary pump delivered the 5% methanol/0.1% FA at a flow rate of 1 mL/min as the loading and washing buffer. After the column was flushed with the loading buffer for 4 min, the valve switched to the injection position (position B) to inject the sample into the LC system. The micropump and autosampler of the high-performance liquid chromatography (HPLC) system are the same as those used for the arecoline and arecaidien study, but a guard column (10- × 2-mm i.d., YMC, Kyoto, Japan) was used here. Isocratic elution using eluent II (85% methanol containing 0.1% FA) was used to separate the analytes. After automatic sample cleanup for 4 min, the sample was automatically eluted from the trap column into the analytical column. The mobile phase was 85% methanol containing 0.1% FA (eluent II) and was delivered at a flow rate of 1 mL/min.

*ESI-MS–MS.* The sample eluting from the HPLC system was introduced into a TurboIonspray source installed on an API 3000 triple-quadrupole MS (Applied Biosystems) that operated in positive mode with a needle voltage of 5.5 kV with nitrogen as the nebulizing gas and turbogas temperature set at 500°C. Data acquisition and quantitative processing were accomplished with Analyst software, Ver. 1.1. For all of the samples, the $(M+H)^+$ ion was selected by the first mass filter. After collisional activation, two fragment ions were selected: the most abundant fragment ion, $(M+H - 116)^+$, was used for quantification (quantifier ion), and the second most abundant ion, $(M+H - 144)^+$, was used for qualification (qualifier ion). The dwell times per channel were set at 150 ms and 150 ms for the analyte and internal standard, respectively. Nebulizer and curtain gas flow rates were set at 12 (arbitrary units). The CAD gas and turbo gas were set at 6 and 8 (arbitrary), respectively. The collision energy was set at 19 eV for the quantifier ion or 45 eV for the qualifier ion with nitrogen as the collision gas. The peak full-width at half-maximum was set to 0.7 Th (thomson) for both Q1 and Q3.

*Statistical analysis.* Mean and standard deviation were used to describe the distribution of the subjects' age and the amount and duration of betel quid consumed. Spearman's correlation was used to study the relationship between different chewing habits (i.e., quantity of betel quid consumed one day before drawing blood, the daily dose measured in quids/day, and total years of consumption) and different biochemical values, including arecoline/arecaidine and 8-OH-dG concentrations. Half of the low detection levels (0.01 ng/mL) were used if the concentrations were below the detection limits. The data were analyzed using the SAS statistical package. All *p* values were two-sided.

## Results and Discussion

### LC–MS and validation

Under the conditions used, the protonated molecule of arecoline at *m/z* 156 fragmented in MS–MS to give a major product ion at *m/z* 44. For arecaidine, the major product ion at *m/z* 44 was a fragment from the protonated molecule at *m/z* 142. The transition ions selected for 8-OH-dG were *m/z* 284.1 → 168.0 for quantification and *m/z* 284.1 → 140.0 for qualification. The optimization of MS parameters for the qualifying and quantifying monitoring ions of arecoline, arecaidene, and

internal standard are shown in Table I. In Figure 2, we presented the LC–MS–MS chromatograms of a real sample from a betel-quid chewer. The spectra of the standard and native arecoline/arecaidine were identical, confirming that there were no interfering substances.

### Limits of quantification (LOQ) and detection (LOD)

The LOQ was defined as the lowest betel alkaloid concentration that could be reliably and reproducibly measured with values for accuracy, intraday, and interday imprecision (< 20%). Using the present method, we determined that the LOQ was 0.5 ng/mL for both arecoline and arecaidine. Hayes et al. reported the first analytical method to quantify plasma arecoline based on gas chromatography (GC)–MS with an LOQ of 2 ng/mL. Similar to our method, they use 1 mL of plasma for each analysis (27). The LOD, defined as the lowest concentration that gave a signal-to-noise ratio of at least 3, was 0.02 ng/mL for both arecoline and arecaidine in our study, which was much lower than that in the previous study (1 ng/mL) (27). In a HPLC–MS-based assay, the LOD for arecoline in meconium was 0.001 µg/g, in cord serum was 0.001 µg/g, and in newborn urine was 0.0004 µg/g (28). Later, similar method was applied to detect arecoline level in hair with the LOQ and LOD of 0.09 ng/mg and 0.3 ng/mg, respectively (29). Another HPLC assay was also reported to efficiently quantify arecoline in human saliva with the minimum detectable amount of 50 pg (30). Recently, LC–MS–MS was used to quantify arecoline in human milk with an LOQ and LOD of 50 ng/mL and 16 ng/mL, respectively (31). To our knowledge, this is the first report to determine arecaidine level in betel quid chewers. In addition, we provided a method with higher sensitivity for quantification of arecoline in plasma.

### Calibration and linearity, recovery, and precision

Calibration curves for arecoline and arecaidine were derived by serial dilution of aqueous calibration solutions. They covered the concentration ranges of arecoline (0.2–100 ng/mL) and arecaidine (0.2–1000 ng/mL) in our study subjects. Linear regression was calculated with nonweighting and non-zero-forced. Two linear equations were obtained: $y = 0.038x + 0.007$; $r^2 = 1$ for arecoline and $y = 0.176x + 0.615$; $r^2 = 0.9995$ for arecaidine. Both $r^2$ were > 99.5%, demonstrating good linearity along the calibration curves. We injected blank samples after the highest point (1000 ng/mL) of the calibration curve and did not find any traces of carryover.

Absolute analytical recoveries were determined by adding 50 ppb, 100 µL of internal standard [$^2H_3$]-arecoline/[$^2H_3$]-arecaidine to three 1-mL drug-free plasma samples and measuring them simultaneously. The recoveries were 85.8% and 85.6% for arecoline and arecaidine, respectively; they were calculated by comparing the peak areas obtained when samples were analyzed by adding the reference standard and internal standard in the plasma before and after the extraction procedure. To evaluate the precision and accuracy of the present method, we performed three replicate determinations of arecoline/arecaidine at different concentrations in the reference standard sample every day for three days (Table II). The intraday and interday variations (CV) were less than 3% in each analysis. The calculated accuracy for arecoline and arecaidine were adequate for the purposes of this study (Table II).

We evaluated the effect of freeze/thaw cycles (stored at –20°C) on the stability of the analytes by repeated analysis of three real blood samples before freeze and 24 h after being thawed. The differences were always less than 3%. When examining the mid-term stability, a difference lower than 10% was always found, assuring the validity of stored sample analysis.

### Biochemical values and their correlation with study subjects' chewing habits

In this study, we collected blood samples in the early morning just before the

**Table I. Optimization of the Mass Spectrometry Parameters**

| Compound | Retention Time (min) | Multiple Reaction Monitoring (*m/z*) | Declustering Potential (V) | Focusing Potential (V) | Collision Energy (eV) | Collision Cell Exit Potential (V) |
|---|---|---|---|---|---|---|
| Arecaidine | 5 | 142/44.2 | 71 | 280 | 33 | 6 |
| Arecaidine-$d_3$ | 5 | 145/47.2 | 20 | 200 | 30 | 15 |
| Arecoline | 7.1 | 156/44.2 | 36 | 240 | 37 | 8 |
| Arecoline-$d_3$ | 7.1 | 159/47.2 | 31 | 170 | 33 | 8 |

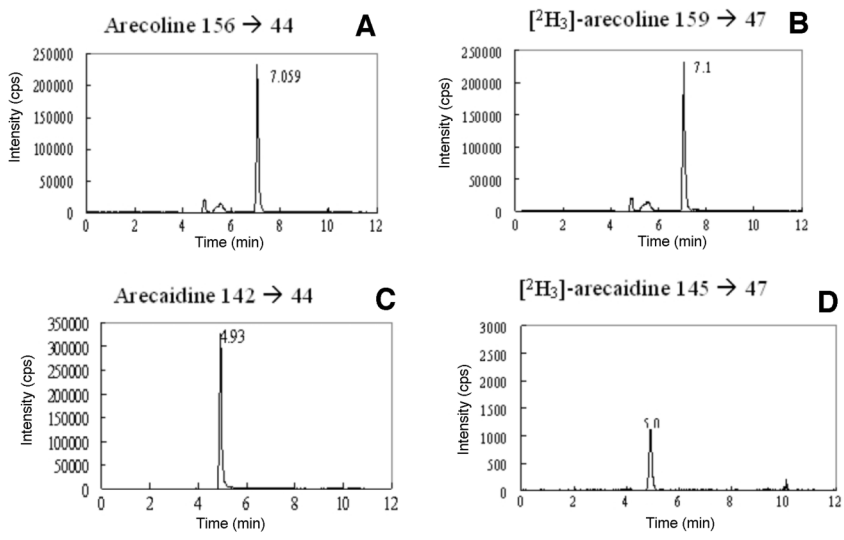


**Figure 2.** LC–MS–MS chromatograms of the real blood sample from a study subject with a chewing habit: arecoline (A), [$^2H_3$]-arecoline (B), arecaidine (C), and [$^2H_3$]-arecaidine (D).

subjects started to work. We used the amount they chewed the day before drawing blood as the information of recent exposure. Five of the 18 subjects did not have the habit of chewing betel quid (5 non-chewers); and one had stopped the habit two years earlier (included in the 13 chewers). Five chewers did not consume betel quid one day before drawing blood while the rest consumed 2–30 pieces the day before (Table III). Chewers had higher mean blood arecoline (7.0 ± 10.7 vs. 0.3 ± 0.2 ng/mL; $p < 0.05$) and arecaidine (142.8 ± 249.3 vs. 1.00 ± 1.3 ng/mL; $p < 0.05$) levels than non-chewers. However, no significant difference was found in mean urine 8-OH-dG levels between the two groups (Table III). In the questionnaire, we asked about the total amount per day but did not have the information about chewing frequency. Therefore, it is unknown whether chewing frequency affects the betel alkaloid levels in blood. There was no significant correlation between different chewing habits and urine 8-OH-dG level (Table IV). Because only one female volunteer participated in this study, the results were not suitable for generalizing to females.

The data in Table IV presents the correlations between chewing characteristics and plasma biochemical values for all study subjects (chewers and non-chewers combined). In this study, although the biochemical levels for betel-related compounds among non-chewers were found to be significantly lower than those for chewers, there still was variation in concentrations observed among non-chewers (Table III). To provide information with regard to the overall relationship between all chewing aspects (even non-chewing) and plasma biochemical levels, we combined the two groups of subjects. The arecoline and arecaidine concentrations in blood were highly correlated to the quantity of betel quid consumed one day before blood was drawn [Spearman correlation coefficient ($r$) = 0.81 and 0.86, respectively; $p$ value < 0.01] and were significantly positively correlated to the average amount consumed per day ($r$ = 0.52 and 0.51, respectively; $p$ value < 0.05) (Table IV). However, there was no significant correlation between the total years of consumption and arecoline ($r$ = 0.43; $p$ value = 0.073) or arecaidine level ($r$ = 0.33; $p$ value = 0.176). Those results suggest that blood alkaloids levels could reflect short-term or recent exposure to betel quids rather than the duration of chewing. The stronger correlations between alkaloids and exposure quantity imply that arecoline and arecaidine are

**Table II. Precision and Accuracy Obtained for Blood Arecoline and Arecaidine**

| Concentration (ng/mL) | Intraday | | | Interday | | |
|---|---|---|---|---|---|---|
| | Estimated mean ± SD | Precision (%) | Accuracy (%) | Estimated mean ± SD | Precision (%) | Accuracy (%) |
| *Arecoline* | | | | | | |
| 20 | 19.4 ± 0.3 | 1.7 | 97.2 | 20.2 ± 0.8 | 3.9 | 101.2 |
| 50 | 52.6 ± 0.4 | 0.8 | 105.3 | 50.6 ± 9 | 1.9 | 101.1 |
| 100 | 105.7 ± 0.6 | 0.5 | 105.7 | 99.6 ± 0.5 | 0.5 | 99.6 |
| *Arecaidine* | | | | | | |
| 20 | 17.4 ± 0.5 | 2.9 | 86.9 | 19.9 ± 0.5 | 2.3 | 99.5 |
| 50 | 48.8 ± 0.7 | 1.5 | 97.7 | 51.0 ± 0.7 | 1.5 | 102.0 |
| 100 | 97.6 ± 2.4 | 2.5 | 97.6 | 99.5 ± 0.3 | 0.3 | 99.5 |

**Table III. Demographic Characteristics of Betel Quid Chewers and Never-Chewers***

| Characteristic | Betel Quid Chewers ($n$ = 13) | Never-Chewers ($n$ = 5) |
|---|---|---|
| Age (years) | 39.4 ± 10.5 | 40.6 ± 11.9 |
| Gender ratio (M/F) | 13/0 | 4/1 |
| *Chewing habits* | | |
| Quantity of chewing before drawing blood (quids) | 6.1 ± 10.8† | 0.00 |
| Quantity of chewing (quids/day) | 22.6 ± 34.7† | 0.00 |
| Duration of chewing (years) | 16.5 ± 10.5† | 0.00 |
| *Biochemical values* | | |
| Arecoline (ng/mL) | 7.0 ± 10.7† | 0.3 ± 0.2 |
| Arecaidine (ng/mL) | 142.8 ± 249.3† | 1.00 ± 1.3 |
| 8-OH-dG (ng/mL) | 3.9 ± 2.6 | 4.6 ± 3.0 |

* Group means are presented with standard deviation (SD). The differences in means ± SD between betel quid chewers and never-chewers were assessed by Mann-Whitney Non-parametric test.
† $p$ value < 0.05.

**Table IV. Correlations Between Chewing Habits and Plasma/Urine Biochemical Values ($n$ = 18)**

| | Spearman's Rank Correlation Coefficient | | |
|---|---|---|---|
| | Quantity of chewing before drawing blood (quids) | Quantity of chewing (quids/day) | Duration of chewing (years) |
| *Plasma* | | | |
| Arecoline (ng/mL) | 0.81* | 0.52† | 0.43 |
| Arecaidine (ng/mL) | 0.86* | 0.51† | 0.33 |
| *Urine* | | | |
| 8-OH-dG (ng/mL) | –0.30 | –0.16 | –0.22 |

* $p$ value < 0.01.
† $p$ value < 0.05.

potentially the most useful biomarkers for the evaluation of short-term exposure quantities. However, other metabolites from areca-nut alkaloids may be more suitable than arecoline/arecaidine in biomarkers of exposure of long-duration chewers.

In humans, little is known about the metabolism and disposition of arecoline/arecaidine and 8-OH-dG. It only has been reported that arecoline level could be determined in human saliva by HPLC (30), in blood (27) and hair (29) by GC–MS, in neonatal biological matrices by HPLC–MS (28), and in human milk by LC–MS–MS (31). Likewise, few studies have compared the urinary 8-OH-dG of betel chewers and non-chewers (32,33). In this study, the concentrations of plasma arecoline/arecaidine, but not urine 8-OH-dG, show a trend toward higher values among "ever" betel chewers (particularly in subjects chewing quids before drawing blood) than those samples among "never" chewers. However, Wu et al. (34) suggested the urine 8-OH-dG was higher in cancer patients than normal control. Those results support the understanding that urine 8-OH-dG is a marker of adverse outcome instead of exposure.

Most of the "never" chewers in this study still measure low amounts of betel alkaloids in blood (ranges: 0–0.63 ng/mL for arecoline and 0–3.12 ng/mL for arecaidine). However, the level was often lower than LOQ (0.5 ng/mL) and was of no clinical significance of exposure. For the two of them with a level slightly higher than LOQ, one plausible hypothesis is that alkaloids may come from drug and food intake. However, betel quid components are not used in Chinese cuisine. Unfortunately, the small number of analyzed samples and the lack of information regarding time elapsed since drug/dietary consumption and sample collection did not provide a definite conclusion to confirm our hypothesis. Despite this, LC–MS–MS is still proven to be a powerful method to quantify plasma arecoline and arecaidine levels. In addition, this is the first report to show the correlation between plasma arecoline/arecaidine levels and chewing habits.

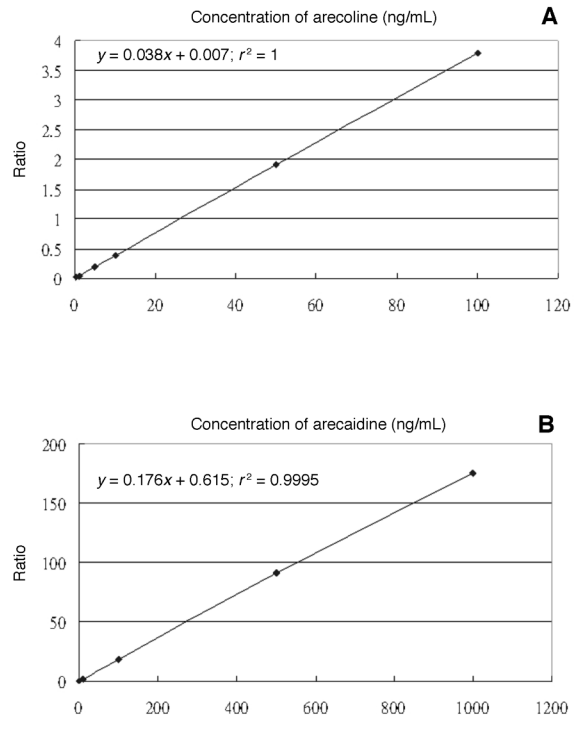


**Figure 3.** Calibration curves for arecoline (A) and arecaidine (B).

Of the chemical ingredients, areca-nut alkaloids and some polyphenols that may have biological activity and adverse effects on tissue have been suggested in previous studies (18,24). Among the areca-nut alkaloids, arecoline is most abundant, whereas arecaidine exists in small quantities. In urinary metabolites in the mouse model, arecoline is metabolized through different pathway and forms various metabolites (35). The majority of arecoline metabolites are formed after the compound is first hydrolyzed to arecaidine (35). The short half-life of arecoline has been attributed to the rapid in vivo enzymatic hydrolysis of the ester functionality to form the carboxylic acid derivative arecaidine, which appears to be a major metabolic product of arecoline in mice (36,37). In human blood, this study showed that the levels of arecaidine were higher than arecoline levels. We suggest that the scale of arecaidine may be more appropriate clinical index of betel quid exposure than arecoline.

## Conclusions

In this study, we provided a sensitive and accurate method to objectively determine the exposure of toxic substances from betel quid consumed. We suggest that plasma arecoline and arecaidine are good biological markers for immediate or short-term betel quid exposure. Arecaidine could be superior to arecoline as a clinical index of betel quid exposure. Urinary 8-OH-dG, known to create adverse effects, in urine is not correlated with betel quid exposure.

## Acknowledgments

This study was supported in part by grants from the National Health Research Institutes (Grant No. NHRI -97A1-PDCO-03-0710-1), the Bureau of Health Promotion, Department of Health, Executive Yuan, Taiwan (Grant No. DOH96-HP-1509), Cancer Medical Treatment Foundation, Kaohsiung Medical University (QC097001), and from the Center of Excellence for Environmental Medicine, Kaohsiung Medical University, Taiwan (Grant No. KMU-EM-98-1-1).

## References

1. B.J. Boucher and N. Mannan. Metabolic effects of the consumption of *Areca catechu*. *Addict. Biol.* **7:** 103–110 (2002).
2. P.C. Gupta and S. Warnakulasuriya. Global epidemiology of areca nut usage. *Addict. Biol.* **7:** 77–83 (2002).
3. Y.C. Ko, T.A. Chiang, S.J. Chang, and S.F. Hsieh. Prevalence of betel quid chewing habit in Taiwan and related sociodemographic

factors. *J. Oral Pathol. Med.* **21:** 261–264 (1992).
4. M.S. Yang, I.H. Su, J.K. Wen, and Y.C. Ko. Prevalence and related risk factors of betel quid chewing by adolescent students in southern Taiwan. *J. Oral Pathol. Med.* **25:** 69–71 (1996).
5. P.S. Ho, Y.C. Ko, Y.H. Yang, T.Y. Shieh, and C.C. Tsai. The incidence of oropharyngeal cancer in Taiwan: an endemic betel quid chewing area. *J. Oral Pathol. Med.* **31:** 213–219 (2002).
6. P.H. Chen, C.C. Tsai, Y.C. Lin, Y.C. Ko, Y.H. Yang, T.Y. Shieh, P.S. Ho, C.M. Li, A. Min-Shan Ko, and C.H. Chen. Ingredients contribute to variation in production of reactive oxygen species by areca quid. *J. Toxicol. Environ. Health A* **69:** 1055–1069 (2006).
7. M.S. Yang, C.H. Lee, S.J. Chang, T.C. Chung, E.M. Tsai, A.M. Ko, and Y.C. Ko. The effect of maternal betel quid exposure during pregnancy on adverse birth outcomes among aborigines in Taiwan. *Drug Alcohol Depend.* **95:** 134–139 (2008).
8. C.F. Lin, J.D. Wang, P.H. Chen, S.J. Chang, Y.H. Yang, and Y.C. Ko. Predictors of betel quid chewing behavior and cessation patterns in Taiwan aborigines. *BMC Public Health* **6:** 271 (2006).
9. A.M. Yen, Y.H. Chiu, L.S. Chen, H.M. Wu, C.C. Huang, B.J. Boucher, and T.H. Chen. A population-based study of the association between betel-quid chewing and the metabolic syndrome in men. *Am. J. Clin. Nutr.* **83:** 1153–1160 (2006).
10. J.Y. Guh, H.C. Chen, J.F. Tsai, and L.Y. Chuang. Betel-quid use is associated with heart disease in women. *Am. J. Clin. Nutr.* **85:** 1229–1235 (2007).
11. T.Y. Lan, W.C. Chang, Y.J. Tsai, Y.L. Chuang, H.S. Lin, and T.Y. Tai. Areca nut chewing and mortality in an elderly cohort study. *Am. J. Epidemiol.* **165:** 677–683 (2007).
12. R.J. Sullivan, S. Andres, C. Otto, W. Miles, and R. Kydd. The effects of an indigenous muscarinic drug, Betel nut (*Areca catechu*), on the symptoms of schizophrenia: a longitudinal study in Palau, Micronesia. *Am. J. Psychiatry* **164:** 670–673 (2007).
13. I.C. Wu, C.Y. Lu, F.C. Kuo, S.M. Tsai, K.W. Lee, W.R. Kuo, Y.J. Cheng, E.L. Kao, M.S. Yang, and Y.C. Ko. Interaction between cigarette, alcohol and betel nut use on esophageal cancer risk in Taiwan. *Eur. J. Clin. Invest* **36:** 236–241 (2006).
14. J.F. Tsai, J.E. Jeng, L.Y. Chuang, M.S. Ho, Y.C. Ko, Z.Y. Lin, M.Y. Hsieh, S.C. Chen, W.L. Chuang, L.Y. Wang, M.L. Yu, and C.Y. Dai. Habitual betel quid chewing and risk for hepatocellular carcinoma complicating cirrhosis. *Medicine (Baltimore)* **83:** 176–187 (2004).
15. C.H. Lee, Y.C. Ko, H.L. Huang, Y.Y. Chao, C.C. Tsai, T.Y. Shieh, and L.M. Lin. The precancer risk of betel quid chewing, tobacco use and alcohol consumption in oral leukoplakia and oral submucous fibrosis in southern Taiwan. *Br. J. Cancer* **88:** 366–372 (2003).
16. Y.C. Ko, Y.L. Huang, C.H. Lee, M.J. Chen, L.M. Lin, and C.C. Tsai. Betel quid chewing, cigarette smoking and alcohol consumption related to oral cancer in Taiwan. *J. Oral Pathol. Med.* **24:** 450–453 (1995).
17. K.W. Lee, W.R. Kuo, S.M. Tsai, D.C. Wu, W.M. Wang, F.M. Fang, F.Y. Chiang, K.Y. Ho, L.F. Wang, C.F. Tai, E.L. Kao, S.H. Chou, C.H. Lee, C.Y. Chai, and Y.C. Ko. Different impact from betel quid, alcohol and cigarette: risk factors for pharyngeal and laryngeal cancer. *Int. J. Cancer* **117:** 831–836 (2005).
18. IARC, Betel-quid and areca-nut chewing and some areca-nut-derived nitrosamines, IARC Monogr Eval Carcinog Risks Hum, 2004.
19. IARC. Betel-quid and areca-nut chewing. *IARC Monogr. Eval. Carcinog. Risk Chem. Hum.* **37:** 145–146 (1985).
20. J.H. Jeng, M.C. Chang, and L.J. Hahn. Role of areca nut in betel quid-associated chemical carcinogenesis: current awareness and future perspectives. *Oral Oncol.* **37:** 477–492 (2001).
21. N.S. Chu. Effects of Betel chewing on the central and autonomic nervous systems. *J. Biomed. Sci.* **8:** 229–236 (2001).
22. M. Dizdaroglu, P. Jaruga, M. Birincioglu, and H. Rodriguez. Free radical-induced damage to DNA: mechanisms and measurement. *Free Rad. Biol. Med.* **32:** 1102–1115 (2002).
23. U. Nair, H. Bartsch, and J. Nair. Alert for an epidemic of oral cancer due to use of the betel quid substitutes gutkha and pan masala: a review of agents and causative mechanisms. *Mutagenesis* **19:** 251–262 (2004).
24. U.J. Nair, G. Obe, M. Friesen, M.T. Goldberg, and H. Bartsch. Role of lime in the generation of reactive oxygen species from betel-quid ingredients. *Environ. Health Perspect.* **98:** 203–205 (1992).
25. H. Kasai. Analysis of a form of oxidative DNA damage, 8-hydroxy-2′-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. *Mutat. Res.* **387:** 147–163 (1997).
26. C.W. Hu, C.J. Wang, L.W. Chang, and M.R. Chao. Clinical-scale high-throughput analysis of urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine by isotope-dilution liquid chromatography-tandem mass spectrometry with on-line solid-phase extraction. *Clin. Chem.* **52:** 1381–1388 (2006).
27. M.J. Hayes, L. Khemani, M. Bax, and D. Alkalay. Quantitative determination of arecoline in plasma by gas chromatography chemical ionization mass spectrometry. *Biomed. Environ. Mass Spectrom.* **18:** 1005–1009 (1989).
28. S. Pichini, M. Pellegrini, R. Pacifici, E. Marchei, J. Murillo, C. Puig, O. Vall, and O. Garcia-Algar. Quantification of arecoline (areca nut alkaloid) in neonatal biological matrices by high-performance liquid chromatography/electrospray quadrupole mass spectrometry. *Rapid Commun. Mass Spectrom.* **17:** 1958–1964 (2003).
29. E. Marchei, A. Durgbanshi, S. Rossi, O. Garcia-Algar, P. Zuccaro, and S. Pichini. Determination of arecoline (areca nut alkaloid) and nicotine in hair by high-performance liquid chromatography/electrospray quadrupole mass spectrometry. *Rapid Commun. Mass Spectrom.* **19:** 3416–3418 (2005).
30. S. Cox, I. Piatkov, E.R. Vickers, and G. Ma. High-performance liquid chromatographic determination of arecoline in human saliva. *J. Chromatogr. A* **1032:** 93–95 (2004).
31. M. Pellegrini, E. Marchei, S. Rossi, F. Vagnarelli, A. Durgbanshi, O. Garcia-Algar, O. Vall, and S. Pichini. Liquid chromatography/electrospray ionization tandem mass spectrometry assay for determination of nicotine and metabolites, caffeine and arecoline in breast milk. *Rapid Commun. Mass Spectrom.* **21:** 2693–2703 (2007).
32. C.Y. Chuang, C.C. Lee, Y.K. Chang, and F.C. Sung. Oxidative DNA damage estimated by urinary 8-hydroxydeoxyguanosine: influence of taxi driving, smoking and areca chewing. *Chemosphere* **52:** 1163–1171 (2003).
33. R.H. Wong, C.W. Hu, C.Y. Yeh, M.R. Chao, C.C. Chen, J.H. Huang, S.H. Chang, S.I. Lee, and H.S. Lee. Sulfotransferase 1A1 and glutathione S-transferase P1 genetic polymorphisms modulate the levels of urinary 8-hydroxy-2′-deoxyguanosine in betel quid chewers. *Arch. Toxicol.* **82(5):** 313–321 (2008).
34. L.L. Wu, C.C. Chiou, P.Y. Chang, and J.T. Wu. Urinary 8-OHdG: a marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics. *Clin. Chim. Acta* **339:** 1–9 (2004).
35. S. Giri, J.R. Idle, C. Chen, T.M. Zabriskie, K.W. Krausz, and F.J. Gonzalez. A metabolomic approach to the metabolism of the areca nut alkaloids arecoline and arecaidine in the mouse. *Chem. Res. Toxicol.* **19:** 818–827 (2006).
36. J. Saunders, G.A. Showell, R.J. Snow, R. Baker, E.A. Harley, and S.B. Freedman. 2-Methyl-1,3-dioxaazaspiro(4.5)decanes as novel muscarinic cholinergic agonists. *J. Med. Chem.* **31:** 486–491 (1988).
37. O. Nieschulz and P. Schmersahl. On the pharmacology of active materials from betel. 2. Transformation of arecoline to arecaidine. *Arzneimittelforschung* **18:** 222–225 (1968).

Manuscript received September 16, 2009;
revision received January 29, 2010.