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Short communication

Improved highly sensitive method for determination of nicotine and cotinine in human plasma by high-performance liquid chromatography

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Abstract

A highly sensitive and reliable method for the determination of nicotine and its metabolite cotinine in human plasma by high-performance liquid chromatography was developed. Nicotine and cotinine were extracted from alkalinized plasma with dichloromethane and the volatility of nicotine was prevented by the addition of conc. HCl to the organic solvent during evaporation. The sensitivity of quantification at 260 nm absorption was improved by using a noise-base clean Uni-3 to 0.2 ng/ml nicotine and 1.0 ng/ml cotinine. The method was validated over linear ranges of 0.2–25.0 ng/ml for nicotine and 1.0–80.0 ng/ml for cotinine. The intra-day precision and accuracy were ≤15.9% relative standard variation (RSD) and 89.9–103.5% for nicotine and ≤8.0% RSD and 98.7–103.0% for cotinine. The inter-day precision and accuracy were ≤17.0% RSD and 94.2–100.9% for nicotine and ≤8.2% RSD and 98.0–105.1% for cotinine.

Keywords: Nicotine; Cotinine

1. Introduction

Nicotine taken up from cigarette smoking is primarily metabolized to cotinine in human liver [1]. Previously, we clarified that the enzyme responsible for the metabolism of nicotine to cotinine is cytochrome P4502A6 (CYP2A6) [2]. Recently, it has been reported that there is a whole deletion allele of the *CYP2A6* gene and that the metabolic capacities in subjects whose *CYP2A6* gene was deleted were poor [3–5]. Therefore, it is suspected that smokers with the deleted *CYP2A6* gene would have a high nicotine plasma concentration and/or negligible cotinine plasma concentration. Thus, the determination of nicotine and cotinine in human plasma or urine is of particular interest to investigators studying the pharmacokinetics of nicotine and cotinine.

Various methods for the determination of nicotine and cotinine in biological specimens have been reported in the literature including radioimmunoassay [6,7], high-performance liquid chromatography (HPLC) [8–10], gas chromatography (GC) using electron-capture detection, flame ionization detection, mass spectrometry (MS) [11–14], and liquid

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chromatography (LC)–MS [15,16]. In this study, we established and evaluated a highly sensitive HPLC procedure for nicotine and cotinine with modifications of the mobile phases, columns, extraction methods, and using a noise-base clean Uni-3.

2. Experimental

2.1. Materials

Nicotine and cotinine were purchased from Sigma (St. Louis, MO, USA). Acetanilide was purchased from Wako (Osaka, Japan). All other chemicals and solvents were of the highest grade commercially available.

2.2. Extraction procedure

For the determination of the nicotine concentration, the plasma sample (1 ml) was alkalinized by 50 μl of 10 *M* NaOH. After the addition of 10 ng of acetanilide as an internal standard, the mixture was extracted with 4 ml of dichloromethane by shaking for 10 min. After centrifugation at 1000 *g* for 10 min, 25 μl of conc. HCl was added to the organic fraction for the determination of the nicotine concentration. The organic fraction was evaporated with a vacuum evaporator at 40°C. The residue was redissolved in 100 μl of the mobile phase and then an 80-μl portion of the sample was subjected to HPLC.

For the determination of the cotinine concentration, the plasma sample (0.5 ml) was alkalinized by 25 μl of 10 *M* NaOH and extracted with 4 ml of dichloromethane by shaking for 10 min. The organic fraction was evaporated with a vacuum evaporator at 40°C without the addition of conc. HCl. The residue was redissolved in 100 μl of the mobile phase and then an 80 μl portion of the sample was subjected to HPLC.

2.3. High-performance liquid chromatography

Chromatography was performed using an L-7100 pump (Hitachi, Tokyo, Japan), an L-7400 UV detector (Hitachi), an L-7200 autosampler (Hitachi), an L-7500 integrator (Hitachi), and an 865-CO column oven (Jasco, Tokyo, Japan). The flow-rate was 1.0 ml/min and the column temperature was 35°C. The eluent was monitored at 260 nm with a noise-base clean Uni-3 (Union, Gunma, Japan). For the determination of the nicotine concentration, the analytical column was a Hichrome 5C18 (150×4.6 mm, 5 μm) column (Tomsic, Tokyo, Japan) and the mobile phase was 7% CH_3OH, 2 m*M* NaH_2PO_4, 0.2% phosphoric acid, and 1 m*M* heptane sulfonate sodium. For the determination of the cotinine concentration, the analytical column was a Capcell Pak C_{18} UG120 (250×4.6 mm, 4 μm) column (Shiseido, Tokyo, Japan) and the mobile phase was 2% CH_3OH, 2 m*M* NaH_2PO_4, 0.1% phosphoric acid, and 1 m*M* heptane sulfonate sodium. Nicotine was quantified by comparison with the standard curves using the HPLC peak height ratios to acetanilide. Cotinine was quantified by comparing the HPLC peak heights to those of authentic standard.

3. Results and discussion

3.1. Assay characteristics

Fig. 1A shows a representative chromatogram of nicotine and acetanilide. Fig. 1B and C represent chromatograms of extracts from human plasma samples before and after smoking, respectively. Fig. 1D shows a representative chromatogram of cotinine. Fig. 1E and F represent chromatograms of extracts from human plasma sample before and after smoking, respectively. None of these chromatograms showed any interfering peaks. For cotinine assay, an appropriate internal standard could not be found. However, the precision and accuracy for cotinine were enough high as described below. The simultaneous determination of nicotine and cotinine has been reported in the literature [17–19]. Similarly, these compounds could be simultaneously analyzed from human plasma samples in our preliminary analysis. However, under the condition of simultaneous analysis, the limit of quantification of nicotine was relatively higher than that in the separate analysis due to the late retention time of nicotine. Therefore, to improve the limit of quantification of nicotine, we analyzed nicotine with a separate C_{18} column and the mobile phase from cotinine.

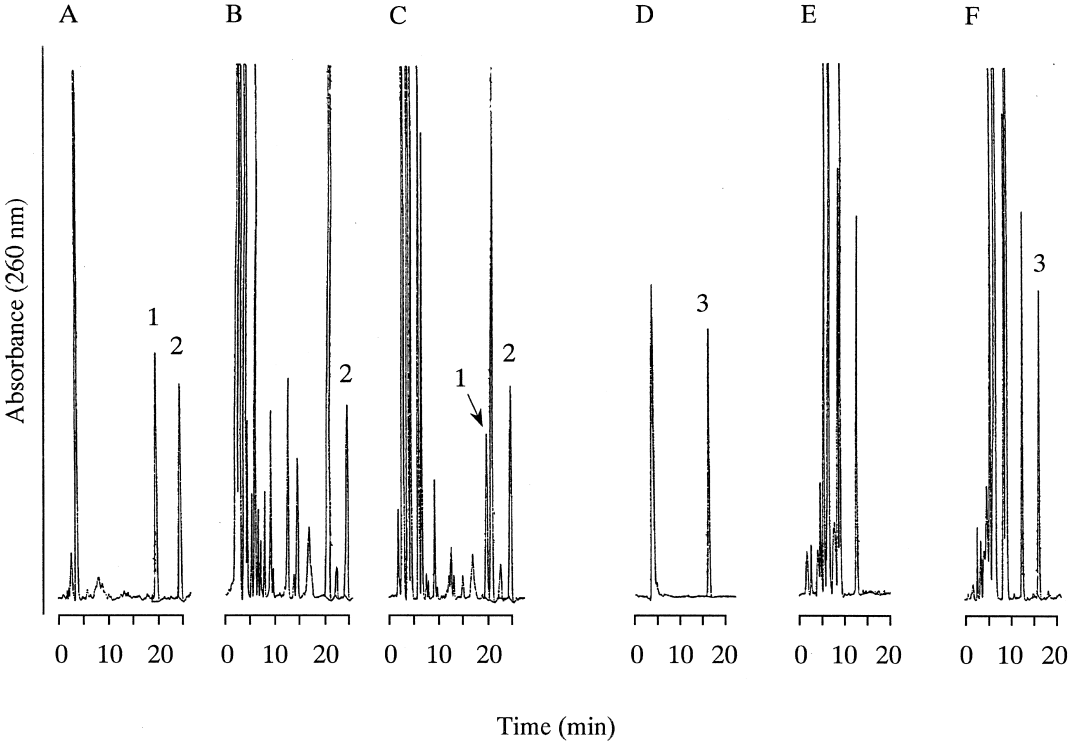

Fig. 1. Representative chromatograms of nicotine and cotinine. (A) Standard solution containing 5 ng nicotine and 5 ng acetanilide. Analysis of nicotine from a plasma sample (B) before and (C) after smoking one cigarette. (D) Standard solution containing 5 ng cotinine. Analysis of cotinine from a plasma sample (E) before and (F) after smoking one cigarette. Peaks: 1, nicotine; 2, acetanilide; 3, cotinine.

3.2. Limits of quantification, calibration curves and recoveries

The limits of quantification of nicotine and cotinine were determined to be 0.2 ng/ml and 1.0 ng/ml plasma, respectively. The limit of quantification of nicotine has been previously reported to be 10 ng/ml [8], 2 ng/ml [20], and 1 ng/ml [9,16]. The limit of quantification of cotinine has been previously reported to be 10 ng/ml [8,16] and 3 ng/ml [9]. In the present study, we modified the mobile phase to provide higher peaks of nicotine or cotinine. A noise-base clean Uni-3 can reduce the noise by integration of output and increase the signal three-fold by differentiation of output and by further amplification five-fold with an internal amplifier, resulted in 15-fold amplification of signal in maximum. Thus, the analytical procedures for nicotine and cotinine established in this study using a noise-base clean Uni-3 were more sensitive than those of previous reports. In the previous reports [9,16,21,22], many investigators extracted nicotine and cotinine from alkalinized biological specimens with dichloromethane or ether. Although nicotine is volatile under the stream, this fact has not been taken into consideration when the organic solvent was evaporated. In the present method, the volatility of nicotine during evaporation was prevented by the addition of conc. HCl to the organic solvent to make nicotine exist as a hydrochloride salt.

The calibration curves were prepared by the addition of nicotine or cotinine to blank plasma samples. The standard curve for nicotine was linear over the concentration range of 0.2–25.0 ng/ml (r^2=0.995), and that for cotinine was linear over 1.0–80.0 ng/ml (r^2=0.994). The recoveries of nicotine and acetanilide were 89.8±10.3% in the range 0.2–25.0 ng/ml and 99.8±2.2% in 10 ng/ml, respectively. The recovery of cotinine was 92.7±11.4% in the range of 1.0–80.0 ng/ml.

Table 1
Intra-day precision and accuracy for nicotine and cotinine ($n=6$)

Nicotine				Cotinine			
Theoretical concentration (ng/ml)	Mean concentration found (ng/ml)	RSD (%)	Accuracy (%)	Theoretical concentration (ng/ml)	Mean concentration found (ng/ml)	RSD (%)	Accuracy (%)
0.20	0.18	5.9	89.9	1.00	1.02	5.2	101.6
0.50	0.52	15.9	103.5	2.00	2.06	7.4	103.0
1.00	0.96	9.7	95.9	5.00	4.93	6.1	98.7
2.00	1.96	3.7	97.9	10.00	10.19	6.4	101.9
5.00	4.97	8.3	99.3	20.00	20.42	8.0	102.1
10.00	10.00	4.8	100.0	40.00	40.35	6.6	100.9
25.00	24.92	6.9	99.7	80.00	79.42	6.6	99.3

3.3. Precision and accuracy

Table 1 shows the intra-day precision and accuracy for each standard concentration. The relative standard deviation (RSD) was 3.7–15.9% and the accuracy was 89.9–103.5% in the range 0.20–25.0 ng/ml nicotine ($n=6$). The RSD was 5.2–8.0% and the accuracy was 98.7–103.0% in the range 1.0–80.0 ng/ml cotinine ($n=6$). Table 2 shows the inter-day precision and accuracy for each standard concentration. The RSD was 0.8–17.0% and the accuracy was 94.2–100.6% in the range 0.20–25.0 ng/ml nicotine ($n=6$). The RSD was 0.7–8.2% and the accuracy was 98.0–105.1% in the range 1.0–80.0 ng/ml cotinine ($n=6$). These data suggested that this HPLC method is very consistent and reliable.

3.4. Applications

This HPLC method was used to study the pharmacokinetic profiles of nicotine and cotinine in human plasma. Ten healthy volunteers smoked one cigarette (Mild Seven, containing 0.9 mg nicotine, Japan Tobacco, Tokyo, Japan) or chewed one piece of nicotine gum (Nicorette, containing 2 mg nicotine, Pharmacia & Upjohn, Tokyo, Japan) after the prohibition of smoking for >7 days. We reported the plasma concentrations of nicotine and cotinine in those volunteers [23]. In that study, we found one subject whose cotinine concentration was extremely low and whose *CYP2A6* gene was completely deleted.

4. Conclusions

A highly sensitive and reliable HPLC method for the determination of nicotine and cotinine was developed and validated. The sensitivity for nicotine and cotinine was high enough for pharmacokinetic studies after the smoking of one cigarette or the chewing of one piece of nicotine gum. This method can be used in the processing and quantification of a large series of plasma samples.

Table 2
Inter-day precision and accuracy of nicotine and cotinine ($n=6$)

Nicotine				Cotinine			
Theoretical concentration (ng/ml)	Mean concentration found (ng/ml)	RSD (%)	Accuracy (%)	Theoretical concentration (ng/ml)	Mean concentration found (ng/ml)	RSD (%)	Accuracy (%)
0.20	0.20	17.0	99.4	1.00	0.98	7.3	98.0
0.50	0.47	14.6	94.2	2.00	2.07	8.2	103.7
1.00	1.01	5.9	100.6	5.00	5.26	5.3	105.1
2.00	2.02	8.2	100.9	10.00	10.08	1.5	100.8
5.00	4.96	6.9	99.3	20.00	20.18	2.0	100.9
10.00	10.00	2.3	100.0	40.00	40.39	1.4	101.0
25.00	24.80	0.8	99.2	80.00	80.11	0.7	100.1

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