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Short communication

High-performance liquid chromatographic determination of arecoline in human saliva

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Abstract

Arecoline (methyl-1,2,5,6-tetrahydro-1-methyl nicotinate) is an alkaloid found in the areca catechu nut which is a major component of the 'betel quid' chewed by a large proporation of the population in India, South Asia and the South Pacific islands. It is commonly associated with the development of oral leukoplakia, oral submucous fibrosis and oral cancer. We have developed a new ion-pairing reversed-phase high-performance liquid chromatographic (HPLC) method for the determination of arecoline in saliva, using arecaidine (1,2,5,6-tetrahydro-1-methylnicotinic acid) as an internal standard. The optimal wavelength was established using UV absorbance scans. It was showed that 215 nm is the optimal wavelength to maximise the signal in detecting arecoline in the mobile phase. Arecoline was extracted from saliva with hexane–isoamyl alcohol (1%) and reconstituted with mobile phase for HPLC analysis. The developed method is an easy and reliable method of determining arecoline concentrations in saliva. Sensitivity, specificity, precision, accuracy and reproducibility of the method were demonstrated to be satisfactory for measuring the arecoline level.
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Keywords: Arecoline; Alkaloids

1. Introduction

It is estimated that at least 200 million people chew the areca catechu nut worldwide, mostly in the Pacific basin and southern Asian countries [1–6]. Numerous epidemiological studies have shown an association between areca nut chewing and the development of oral cancer [1,2,6], as well as the development of the leukoplakia and oral submucous fibrosis [7–12]. Arecoline (methyl-1,2,5,6-tetrahydro-1-methylnicotinate) is a cholinomimetic alkaloid found in the areca nut and acts on both nicotinic and muscarinic receptors. As such it stimulates both the sympathetic and parasympathetic nervous systems—smooth muscle of the intestinal tract, constricts the bronchial smooth muscle, it is a potent diaphoretic, causes nausea, vomiting and hiccough. Arecoline stimulates the lacrimal, gastric, pancreatic and intestinal glands, as well as the mucous cells of the respiratory tract [4,10,13–21].

Different chemical compounds have been identified in the areca nut [16] but arecoline has been shown to be implicated in the pathogenesis of these diseases because of it genotoxic, mutagenic and carcinogenic potential [9,22,23].

It is therefore important to be able to determine the concentration of arecoline in saliva, as this would give an indication of the concentration of arecoline being administered to the keratinocytes and fibroblasts in the mucosa. Previous reports on the concentration of arecoline in saliva involved the use of gas chromatography and had small numbers [24], or sought to differentiate the various alkaloids in the areca nut [25]. This is the first paper to present an easy and reliable method of determining arecoline concentrations in saliva utilizing high-performance liquid chromatography (HPLC).

2. Materials and methods

2.1. Instrumentation

A Shimadsu HPLC system was used for the detection of arecoline. It consists of an LC-10AD pump, DGU-3A

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degaser, SIL-10A automated injector, CBM-10A controller, CTO-10A oven and SPD-10AV–UV detector. (Japan) The C_{18} reversed-phase column (Waters, Sydney, Australia) was Luna 5 μm, 250 mm × 4.6 mm.

2.2. Reagents

Arecoline (methyl-1,2,5,6-tetrahydro-1-methylnicotinate), arecaidine (1,2,5,6-tetrahydro-1-methylnicotinic acid) and caffeine were from Sigma, acetonitrile (HPLC grade) from Unichrom Co. and triethylamine from Sigma–Aldrich.

Calibration solutions in concentration from 50 to 500 ng were prepared in Milli-Q water. Control samples were arecoline-spiked saliva from individuals with no chewing habits.

2.3. Sample preparation

The saliva was collected from the individuals with a regular, semi-regular habit of chewing the areca nut in one form or another: betel quid, areca nut alone, areca nut with other spices. Control saliva was collected from the individuals with no previous areca nut chewing habits.

After chewing the nut saliva was collected in 10 ml polyethylene tubes with screw tops. Samples were frozen and stored at −80 °C.

Examination involved 0.5 ml of saliva being mixed with 0.5 ml 1% iso-amyl alcohol in hexane and 0.2 g NaCl in eppendorf disposable polypropylene vial. Samples were vortexed for 30 s, centrifuged at 3000 rpm and frozen. In some samples gel substances developed between the two phases of organic solvent and saliva, which complicated the accurate collection of the exact amount of organic solvent (to overcome this problem we have used the internal standard calculation method). Freezing of the samples with repeated vortexing and centrifugation appeared to reduce size of the gel layer.

After freezing, hexane solution was decanted into the glass HPLC vials and evaporated under the nitrogen. Samples were reconstituted in 100 μl of mobile phase and analysed on the HPLC.

2.4. Internal standards

We found that arecaidine (1,2,5,6-tetrahydro-1-methylnicotinic acid) and caffeine were both suitable for use as alternative internal standards in saliva. Neither of these substances was found in saliva of subjects who participated in the research.

2.5. HPLC method

Reversed-phase chromatography was carried out using a mobile phase composed of 50% acetonitrile and 50% 0.01 M sodium hydrogenphosphate with 0.01% triethylamine. The flow rate was 1.2 ml/min passing through the Luna C_{18} column with column temperature 30 °C. The UV detector was set up at 215 nm.

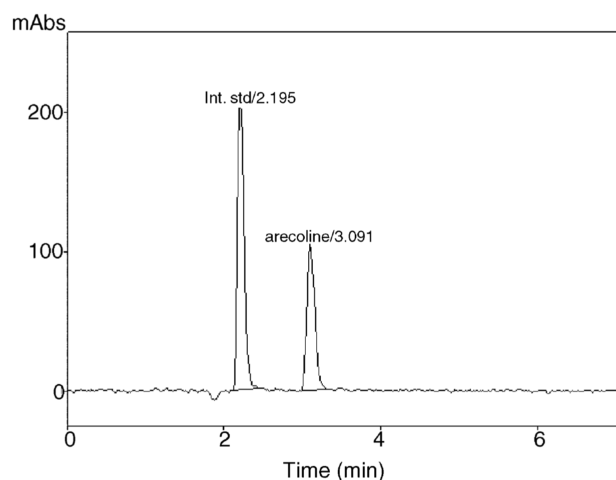

Fig. 1. Chromatogram of arecoline (500 ng/ml). Column: Luna C_{18}. Flow rate: 1.2 ml/min. Injection: 10 μl. 50% acetonitrile.

The optimal wavelength for arecoline detection was established using two UV absorbance scans over the range of 190–360 nm, one scan of the mobile phase and the second of arecoline in the mobile phase. It was shown that 215 nm is the optimal wavelength to maximise the signal. Signal/noise ratio 0.33:0.005 mAV, which is equivalent to 22:1.

As arecoline can be present in the ionized and no-ionized forms (pK_a 6.8), influence of pH on the method sensitivity was examined. Taking in account the column efficiency we found that pH 7.8 is the optimal level for this method.

3. Results and discussion

3.1. Analyte identity

Retention time (t_R) for the arecoline standard was 3.1 min with the internal standard, arecaidine (1,2,5,6-tetrahydro-1-methylnicotinic acid) being 2.2 min (Fig. 1).

A stopped flow method with spectral scans over the absorbance range 200–300 nm on the three different positions of the peaks were used to compare peak homogeneity of the arecoline standard and native peaks. The spectra of the standard arecoline and native arecoline were identical, confirming that there were no coeluting substances (Fig. 2).

3.2. Validation of method

A validation curve over the range of 0.1–500 ng/ml of arecoline (pH 7) was prepared with three repetitions each at 0.1, 0.5, 1.0, 10, 50, 100, 200, 300, 400, 500 ng/ml. The calibration curve was accepted as linear over the range 0.1–100 ng. $R^2 = 0.9995$; $y = 5746.7x$; R.S.D. less than 10%, STEYX (standard error of the predicted y-value for each x in the progression) = 1.0.

The minimum detectable amount was 50 pg, based on the sample preparation method described above.

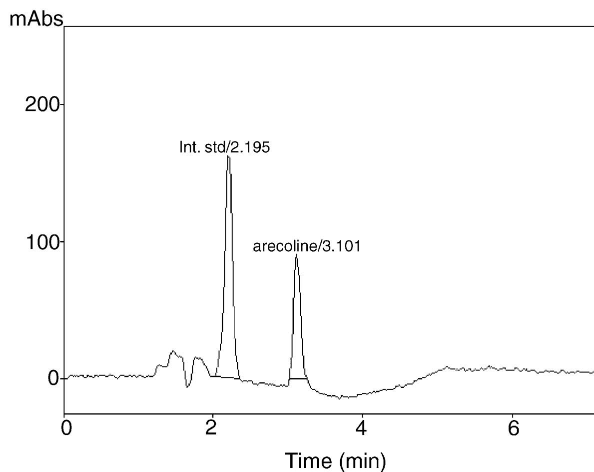

Fig. 2. Chromatogram of arecoline in saliva. Column: Luna C_{18}. Flow rate: 1.2 ml/min. Injection: 10 μl. 50% acetonitrile.

3.3. Recovery, accuracy and precision

Saliva sample spiked with 50 ng of arecoline was used for calculation of accuracy and precision. The precision within and between the run were 1.2 and 2.5% R.S.D. ($n = 10$).

Recovery was estimated by using four different concentrations in duplicate spiked saliva: 100, 150, 200, 250, 300 ng/ml. Average rate of recovery was 93.4%.

4. Conclusion

Areca nut is chewed by a 10th of the world's population [6]. A strong relationship exists between use of the areca nut and oral pathology. Further research into the oncogenic affects of arecoline will require an easy and reliable method of monitoring its concentration in saliva.

We have presented an easy, reliable, cost-effective and efficient method for the determination of arecoline concentration in saliva using HPLC.

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