Int J Leg Med (1994) 106:232–236

International Journal of
Legal Medicine

Original articles

Simultaneous determination of nicotine and cotinine in various human tissues using capillary gas chromatography/mass spectrometry

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Received November 24, 1993

Summary. A reliable and sensitive method for the simultaneous determination of nicotine and cotinine concentrations in various human tissues was developed using capillary gas chromatography/mass spectrometry. Nicotine and cotinine were extracted using a 3-step solvent extraction procedure and quinoline as an internal standard. Quantification was carried out by single ion monitoring using ions of m/z 133 for nicotine, m/z 176 for cotinine and m/z 129 for quinoline. The lower limit of detection was 5 ng/g for nicotine and 10 ng/g for cotinine, in each tissue sample. The calibration curves of various tissues were linear in the concentration range from 5–1,200 ng/g for nicotine and 10–1,500 ng/g for cotinine. The accuracy and precision of this method were examined using human tissues and the results were satisfactory. The distribution of nicotine and cotinine was measured in tissues from 10 human autopsies. Nicotine was detected in every tissue examined at a level seen in habitual smokers. The nicotine concentration was high in the liver, kidney, spleen and lung, and low in adipose tissue. The cotinine level was highest in the liver. The tissue/blood concentration ratios of nicotine and cotinine were most stable in skeletal muscle, where the level of these drugs was close to that in whole blood. Skeletal muscle is, therefore, considered to be the most suitable tissue sample for toxicological examination, when acquisition of blood samples is not feasible.

Key words: Nicotine – Cotinine – Gas chromatography/mass spectrometry – Human Tissue – Distribution – Smokers' level

Zusammenfassung. Zur gleichzeitigen Bestimmung von Nikotin und Cotinin in verschiedenen Körpergeweben wurde eine zuverlässige und sensitive Methode mittels der Kapillar-Gas-Chromatographie/Massenspektrometrie entwickelt. Nikotin und Cotinin wurden durch einen 3-stufigen Extraktionsvorgang mit Chinolin als internem Standard isoliert und die Quantifizierung mittels der single ion monitoring-Technik durchgeführt, wobei für Nikotin das Ion m/z 133, für Cotinin m/z 176 und für Chinolin m/z 129 verwendet wurde. Die Detektionsgrenze lag in allen Geweben für Nikotin bei 5 ng/g und für Cotinin bei 10 ng/g, die Kalibrierung erbrachte lineare Verhältnisse im Bereich von 5–1.200 ng/g für Nikotin und im Bereich von 10–1.500 ng/g für Cotinin. Die Genauigkeit und Präzision der Methode wurde an verschiedenen Körpergeweben ausreichend bewiesen. Die Verteilung der beiden Verbindungen in verschiedenen Geweben wurde in 10 Fällen bestimmt. Die festgestellten Nikotinspiegel lagen hierbei bei Konzentrationen, die bei üblichen Tabakrauchern gemessen werden. Hohe Nikotinspiegel wurden in Leber, Niere, Milz und Lunge, niedrige Konzentrationen im Fettgewebe festgestellt. Die Cotinin-Konzentration lag in der Leber am höchsten. Das Gewebe-Blut-Verteilungsverhältnis für Nikotin und Cotinin war im Skelettmuskelgewebe am konstantesten, wobei die Konzentrationen hier jeweils nahe an den Konzentrationen im Blut lagen. Der Skelettmuskel ist somit das geeignetste Gewebe für toxikologische Untersuchungen, wenn die Asservierung von Blut nicht möglich ist.

Schlüsselwörter: Nikotin – Cotinin – Gaschromatographie/Massenspektrometrie – Menschliches Gewebe – Verteilung – Tabakraucher

Introduction

Poisoning by nicotine often occurs when victims ingest cigarettes or tobacco perorally, either by mistake or for purposes of intentional suicide. Paralysis of respiratory muscles can occur, followed by death. In addition to the

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poisoning, a forensic diagnosis is often required as to whether the patient or victim in question was a smoker. To deal with both clinical and forensic problems, a sensitive and reliable analysis of nicotine together with its major metabolite, cotinine, in body tissues is important.

Reports concerning analyses of nicotine and/or cotinine have mainly focused on the determination of these substances in biological fluids [1–7]. Chaturvedi et al. [8] reported a case of multichemical death, where only nicotine was analyzed in the blood, urine and liver. Deutsch et al. [9] described the analysis of nicotine and cotinine by gas chromatography/mass spectrometry (GC/MS) in rat plasma and brain. Thompson et al. [10] analyzed substances in mouse liver, blood, urine and carried out a pharmacokinetic study of the liver. Kintz et al. [11] examined nicotine in human hair. We have found no reports on the simultaneous analysis of these drugs in human solid tissues using GC/MS and described with detailed accuracy and precision. The distribution of nicotine in experimental animals was examined using radioimmunoassay methods [12–15], and in poisoned humans using photometric analysis [16]. Data on humans with nicotine present in tissues at the habitual smoker's level, have apparently not been documented. In forensic practice, blood is not always available in case of advanced post-mortem change, including putrefaction and here, the blood level has to be assumed following analysis of other tissues. We, therefore, attempted to set up a sensitive and reliable method to analyze both nicotine and cotinine, simultaneously, in various human tissue samples, inclusive of visceral as well as muscle tissues. The examination was also made on the distribution of nicotine and cotinine in the tissues collected from 10 autopsied humans and suitable tissue samples for toxicological analysis were given close attention.

Materials and methods

Reagents. Nicotine and quinoline were purchased from Ishizu Pharmaceutical Co., LTD. (Osaka, Japan), cotinine from Aldrich Chem. Co. tert-Butyl methyl ether and dichloromethane were purified by distillation. Sodium hydroxide and potassium carbonate used as alkalizing reagents were washed with dichloromethane immediately before use to prevent contamination by nicotine [7]. A Shimadzu CBP1 capillary column was purchased from Shimadzu Co. (Kyoto, Japan). All chemicals used in this study were of analytical reagent grade.

Biological samples. Human tissue samples were obtained at the time of autopsy from adult, Japanese patients. The autopsies were carried out 15–48 hours after death and whole blood, brain, lung, liver, spleen, kidney, skeletal muscle and adipose tissue were examined. A blank sample was obtained from a child. Outdated whole blood from a blood bank was also used as control samples. The samples were stored at –20°C until analysis.

Standard solutions of nicotine, cotinine and quinoline. Nicotine (15 mg) was dissolved in methanol and the volume was adjusted to 10 ml to give a concentration of 1,500 ng/μl. This solution was further diluted to the required concentrations. Standard solutions of cotinine and quinoline, internal standard (I.S.), were prepared in the same manner.

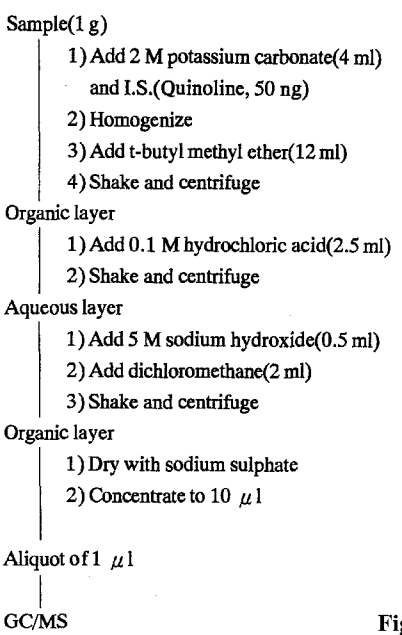

Fig. 1. Extraction procedure

Extraction procedure. The extraction procedure is summarized in Fig. 1. Approximately 1 g whole blood or tissue was weighed and homogenized in a mixture of 4 ml of potassium carbonate solution (2 M) and 0.5 μl of I.S. solution (50 ng of quinoline) in a 30-ml centrifuge tube. After a 12-ml volume of tert-butyl methyl ether was added, the preparation was shaken for 10 min and centrifuged at 850 g for 10 min. The solvent layer was transferred into a 30-ml centrifuge tube containing 2.5 ml of 0.1 M hydrochloric acid. The mixture was then shaken and centrifuged at 850 g for 10 min (back-extraction). The aqueous layer was transferred into a 10-ml centrifuge tube and made alkaline by adding 0.5 ml of 5 M sodium hydroxide solution. To the solution was added 2 ml of dichloromethane and the preparation was shaken for 10 min (re-extraction). After centrifugation, the solvent layer was dried over sodium sulphate and concentrated to approximately 10 μl by evaporation at 30°C under a gentle stream of nitrogen. A 1 μl aliquot of the solution was injected into the gas chromatograph-mass spectrometer.

To avoid external contamination with nicotine, smoking was strictly forbidden in the laboratory, and all reagents were purified before use.

GC/MS conditions. The apparatus used was a Shimadzu QP-1000 gas chromatograph-mass spectrometer, a quadrupole type with EI mode, equipped with a multi-ion detector. The fused-silica capillary column (12 m × 0.53 mm I.D., 1.0 μm film thickness) was coated with Shimadzu CBP1 bonded methylsilicone stationary phase. Helium was used as the carrier gas, at a flow rate of 40 ml/min. The operating temperatures were as follows: columns 90–220°C (40° C/min), injection port 240°C, separator 250°C, ion source 270°C. The ionization energy was 20 eV. Multi-ion detectors were set to the ions at m/z 133 (nicotine), 176 (cotinine) and 129 (quinoline).

Preparation of calibration curves. Calibration curves were prepared by adding the standard solutions of nicotine and cotinine to the tissue samples at concentrations of 5–1,200 ng/g for nicotine and 10–1,500 ng/g for cotinine, containing I.S. of 100 ng/g. The samples were extracted at each concentration in the same manner. Calibration curves were obtained by plotting the peak area ratio of nicotine (or cotinine) to I.S. (quinoline) versus the amount of nicotine (or cotinine), measured by a data system built into the gas chromatograph/mass spectrometer.

Distribution of nicotine and cotinine in autopsied human tissues. Human tissues obtained at autopsy were analyzed to examine the

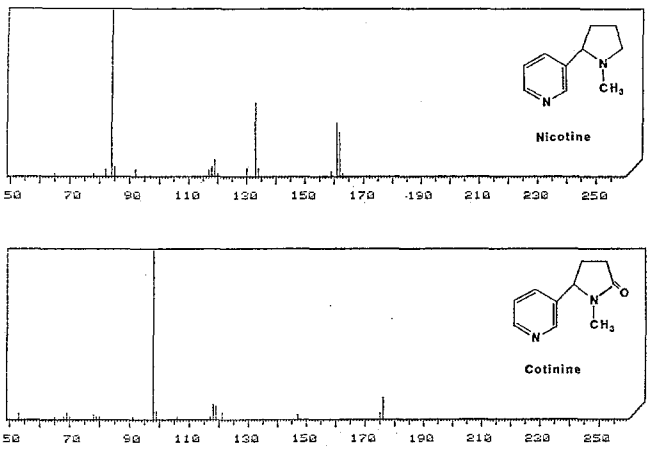

Fig. 2. Mass spectra of nicotine and cotinine

distribution of nicotine and cotinine. Whole blood and liver were analyzed for confirmation of the presence of nicotine and cotinine. As 10 of the 20 subjects were found to be tobacco smokers, tissue distribution in these 10 was examined. Tissues analyzed were whole blood, brain, lung, liver, spleen, kidney, abdominal and thigh muscles and adipose tissue.

Results and discussion

Extraction procedure

Sodium hydroxide is usually used as the alkalizing reagent at the first step of extraction step [2, 3, 5–7, 9, 10]. This reagent, however, is not suitable for solid tissue samples, especially in the case of adipose tissue, because a dense emulsion forms. We, then, examined various alkalizing reagents, and potassium carbonate used for plasma and urine analysis [4] was found to be suitable. Sodium hydroxide and potassium carbonate can be used for the re-extraction step.

Determination of nicotine and cotinine

The mass spectra of nicotine and cotinine obtained by GS/MS are shown in Fig. 2. Nicotine showed ions at m/z 84 (base peak), 133 (relative abundance, 50%) and 162 (molecular peak), cotinine at m/z 96 (base peak) and 176 (molecular peak). Based on analysis of tissue extracts, ions at m/z 133 for nicotine and m/z 176 for cotinine were determined to be appropriate for single ion monitoring (SIM) mass spectrometry. The SIM chromatograms of the extracts from tissues containing 100 ng each of nicotine, cotinine and I.S. are shown in Fig. 3. Each peak was clearly separated on the chromatograms. Few or no interfering peaks appeared in the chromatograms of the nicotine-free human tissues used as blank tissues.

Calibration curves of nicotine and cotinine

The standard calibration curves were linear in the concentration range 5–1,200 ng/g for nicotine and 10–1,500 ng/g for cotinine in human tissues. Correlation coefficients, r^2, in all cases were better than 0.99. The calibration curves of the tissues tested were similar except that of brain, the slope of which was nearly twice as high. The brain level, therefore, should be calculated using a separate calibration curve. The lower limit of detection was 5 ng/g for nicotine and 10 ng/g for cotinine, in every tissue sample examined.

Recoveries and precision

Recoveries of nicotine and cotinine were determined by comparing the peak area of the sample to that of the saline solution spiked with 100 ng each of nicotine and cotinine. The calculated recoveries were 87.0–105.5% for nicotine and 92.9–103.2% for cotinine. The precision and accuracy of the method were evaluated in a whole blood sample, at 4 different concentrations, 5, 20, 110, 1,100 ng/g for nicotine and 10, 50, 400, 1,000 ng/g for cotinine. The coefficient of variation for nicotine ranged from 3.4 to 10.1% for the within-day and from 3.6 to 8.5% for the between-day precision, and for cotinine from 2.6 to 8.6% and from 2.7 to 13.6% respectively. The precision of this method in various human tissues is summarized in Table 1. The coefficient of variation of nicotine (60 ng/g) ranged from 1.9 to 8.5% and that of cotinine (100 ng/g) from 2.2 to 7.9%.

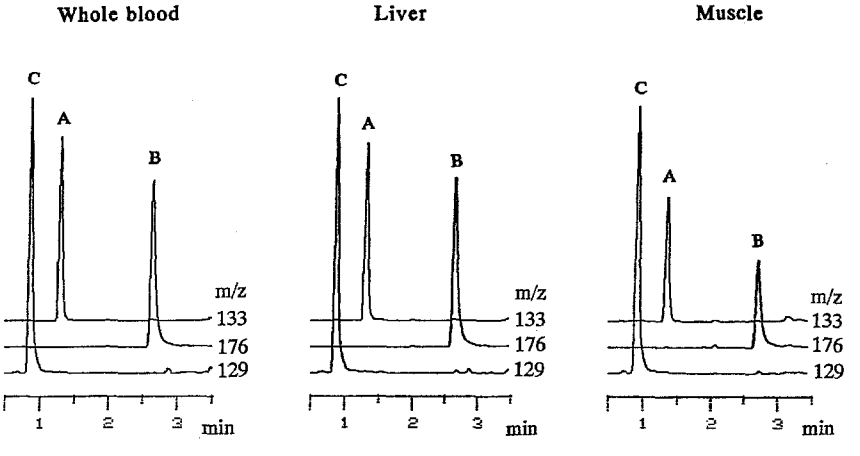

Fig. 3. SIM chromatograms of extracts from whole blood, liver and skeletal muscle containing 100 ng/g each of nicotine (A), cotinine (B) and quinoline (C)

Distributions of nicotine and cotinine in autopsied human tissues

The amounts of nicotine and cotinine in tissues of 10 autopsied human bodies are shown in Table 2. Nicotine concentrations in the whole blood ranged from non-detectable to 36.2 ng/g and those for cotinine from 74.2 to 272.6 ng/g, thus the nicotine concentration in the blood was considered to be at a level of a habitual smoker [17]. As nicotine and cotinine are always present in the tissues of non-smokers originating from environmental tobacco smoke or from dietary vegetables [18], interpretation of the values must be made with caution.

The concentrations of nicotine in the liver, kidney, spleen and lung were relatively high and were similar to those in the brain and muscle, but low in the adipose tissue, as compared to findings in whole blood.

These results are similar to findings in monkey and dog [14] and in cases of nicotine poisoning [16]. The highest concentration of cotinine was found in the liver, an organ in which nicotine is probably oxidized. The concentration of cotinine was also lowest in adipose tissue. Nicotine has a short half-life and was not detected, in a few cases, in the blood, muscle and adipose tissue. Nicotine derived from the environment may contaminate stored tissues. To confirm nicotine intake, analyses of cotinine in the liver as well as nicotine in the blood are recommended.

The tissue/blood concentration ratios of nicotine and cotinine are shown in Fig. 4. The ratios of nicotine in the liver, kidney, spleen and lung showed a wide variation. On the other hand, the ratios of abdominal and thigh muscles were stable, presenting values close to 1. The ratios of nicotine and cotinine varied widely in the liver. With respect to other tissues, the lung, abdominal and thigh muscle showed stable ratios and the values were close to 1.

Based on the above data, skeletal muscle is considered to be the most suitable tissue sample to determine concentrations of nicotine and cotinine, when blood is not available. This result is consistent with reports

Table 1. Precision and accuracy for assessing nicotine and cotinine concentrations in tissues

Tissue	Nicotine		Cotinine	
	Determined (mean ± S.D.) (ng/g)	C.V. (%)	Determined (mean ± S.D.) (ng/g)	C.V. (%)
Whole blood	58.75 ± 1.57	2.7	104.23 ± 2.31	2.2
Brain	62.95 ± 3.99	6.3	107.59 ± 5.31	4.9
Lung	64.29 ± 1.21	1.9	112.13 ± 6.33	5.6
Liver	58.35 ± 3.48	6.0	107.82 ± 6.81	6.3
Spleen	57.09 ± 1.45	2.5	107.07 ± 8.43	7.9
Kidney	64.44 ± 5.32	8.3	104.51 ± 3.97	3.8
Adipose	56.99 ± 4.83	8.5	95.54 ± 4.34	4.5
Skeletal muscle	56.95 ± 2.12	3.7	106.34 ± 4.01	3.8

Added amount: nicotine 60 ng/g, cotinine 100 ng/g, $n = 5$

Table 2. Concentrations of nicotine (cotinine) in human autopsy tissue samples (ng/g)

Tissues	Case No.									
	1	2	3	4	5	6	7	8	9	10
Blood	32.8 (272.6)	N.D. (74.2)	11.2 (99.7)	12.6 (115.2)	27.7 (270.3)	N.D.(106.5)	33.1 (193.8)	36.2 (239.8)	36.2 (241.2)	26.2 (112.6)
Brain	44.6 (115.8)	N.D. (56.5)	1.1 (116.4)	6.5 (108.9)	7.6 (166.6)	31.2 (111.4)	32.2 (264.8)	32.6 (295.6)	54.4 (365.2)	89.7 (88.4)
Lung	38.4 (287.1)	12.3 (78.0)	20.9 (164.5)	8.6 (75.3)	28.1 (210.1)	49.2 (143.1)	184.0 (332.4)	64.3 (189.0)	66.9 (229.2)	83.4 (139.0)
Liver	126.2 (1493.8)	17.5 (411.0)	13.9 (349.0)	26.4 (259.8)	51.8 (470.2)	58.0 (884.6)	325.0 (1586.1)	103.3 (1028.3)	73.2 (708.8)	135.3 (504.5)
Spleen	69.8 (273.3)	10.4 (172.9)	10.4 (104.5)	24.7 (130.7)	29.1 (211.2)	17.8 (82.9)	86.9 (299.0)	96.3 (241.0)	71.3 (191.2)	139.8 (115.8)
Kidney	67.4 (303.8)	6.8 (97.2)	13.4 (95.7)	24.3 (103.8)	35.6 (214.8)	52.2 (274.1)	104.8 (304.9)	73.0 (230.6)	59.5 (233.8)	164.9 (173.3)
Abdominal Ms	32.1 (321.6)	N.D. (58.3)	12.2 (95.7)	12.4 (90.4)	20.5 (145.2)	N.D.(152.2)	3.3 (198.2)	30.0 (130.5)	N.D.(216.5)	51.7 (130.5)
Thigh Ms	35.0 (302.7)	9.6 (66.9)	4.7 (74.2)	10.5 (94.6)	18.9 (164.8)	N.D.(107.3)	N.D. (178.2)	32.1 (167.3)	25.1 (169.4)	33.7 (112.0)
Adipose	N.D. (N.D.)	N.D. (9.2)	N.D. (8.0)	N.D. (29.6)	1.5 (46.6)	5.4 (N.D.)	N.D. (65.8)	N.D. (26.8)	N.D. (40.3)	25.7 (23.7)

N.D.: not detectable

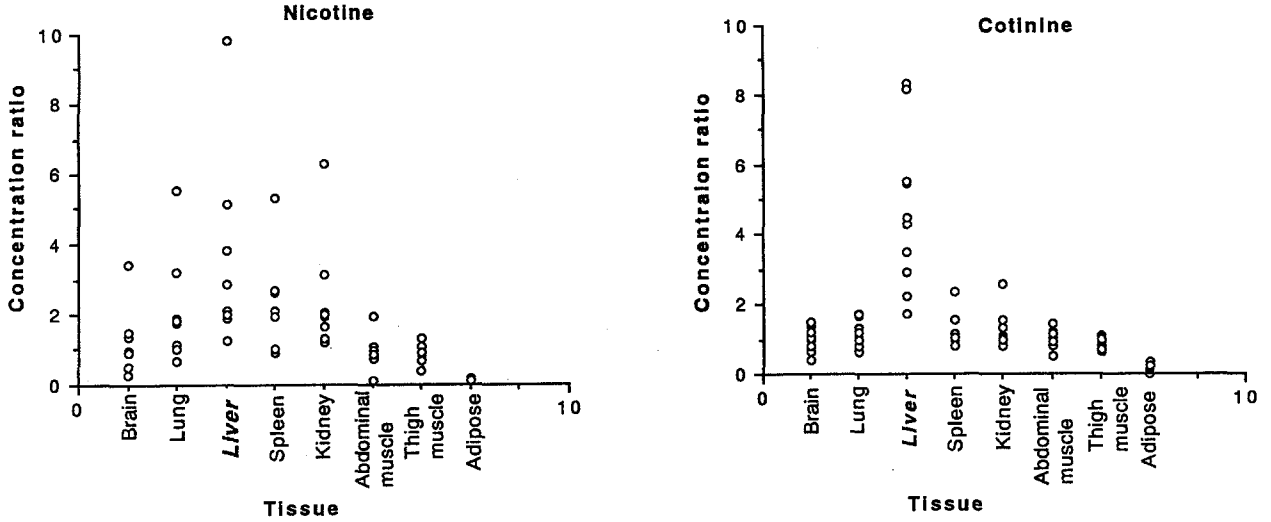

Fig. 4. The tissue/blood concentration ratios of nicotine and cotinine in human tissues

on methamphetamine [19], thinner [20] and triazolam [21].

Conclusion

A sensitive and reliable method was developed for the simultaneous determination of nicotine and cotinine in human tissue samples using GC/MS, at the nanogram level.

The findings of nicotine and cotinine distribution in human body tissues showed that skeletal muscle was the most suitable sample to use when blood samples are not available.

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