The Novel Assay Method for Nicotine Metabolism to Cotinine Using High Performance Liquid Chromatography

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Nicotine is the primary psychoactive component in tobacco. It is taken into the body by tobacco smoking, and mainly metabolized to cotinine in the hepatic cytochrme P450 (CYP) 2A6. The objective of this study was to develop a sensitive method for the determination of nicotine metabolism to cotinine using HPLC. The internal standard, *trans*-4′-carboxycotinine methyl ester was synthesized with a simple method. The nicotine and cotinine were separated completely and detected by C_{18} 5-μm analytical column (L-column Octa decyl silyl (ODS), 150 mm×4.6 mm i.d.) equipped with a C_{18} 5-μm guard column (L-column ODS, 10 mm×4.6 mm i.d.) and ultraviolet detection at 260 nm. The detection limit of the assay was 0.05 μM for cotinine (n=5, R.S.D) and 0.1 μM for nicotine. Thus the present results provided a sensitive and useful method for the determination of nicotine metabolism catalyzed by CYP2A6.

Key words *trans*-4′-carboxycotinine methyl ester; nicotine; metabolism; inhibition; cytochrome P450 2A6

Tobacco smoking is the single most preventable cause of several adverse health effects to both active and passive smokers in the world today. The health consequences of smoking include respiratory, cardiovascular and cerebrovascular disorders and cancer.[1—3] The most well-established association between smoking and disease is that for cancer, which is also the most widespread smoking-related disease.[4,5] Nicotine is the primary psychoactive component in tobacco responsible for the addictive properties of cigarettes.[2,6,7] The major pathway of nicotine metabolism in humans is a C-oxidation to form cotinine, which is *via* two-step process. The first step is catalization by cytochrome P450 (CYP) 2A6 to produce the intermediate nicotine-$\Delta^{1'(5')}$-iminium ion, which is further oxidized to cotinine by cytosolic aldehyde oxidase[5—10] (Fig. 1).

So far, detection for nicotine metabolism was carriedout using high-performance liquid chromatography (HPLC),[11—16] radiometric high-performance liquid chromatography,[12] and gas chromatography-mass spectrometry.[17,18] In this study, we report a simple and sensitive method for the determination of nicotine metabolism to cotinine using new column ((L-column octa decyl silyl (ODS), 150 mm×4.6 mm i.d., Chemicals Evaluation and Research Institute, Japan) equipped with a C_{18} 5-μm guard column (L-column ODS, 10 mm×4.6 mm, Chemicals Evaluation and Research Institute)), on HPLC. The assay method using high-performance liquid chromatography, Ketamine had been used as an internal standard before.[2,3] However, it was omitted from drug designation in Japan in 2007 (Ministry of Health, Labour and Welfare, Japan). Thus, the novel internal standard was needed, and we newly synthesized *trans*-4′-carboxycotinine methyl ester with a simple method as an internal standard in this assay.

Experimental

Chemicals *S*-(−)-Nicotine and *S*-(−)-cotinine were obtained from Sigma (St. Louis, MO, U.S.A.), and *trans*-4′-carboxycotinine was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

Enzyme Preparation Human recombinant CYP2A6 in a baculovirus system co-expressing human reduced nicotinamide adenine dinucleotide phosphate (NADPH)-P450 reductase and pooled human liver cytosol were purchased from BD Gentest Corporation (Woburn, MA, U.S.A). Nicotinamide adenine dinucleotide phoshate ($NADP^+$), glucose 6-phosphate, glucose 6-phosphate dehydrogenase were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan).

Metabolism of Nicotine to Cotinine Nicotine C-oxidation activities were determined by the method of Yamazaki *et al.* with slight modifications.[6] The standard incubation mixture (final volume of 250 μl) contained recombinant human CYP2A6 (20 pmol/ml), liver cytsol (0.3 mg/ml), 50 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system consisting of 0.5 mM $NADP^+$, 5 mM glucose 6-phosphate, 0.5 unit/ml glucose 6-phosphate dehydrogenase, and 50 μM nicotine. Incubations were carried out at 37 °C for 30 min, and then the reaction was terminated by adding 50% acetonitrile/methanol. *trans*-4′-carboxycotinine methyl ester was added as an internal standard to this solution at final concentration of 25 μM.

Preparation of *trans*-4′-Carboxycotinine Methyl Ester To 30 mg of *trans*-4′-carboxycotinine was dissolved in 20 ml of acetone and added 2 ml of diazomethane. The mixture was made to react for 20 min at the room temperature. After distilling away the mixture, 39 mg of *trans*-4′-carboxycotinine methyl ester was obtained (Fig. 2). Electron ionization-mass spectra (EI-MS) *m/z* (rel. int.): 234 (100), 219 (19), 206 (40), 191 (73), 175 (97), 147 (92), 121 (69), 119 (96), 97 (16), 91 (19), 78 (32), 68 (34), 55 (50), 42 (64). ^{1}H-NMR (400.00 MHz, in $CDCl_3$, tetramethylsilane (TMS) as internal standard): δ: 2.89 (3H, s), 2.80 (2H, dd, J=9.3, 17.6 Hz), 2.88 (2H, dd, J=8.3 17.6 Hz), 3.05 (1H, ddd, J=6.4, 8.0, 9.3 Hz), 4.83 (1H, d, J=6.4 Hz), 7.37 (1H, dd, J=4.7, 7.7 Hz), 7.57 (1H, ddd, J=2.4, 2.4, 7.7 Hz), 8.56 (1H, d, J=2.4), 8.63 (1H, dd, J=2.4, 4.7 Hz).

HPLC System After terminating the enzyme reaction by adding 50% acetonitrile/methanol, the denatured protein was removed by centrifugation

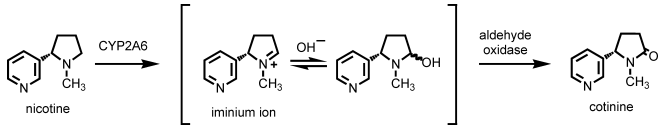

Fig. 1. Metabolic Pathway of Nicotine by CYP2A6 and Aldehyde Oxydase

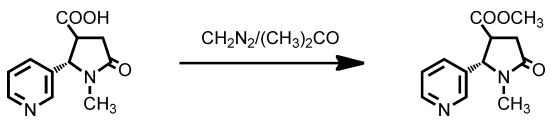

Fig. 2. The Reaction of *trans*-4′-Carboxycotinine Methyl Ester with Diazomethane in the Presence of Acetone at Room Temperature for 30 min

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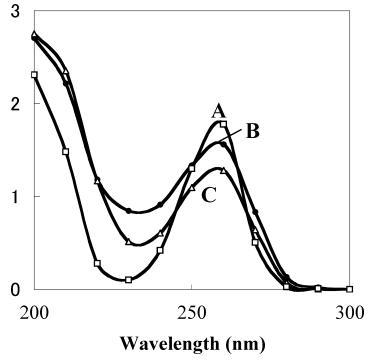

Fig. 3. UV Spectra of A: *trans*-4′-Carboxycotinine Methyl Ester, B: Nicotine C: Cotinine

Concentrations of these chemicals examined were all 250 μM.

at 3000 rpm at 4 °C for 10 min and the supernatant was used for HPLC analysis with a LC-CCPS system (Tosoh, Tokyo, Japan) equipped a detector UV-8020. The separation was done with a C_{18} 5-μm analytical column (L-column ODS, 150×4.6 mm i.d., Chemicals Evaluation and Research Institute, Japan) equipped with a C_{18} 5-μm guard column (L-column ODS, 5—4.6 mm, Chemicals Evaluation and Research Institute). The eluent consisted of a mixture of 10% acetonitrile (v/v) containing 25 mM potassium phosphate buffer (pH 7.0). The flow-rate was 1.0 ml/min and the UV detection was done at 260 nm (Fig. 3). Peak areas thus obtained were integrated with a Chromatopac Instrument (C-R6A Chromatopac, Shimadzu, Kyoto, Japan).

Inhibitory Effect of (+)-Menthofuran on Nicotine Metabolism The standard incubation mixture (final volume of 250 μl) contained recombinant human CYP2A6 (20 pmol/ml), liver cytsol (0.3 mg/ml), an NADPH-generating system consisting of 0.5 mM $NADP^+$, 5 mM glucose 6-phosphate, 0.5 unit/ml glucose 6-phosphate dehydrogenase, and 50 μM nicotine, 1 μM (+)-menthofuran (dissolved in dimethyl sulfoxide (DMSO)), 50 mM potassium phosphate buffer (pH 7.4). Incubations were carried out at 37 °C for 30 min, and then the reaction was terminated by adding 50% acetonitrile that were dissolved in methanol (v/v). *trans*-4′-Carboxycotinine methyl ester was added as an internal standard to this solution at final concentration of 25 μM.

Results

Standard Curve of Cotinine and Nicotine Standard curve was constructed by plotting peak area (PA) *versus* concentration of the cotinine and nicotine. Within-day precision of assay was determined by analysis of replicate (n=5) samples of 5 different concentrations on the same day. Standard curves for cotinine were liner over the concentration range of 0.05—50 μM and and these for nicotine were 0.1—250 μM. The cotinine concentration metabolized from nicotine was determined by comparison to a standard curve. UV intensities increased linearly between 0.05 and 50 μM cotinine in the HPLC method (Fig. 4).

Detection of *trans*-4′-Carboxycotinine Methyl Ester by HPLC To detect *trans*-4′-carboxycotinine methyl ester by HPLC, *trans*-4′-carboxycotinine was methylated by diazomethane and produced to *trans*-4′-carboxycotinine methyl ester. Both *trans*-4′-carboxycotinine and *trans*-4′-carboxycotinine methyl ester were dissolved in 50 mM potassium phosphate buffer (pH 7.4), and analyzed by HPLC (Fig. 5). The peaks of *trans*-4′-carboxycotinine and *trans*-4′-carboxycotinine methyl ester were detected.

HPLC Analysis for the Detection of Nicotine Metabolism by CYP2A6 The cotinine produced from nicotine metabolism was separated from nicotine and *trans*-4′-carboxycotinine methyl ester as an internal standard by HPLC. Furthermore, the inhibitory effect of (+)-menthofuran, which is known as a major inhibitor of CYP2A6 activity, on nicotine

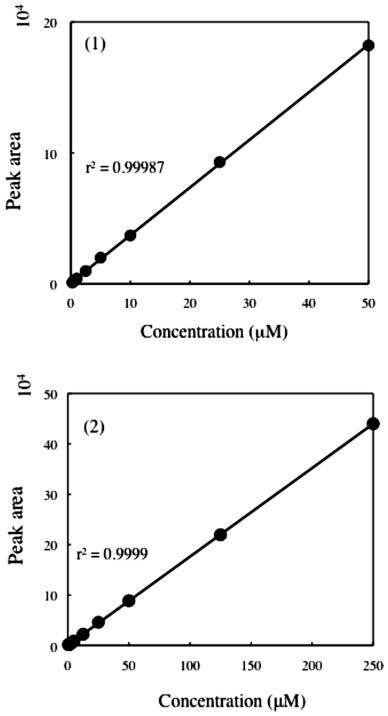

Fig. 4. Standard Curve of Cotinine (1) and Nicotine (2) by HPLC Methods

Cotinine was dissolved in the 10% acetonitrile (v/v) containing 25 mM potassium phosphate buffer (pH 7.0).

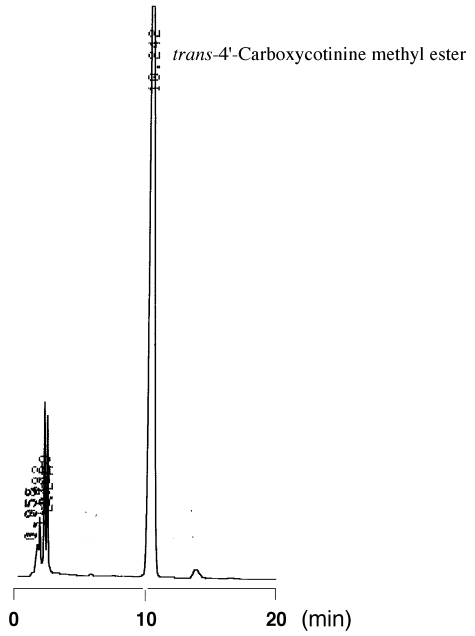

Fig. 5. HPLC Analysis for 50 μM *trans*-4′-Carboxycotinine Methyl Ester

trans-4′-Carboxycotinine methyl ester was dissolved in 50 mM potassium phosphate buffer (pH 7.4).

metabolism was investigated by using this method. The cotinine produced by nicotine metabolism was hardly detected by 1 μM (+)-menthofuran (Fig. 6).

Discussion

In this work, we showed to be able to quantitatively determine nicotine metabolism to cotinine by using C_{18} 5-μm ana-

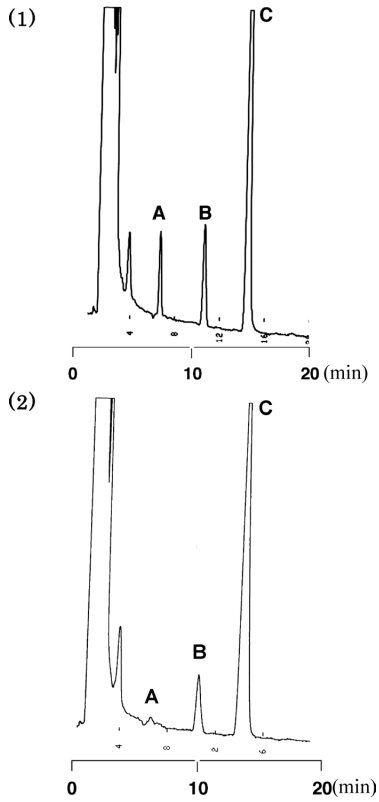

Fig. 6. Inhibition of Nicotine Metabolism by (+)-Menthofuran

Inhibitor was not added (**1**), and 1 μM (+)-menthofuran was added as an inhibitor (**2**). Peak A: Cotinine, B: *trans*-4′-carboxycotinine methyl ester, C: nicotine.

lytical column (L-column ODS, 150×4.6 mm i.d., Chemicals Evaluation and Research Institute, Japan) equipped with a C_{18} 5-μm guard column (L-column ODS, 5—4.6 mm, Chemicals Evaluation and Research Institute) on HPLC. So far, the assay method using high-performance liquid chromatography, Ketamine had been used as an internal standard before,[2,3] but this chemical was omitted from drug designation in 2007 (Ministry of Health, Labour and Welfare, Japan). Therefore, we need to find novel internal standard material. *trans*-4′-Carboxycotinine was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and measured retention time at HPLC, which was detected at 2 min. On purpose of increasing hydrophobicity, we methylated carboxyl group of *trans*-4′-carboxycotinine and obtained *trans*-4′-carboxycotinine methyl ester at high purity (over 98%). We found to be able to use *trans*-4′-carboxycotinine methyl ester as a novel internal standard (retention time of 10 min) (Fig. 5).

Nicotine is metabolized by CYP2A6 to produce the intermediate nicotine-$\Delta^{1'(5')}$-iminium ion, which is further oxidized to cotinine by cytosolic aldehyde oxidase. The cotinine detected at the concentrations range of 0.01—50 μM, and nicotine was 0.05—250 μM in the HPLC method. Detection limit of cotinine was 0.05 μM and nicotine was 0.1 μM. These results suggest that we have developed a high throughput method for simultaneous quantification of nicotine and cotinine, and this method could be useful to study the effect of nicotine metabolism to cotinine.[19]

This HPLC method gives a good resolution of nicotine metabolism within a short analysis time (20 min), and the sample preparation is very simple without need for evaporation. We hypothesized that the administration of chemicals that strongly and specifically inhibited the nicotine metabolism catalyzed by CYP2A6 activity might result in an inhibition of nicotine concentration reduction.[20,21] Some chemicals such as (+)-menthofuran, and tranylcypromine were reported to strongly inhibit CYP2A6.[3,8] One of inhibitory assay, we investigated the ability of (+)-menthofuran to inhibit nicotine metabolism on CYP2A6 using this method. As a result, the cotinine produced by nicotine metabolism was hardly blocked by 1 μM of (+)-menthofuran (Fig. 6). These results suggest that this method of nicotine metabolic assay could be useful to study the effect of nicotine metabolism to cotinine.

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