ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
20 (1999) 195–202

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Simultaneous GC–MS determination of nicotine and cotinine in plasma for the pharmacokinetic characterization of nicotine in rats

Byung Hwa Jung [a,b], Bong Chul Chung [b], Suk-Jae Chung [a], Min-Hwa Lee [a], Chang-Koo Shim [a,*]

[a] *Department of Pharmaceutics, College of Pharmacy, Seoul National University, Seoul 151-742, South Korea*
[b] *Bioanalysis and Biotransformation Research Center, Korea Institute of Science and Technology, PO Box 131, Cheongryang, Seoul 130-650, South Korea*

Received 7 September 1998; received in revised form 16 November 1998; accepted 3 December 1998

Abstract

A gas–liquid chromatography/mass spectrometry assay method was developed for the simultaneous determination of nicotine and its major metabolite, cotinine, in rat plasma. Of particular interest was improving the low and variable extraction recovery for the parent drug and the metabolite. In addition, the feasibility of this assay method for pharmacokinetic studies of nicotine and cotinine after intravenous (iv), oral, and intraperitoneal (ip) administration of 1 mg kg^{-1} of nicotine was tested. The low (30 and 48% for nicotine and cotinine, respectively) and variable (25 and 22% coefficient of variation for nicotine and cotinine, respectively) extraction recovery for nicotine and cotinine into dichloromethane was significantly improved by the addition of NaCl to the plasma. As a result, the recoveries for nicotine and cotinine were improved to 68 and 65%, respectively. The coefficient of variation was less than 10% in the 50–500 ng ml^{-1} range and less than 16.58% at 10 ng ml^{-1} for both nicotine and cotinine, indicating that the reproducibility of the assay was also improved by the extraction procedure. When injected intravenously at a dose of 1 mg kg^{-1}, the temporal profile of plasma concentration for nicotine followed a bi-exponential decline. Moment analysis revealed that pharmacokinetic parameters for nicotine (i.e. Cl, 46.30 ml min^{-1} kg^{-1}; V_{ss}, 2.77 l kg^{-1}) was similar to those reported in studies using ^{14}C-nicotine. Absolute bioavailabilties of nicotine for ip and oral administration were 87.0 and 80.4%, respectively. The concentration of the metabolite increased up to 4 h to reach C_{max} after ip and oral administrations and then declined slowly with time. These results indicate that this convenient analytical procedure is readily applicable to pharmacokinetic studies of nicotine and cotinine involving small laboratory animals with a sensitivity comparable with that reported for studies using ^{14}C-nicotine.

Keywords: Nicotine; Cotinine; Extraction; GC–MSD; Pharmacokinetics

* Corresponding author. Tel.: +82-2-880-7873; fax: +82-2-885-8429.
E-mail address: shimck@plaza.snu.ac.kr (C.-K. Shim)

0731-7085/99/$ - see front matter
PII: S0731-7085(99)00020-5

1. Introduction

Nicotine (Fig. 1A) is a major pharmacologically active component in tobacco smoke and the primary cause of tobacco addiction [1]. Current researches regarding nicotine have focused on replacement therapy, in order to alleviate withdrawal effects which are associated with smoking cessation. In addition, the therapeutic value of nicotine in the treatment of the Alzheimer's disease is currently under investigation [2].

Regardless of the potential use of nicotine, a pharmacokinetic characterization of the drug and cotinine, a major metabolite of nicotine (Fig. 1B), is necessary in order to optimize the dosage regimen of nicotine. Since pharmacokinetic studies are based on the quantitative determination of nicotine and cotinine in biological fluids, a large number of publications have appeared relative to such a determination [3–7].

In determining a level of a drug and its metabolites in biological samples, it is logical to consider an extraction with an organic solvent followed by a chromatographic separation. For the extraction of nicotine from human plasma, dichloromethane is known to be superior to other solvents [3]. However, in our preliminary studies using gas chromatography (GC) and mass spectrometry (MS), extraction recovery with dichloromethane from rat plasma was fairly low and variable for both nicotine ($30 \pm 25\%$, $n = 3$) and cotinine ($48 \pm 22\%$, $n = 3$). Poor extraction could be the result of the formation of an emulsion of plasma and dichloromethane [8]. In such a situation, the interface of the two phases becomes obscure, thereby rendering the collection of the organic phase problematic. In order to circumvent this problem, a two-step extraction (i.e. extraction with dichloromethane followed by re-extraction at a different pH using another organic solvent, such as hexane or isobutyl chloride) technique has been proposed [4]. However, nicotine contamination from the environment during the assay procedure often leads to extremely erroneous results [3–5,8], especially for samples which contain nicotine concentrations in the ng/ml level. In this sense, a single step extraction technique appears to be advantageous over a two-step extraction technique [4].

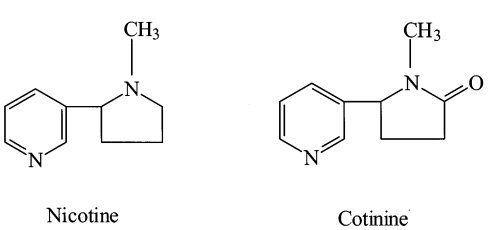

Fig. 1. Chemical structures of nicotine (A) and cotinine (B).

A separate assay method for nicotine and cotinine has also been reported [5]. This assay is essentially based on the separate extraction of nicotine and cotinine with different solvents (e.g. ether for the extraction of nicotine and dichloromethane for cotinine) at an alkaline pH. However, since routine pharmacokinetic studies generate a large number of samples, assay methods that involve a lengthy and complicated processing procedure such as separate extraction [5] are not readily applicable for routine studies. In addition to GC–MS methods, for samples collected from small experimental animals, a simultaneous assay method for nicotine and cotinine has also been reported for ^{14}C-nicotine by separation with thin layer chromatography followed by determination by liquid scintillation counting [9,10]. However, the use of radiolabeled compounds is accompanied by some risk in terms of radiation hazard, as well as difficulty in disposing wastes and the handling of radioactive compounds.

The objective of the present study was to develop a simultaneous assay method for nicotine and cotinine in rat plasma samples. We were particularly interested in simplification of the extraction procedure in order to reduce the possibility of contamination from environmental nicotine and in improving the extraction efficiency of the drug and the metabolite. As a result, we developed and validated a simultaneous GC–MS assay method for nicotine and cotinine in rat plasma using a single-step extraction technique. We report that nicotine and cotinine in the plasma can be extracted with improved efficiency and determined simultaneously in a simple, straightforward manner, with favorable accuracy and precision. This analytical method could be applied to phar-

macokinetic studies of nicotine and cotinine in the plasma following intravenous (iv), oral (po), and intraperitoneal (ip) administration of nicotine hydrogen tartarate to rats.

2. Experimental

2.1. Materials

(−)-Nicotine hydrogen tartarate, (±) d_3-nicotine salicylate, (−)-cotinine and (±) d_3-cotinine were obtained from Sigma (St. Louis, MO, USA). NaOH and NaCl were supplied from Junsei Chemical Co. (Tokyo, Japan). All solvents were HPLC grade and were used without further purification. Adult male Sprague Dawley rats, 220–270 g in body weight, were obtained from DaeHan Laboratory Animal Research Center (Eumsung, Choongbuk, Korea).

2.2. Extraction of nicotine and cotinine from rat plasma

To 200 μl of a plasma sample, 20 μl of a methanol solution containing 1 ng ml^{-1} of (±) d_3-nicotine salicylate and d_3-cotinine, internal standards for nicotine and cotinine, and 200 μl of alkalizing solution (5 M NaOH) containing varying amounts of NaCl were added and the solution mixed. The final concentration of NaCl in the aqueous plasma phase varied over a range of 0.05–1 M. Then 700 μl of dichloromethane were added to the aqueous phase followed by vortexing for 1 min and then centrifugation for 5 min at 12 000 rpm. Dichloromethane (700 μl) was added to the mixture which was then vortexed for 1 min. After removing the aqueous phase through aspiration, the remaining organic layer was collected and evaporated on a rotary vacuum evaporator and further dried by vacuum desiccation over P_2O_5/KOH for 30 min. The residue was then dissolved in methanol (25 μl) and an aliquot (2 μl) was injected onto the GC–MS system.

All glassware used in this process was acid-washed (6 M HCl at 105°C) and silanized with MSTFA (*N*-methyl-*N*-trimethylsilyl-trifluoroacetamide) to prevent nicotine adsorption from the sample and environment.

2.3. Gas chromatography (GC) and mass spectrometry (MS)

GC–MS analysis was carried out on an HP 5972 gas chromatograph, equipped with an HP 5989B Mass Engine (Hewlett Packard, USA). The separation of nicotine and cotinine from endogenous substances was performed on Hewlett Packard fused-silica capillary column (Ultra 2, cross-linked 5% phenylmethyl silicone, 25 m length × 0.2 mm ID, 0.33 μm film thickness). Temperatures of the injector, source, quadruple and transfer line were 250, 250, 100 and 300°C, respectively. The oven temperature was increased from 80 to 250°C at a rate of 15°C min^{-1}, then to 300°C at a rate of 50°C min^{-1}, and thereafter maintained at 300°C. The selected ion monitoring (SIM) mode was used in mass analysis. As selected ion, m/z of 84, 87, 98 and 101 was used for nicotine, d_3-nicotine, cotinine and d_3-cotinine, respectively. Samples were injected using the split mode with a split ratio of 10:1 via an autoinjector. The split mode injection appears to improve separation of nicotine and cotinine, and may also reduce contamination of nicotine in the MS source following the injection of subsequent samples. The contamination from the MSD source was also minimized by maintaining the source temperature at 250°C.

2.4. Pharmacokinetic study of nicotine in rats

Rats were subjected to a surgical procedure involving catheterization of the femoral artery under sodium pentobarbital (50 mg kg^{-1}) anesthesia. Rats were placed on a surgery table in a supine position. Under the anesthesia, heparin-filled (100 IU ml^{-1}) PE-50 tubing (Intramedic, Clay Adams, Sparks, MD) was gently inserted into the left femoral artery of the rat for blood sampling. For iv administration of nicotine, the adjacent femoral vein was additionally catheterized with the PE-50 tubing, and a saline solution of nicotine hydrogen tartarate (10 mg ml^{-1}) was injected at a dose of 1 ml kg^{-1} (1 mg kg^{-1} as nicotine) through the catheter. For the po administration study, a curved needle (zonde) was intubated to the stomach, and the nicotine hydrogen

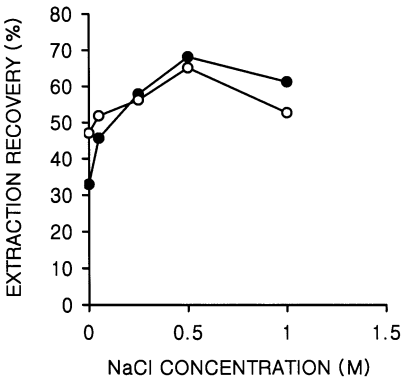

Fig. 2. Effect of NaCl concentration in the aqueous plasma phase on the extraction recovery (%) of nicotine (●) and cotinine (○) to dichlomethane. The aqueous phase was prepared by diluting the plasma sample with an equivalent volume of 5 M NaOH.

tartarate solution was administered at the same dose. For the ip administration study, the same solution was injected at the same dose into the intraperitoneal space.

Approximately 400 μl of blood samples were withdrawn from the femoral artery through the arterial catheter at 0 (predose), 5, 15, 30, 60, 90, 120, 180 and 240 min into polypropylene tubes (Sarstedt, Germany). Blood samples were centrifuged at 10 000 rpm for 1 min to separate plasma. Plasma samples were then stored at −20°C before GC–MS determination for nicotine and cotinine. Pharmacokinetic parameters including the area under the plasma concentration–time curve from time zero to infinity (AUC), systemic clearance (Cl) and distribution at steady-state (V_{ss}) were calculated according to the standard methods [15].

3. Results and discussion

3.1. Extraction of nicotine and cotinine

The addition of NaCl to plasma samples greatly improved phase separation between dichloromethane and aqueous phase. A nearly clear dichloromethane layer was separated from the aqueous phase, which contained NaCl. This observation indicates that NaCl inhibits the formation of an emulsion between dichloromethane with plasma. NaCl also influenced the extraction efficiency (i.e. recovery) of nicotine and cotinine. As shown in Fig. 2, the recovery increased from 33 up to 68% as the NaCl concentration increased from 0 to 0.5 M. Salting out, in addition to the improving separation may also contribute to the improved recovery. However, the recovery was 61% at a NaCl concentration of 1.0 M. Therefore, the extraction of nicotine and cotinine from the plasma sample in subsequent studies was carried out with NaCl added to a final concentration of 0.5 M in the aqueous plasma phase. Other procedures (e.g. extraction procedures and other handling processes) were identical to those described in Section 2.

Total ion chromatograms of GC–MS analysis are shown in Fig. 3. Blank plasma showed negligible peaks for nicotine and cotinine (Fig. 3A), indicating that contamination from the environment was below the detection limit under these assay conditions. Tables 1 and 2 show inter- and intraday variations of the assay for nicotine and cotinine. Coefficients of variations (CV%) were below 10% for 50–500 ng ml^{-1} of nicotine and cotinine, and below 20% for 10–50 ng ml^{-1} for both compounds. Both calibration curves for the compounds were linear over 10–500 ng ml^{-1} with a correlation coefficient of 0.999.

3.2. Pharmacokinetic study

Temporal profiles of plasma nicotine level following the administration of nicotine hydrogen tartarate (1 mg kg^{-1} as nicotine) through various routes are shown in Fig. 4. The plasma concentration of nicotine following iv administration declined bi-exponentially as a function of time, consistent with previous reports [9–13]. Calculated systemic clearance (Cl) and volume of distribution at steady state (V_{ss}) for nicotine are summarized in Table 3. These values were comparable to those reported by Kyerematen et al. [10], for a study using ^{14}C-labeled nicotine in rats. This observation indicates that the GC–MS assay in the present study may replace previous assay methods, and is applicable to routine pharmacokinetic studies of nicotine in small animals.

Temporal profiles of nicotine from po and ip administration routes followed an apparent mono-exponential decline with first-order absorption. Calculated pharmacokinetic parameters for the routes are also shown in Table 3, in which all the parameters appear to be consistent with Fig. 4, except for T_{max} for ip administration. It was calculated to be much larger than expected from the figure due to the profound inter-individual variation in plasma nicotine profiles of rats.

By comparing AUC values, the bioavailabilities (BA) of nicotine from po and ip routes were calculated to be 80.4 and 87.0%, respectively, indicating that nicotine is absorbed fairly well from the GI tract and the peritoneal space. In addition, relatively small T_{max} values for both administration routes indicate a rapid absorption of nicotine. Bioavailabilities of nicotine in human following po administration of nicotine tartarate salt and (*S*)-nicotine-3′,3′-D_2 were reported to be 20 [14] and 44% [6], respectively. These values are much lower than those found herein. The underlying mechanism for this discrepancy is not known. However, some degree of species difference might have contributed to the discrepancy.

Plasma profiles of cotinine following administration of nicotine hydrogen tartarate solution (1 mg kg^{-1} as nicotine) through iv, po and ip routes

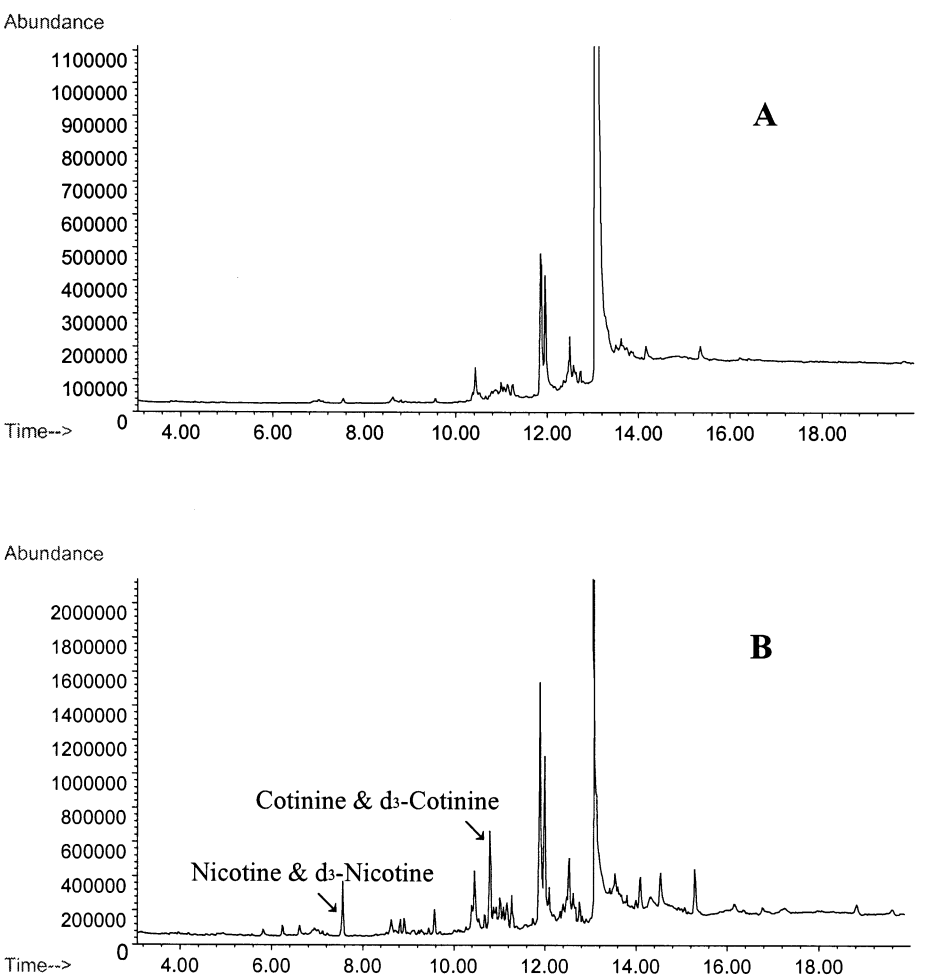

Fig. 3. GC–MSD chromatograms of nicotine and cotinine following extraction with dichloromethane: (A) blank plasma; (B) rat plasma collected at 2 h after iv injection of nicotine hydrogen tartarate at nicotine dose of 1 mg kg^{-1}.

Table 1
Within-day variation of nicotine and cotinine ($n = 4$)

Spiked concentration	Nicotine			Cotinine		
(ng ml^{-1})	Mean founded concentration (ng ml^{-1})	SD	Coefficient of variation (%)	Mean founded concentration (ng ml^{-1})	SD	Coefficient of variation (%)
10	10.30	1.45	14.10	11.35	1.83	16.17
50	45.39	3.87	8.53	47.23	2.70	5.72
100	99.77	2.73	2.74	98.36	0.88	0.90
200	212.45	11.24	5.29	200.38	6.78	3.39
300	299.14	2.36	0.79	297.59	3.33	1.12
400	395.82	7.55	1.91	399.88	2.28	0.57
500	481.17	11.62	2.41	494.68	2.46	0.50

Table 2
Day to day variation of nicotine and cotinine ($n = 4$)

Spiked concentration	Nicotine			Cotinine		
(ng ml^{-1})	Mean founded concentration (ng ml^{-1})	SD	Coefficient of variation (%)	Mean founded concentration (ng ml^{-1})	SD	Coefficient of variation (%)
10	9.96	1.39	13.95	10.13	1.68	16.58
50	47.94	1.13	2.35	46.03	3.79	8.24
100	102.40	4.52	4.41	101.29	3.93	3.88
200	198.46	6.08	3.06	202.60	1.16	0.57
300	293.87	13.57	4.62	299.11	4.24	1.42
400	408.16	12.54	3.07	407.19	10.81	2.66
500	493.42	8.43	1.71	495.69	6.79	1.37

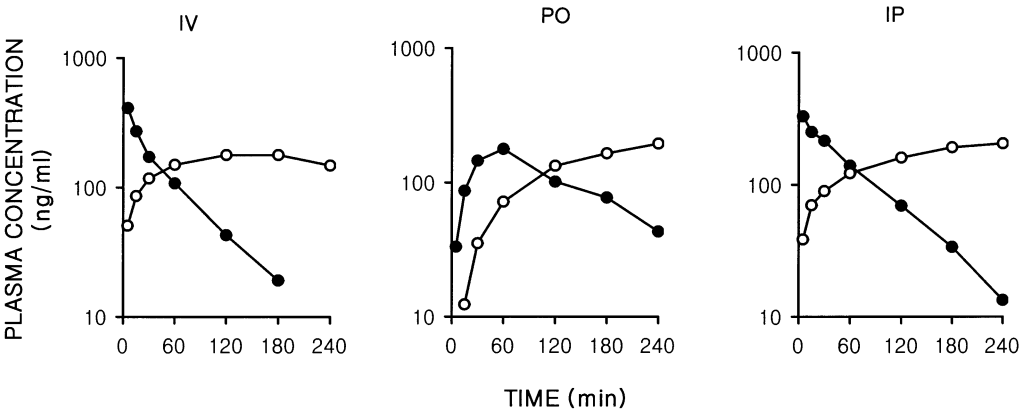

Fig. 4. Temporal profiles of nicotine (●) and cotinine (○) in plasma following iv, po and ip administration of nicotine hydrate tartarate (1 mg kg^{-1} as nicotine) to rats. Each point represents mean ± SE of four determinations.

are also shown in Fig. 4. The formation of cotinine appears rapid. The concentration of cotinine exceeded that of nicotine in 60 (for iv and ip administrations) and 120 min (for po administration) after administrations. The AUC of cotinine during 4 h, $AUC_{(0-4),}$ were comparable with those of nicotine yielding a similar AUC ratio of cotinine over nicotine regardless of the route used for administration (i.e. 0.73, 0.79, 0.69 for iv, po and ip routes). Thus, the accumulation of the metabolite should not be neglected in understanding the adverse effects of nicotine, since cotinine has been reported to play a role in depressed blood pressure, arterial thrombosis and cardiovascular disease [16,17] after the administration of nicotine.

4. Conclusion

A single step liquid–liquid extraction followed by GC–MS determination of low levels of nicotine and cotinine in rat plasma was developed and validated. The extraction of the compounds by methylene chloride from the alkalinized plasma was significantly improved (from 30 to 68%) by adding NaCl to the aqueous phase. As a result, it was possible to determine nicotine and cotinine in a plasma concentration range from 10 to 500 ng ml^{-1} simultaneously with adequate reproducibility, precision and accuracy. This method was found readily applicable to a pharmacokinetic study of nicotine and cotinine following administration of nicotine in small animals.

Table 3
Pharmacokinetic parameters (mean ± SD, $n = 4$) after iv, ip and po administration of nicotine hydrogen tartarate at a nicotine dose of 1 mg kg^{-1}

Route	iv	ip	po
Parameter			
AUC (μg · min ml^{-1})	27.7 (±9.31)	24.1 (±2.78)	22.3 (±9.65)
Cl (ml min^{-1} kg^{-1})	46.3 (±7.51)	–	–
V_{ss} (l kg^{-1})	2.77 (±0.98)	–	–
C_{max} (ng ml^{-1})	–	280.4 (±130.3)	152.1 (±78.0)
T_{max} (min)	–	21.3 (±26.3)	48.8 (±22.5)
BA (%)	–	87.0	80.4

Cotinine concentrations following iv, po and ip administration of nicotine hydrogen tartarate were comparable with those of nicotine, suggesting a potential contribution of cotinine to the adverse effects of nicotine.

Acknowledgements

This work was supported by a grant from the Research Center for New Drug Development (RCNDD) of KOSEF, Seoul National University, South Korea.

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