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Electron-impact and chemical ionization detection of nicotine and cotinine by gas chromatography–mass spectrometry in rat plasma and brain

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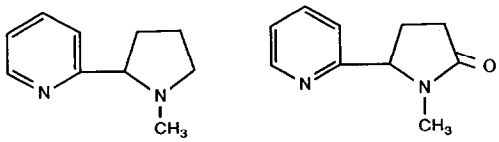
ABSTRACT

Nicotine and its metabolite, cotinine, were measured in rat plasma and brain by gas chromatography–mass spectrometry. Both agents were extracted from plasma and brain, separated on a capillary column, and quantified by single-ion monitoring. The major fragment ions of nicotine and cotinine at *m/z* 84 and *m/z* 98, respectively, were monitored by electron-impact ionization detection and the protonated molecular ions at *m/z* 163 and *m/z* 177, respectively, were monitored by chemical ionization detection. Both compounds were quantified using deuterium-labeled nicotine and cotinine, respectively, as internal standards.

INTRODUCTION

Nicotine (Fig. 1) is a naturally occurring alkaloid obtained from the tobacco plant. Due to its widespread use, its pharmacokinetics in rats [1–3], dogs [4] and humans [5] have been reported. Nicotine stimulates a subclass of acetylcholine receptors in brain [6], and may have therapeutic value in the treatment of Alzheimer's disease, which involves degeneration of basal forebrain cholinergic neurons. To ameliorate symptoms of this disorder, potential therapeutic agents, such as nicotine, must be administrated at an optimal dose to be effective. A rapid and simple assay for nicotine and its metabolite, cotinine (Fig. 1), might facilitate such therapy by combining pharmacodynamic observations with pharmacokinetic measurements in human subjects.

High-performance liquid chromatography (HPLC) [7,8] and gas chromatography (GC) [9–11], have been widely employed for the determination of nicotine and cotinine. The most sensitive methods, however, use gas chromatography–mass spectrometry (GC–MS) [3,5,12–16]. We wished to develop a simple assay to quantitate nicotine and cotinine in plasma and brain tissue with sufficient sensitivity to determine their pharmacokinetics in rodents prior to human studies.



a Nicotine b Cotinine

Fig. 1. Structures of nicotine (a) and cotinine (b).

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We report here a rapid GC–MS method in which nicotine and cotinine are extracted from biological samples and quantified together, using deuterium labeled (N-methyl-d_3) nicotine and cotinine as internal standards.

EXPERIMENTAL

Materials

Deuterium-labeled (N-methyl-d_3) nicotine and cotinine were obtained from MSD Isotopes (Montreal, Canada). Nicotine hydrogen(+)tartrate was from BDH (Poole, UK). (−)-Cotinine was from Sigma (St. Louis, MO, USA). All solvents were of HPLC grade. Adult male Fischer-344 rats, three months old and approximately 240 g weight, were from Charles River Breeding Labs. (Wilmington, MA, USA).

Plasma extraction

Plasma (1.0 ml), spiked with deuterium-labeled nicotine (20–100 ng) and deuterium-labeled cotinine (200 ng), was treated with 5 *M* NaOH solution (0.5 ml) and extracted with *n*-butyl chloride (3.0 ml). The *n*-butyl chloride extract was acidified with 0.2 *M* HCl (0.5 ml), and the organic solvent was removed. To the remaining aqueous phase, 5 *M* NaOH solution (100 μl) was added, and it was extracted with chloroform (50–100 μl). After centrifugation the lower phase (1–3 μl) was injected into the GC–MS system. At concentrations below 20 ng/ml, the chloroform was removed in a centrifugal evaporator under vacuum. The remaining material was redissolved in toluene (20 μl), and 1–3 μl were injected into the GC–MS system.

Brain extraction

Sonicated brain (Heat Systems-Ultrasonics, Farmingdale, NY, USA) (1.0 g), spiked with deuterium-labeled nicotine (100–200 ng) and deuterium-labeled cotinine (200 ng), was treated with 5 *M* NaOH solution (0.5 ml) and extracted with *n*-butyl chloride (3.0 ml). The *n*-butyl chloride extract was acidified with 1 *M* HCl (0.5 ml) and the organic phase was removed. To the remaining solution, 5 *M* NaOH (100 μl) was added, and then it was extracted with chloroform (50–100 μl). After centrifugation, 1–3 μl of the lower phase were injected into the GC–MS system.

Chromatography

Samples were injected onto an HP 5890 gas chromatograph (Hewlett Packard, Avondale, PA, USA), equipped with a Hewlett Packard fused-silica capillary column of cross-linked 5% phenylmethylsilicone, 25 m × 0.20 mm I.D., 0.33 μm film thickness. Samples were injected, with splitless valve on, for 0.8 min and were run with the oven temperature at 80°C for 1 min followed by a 50°C/min increase up to 250°C. The temperature of the injector and detector were 250 and 280°C, respectively. The retention time of nicotine was 5.1–5.8 min and of cotinine 6.3–7.0 min, at 35 kPa helium pressure on the top of the column.

Mass spectrometry

GC–MS measurements were carried out on an HP 5971A (Hewlett Packard) mass-selective detector. For electron-impact ionization detection the selective-ion monitor (SIM) device was set to monitor the ions *m/z* 84, 87, 98 and 101. For chemical ionization (CI) detection the ion source was replaced with a Hewlett Packard CI ion source. The CI was performed with ammonia at 4.6–5.3 Pa and the SIM was set to monitor the ions *m/z* 163, 166, 177 and 180.

Animal studies

Rats were injected intraperitoneally with nicotine hydrogen(+)tartrate, 1.0 mg/kg body weight, in distilled water (1.0 ml/g). At times between 5 and 120 min, two animals per time point were killed by decapitation. Blood was collected, centrifuged (10 000 *g*, 60 s), and plasma and brain samples were immediately mixed with 0.2 *M* HCl (0.6 ml/ml or ml/g) and stored at −70°C.

RESULTS

Yields and stability

n-Butyl chloride extraction of alkalinized blank rat plasma and brain that were spiked with

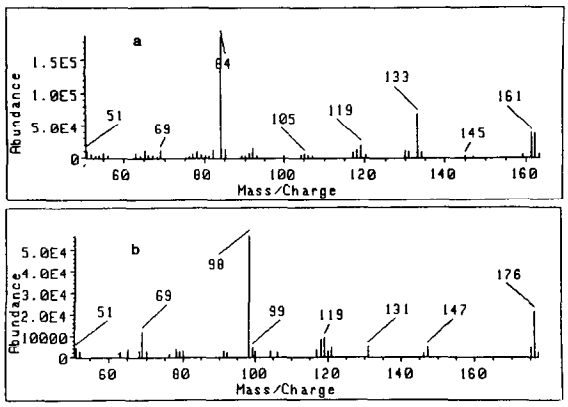

Fig. 2. Electron-impact mass spectra of nicotine (a) and cotinine (b).

nicotine base yielded 95–98% extraction from plasma and 80–85% extraction from brain. The extraction yields of cotinine from plasma and brain were 58 and 61%, respectively. Stability studies of nicotine in plasma and brain stored at −70°C demonstrated a loss of approximately 10% within 24 h. Acidification of plasma and brain with 0.2 *M* HCl solution reduced this loss to less than 5% over one week. Stability of cotinine was poor. Cotinine plasma and brain levels (100 ng/ml or 100 ng/g) were undetectable after one week at −70°C.

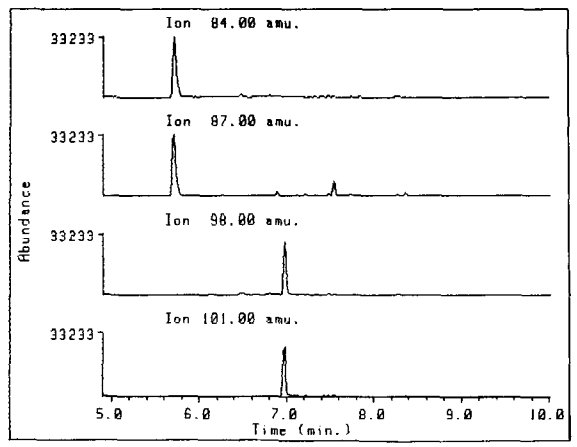

Fig. 3. EI SIM profiles obtained from 1.0 g of brain spiked with nicotine, cotinine and the deuterium-labeled (N-methyl-d_3) analogues (50 ng each). Upper is due to nicotine (*m/z* 84), whereas lower profiles are due to deuterium-labeled nicotine (*m/z* 87), cotinine (*m/z* 98), and deuterium-labeled cotinine (*m/z* 101).

Mass spectrometry

EI of nicotine produced a minor molecular ion at *m/z* 162 and a large peak at *m/z* 84 (Fig. 2a). Both peaks were shifted by three units to *m/z* 87 and 165, respectively, when deuterium-labeled (N-methyl-d_3) nicotine was measured. The mass spectrum of cotinine (Fig. 2b) showed a molecular ion at *m/z* 176 and a large peak at *m/z* 98. Both peaks were shifted by three units when the deuterium-labeled cotinine was measured. The EI SIM profiles obtained from extracted brain, spiked with nicotine, cotinine and the deuterium-labeled (N-methyl-d_3) nicotine and cotinine (50 ng/g each), are shown in Fig. 3.

CI of nicotine and cotinine produced protonated molecular peaks at *m/z* 163 and 177, respectively. Both peaks were shifted by three units to *m/z* 166 and 180, respectively, when the deuterium-labeled analogues were measured. Relative to the EI, the molecular ions appeared at a higher mass range, yielding more selectivity, but the ion yields were slightly lower. The CI SIM monitoring profiles obtained from brain (1.0 g) spiked with nicotine, cotinine and the deuterium-labeled (N-methyl-d_3) analogues (50 ng/g each) are shown in Fig. 4.

Detection in plasma

The estmated limits of detection in plasma (based upon a signal-to-noise ratio of 2:1) using EI or CI were 1 ng/ml for nicotine and 10 ng/ml for cotinine. The limit of quantitation was defined by a mean accuracy between 95 and 105% and a coefficient of variation (standard deviation/mean) lower than 10%. The estimated limits of quantitation in plasma using EI or CI were 5 ng/ml for nicotine and 50 ng/ml for cotinine. Two standard curves were constructed by spiking 1.0 ml aliquots of plasma: (a) 1–10 ng nicotine and deuterium-labeled nicotine (20 ng) and (b) 50–300 ng nicotine and cotinine and deuterium-labeled nicotine and cotinine (100 ng). The linear regression coefficient of each standard curve was 0.99. The coefficient of variation for five replicate measurements of nicotine (100 ng/ml) was 5.0% and for cotinine (100 ng/ml) was less than 10%.

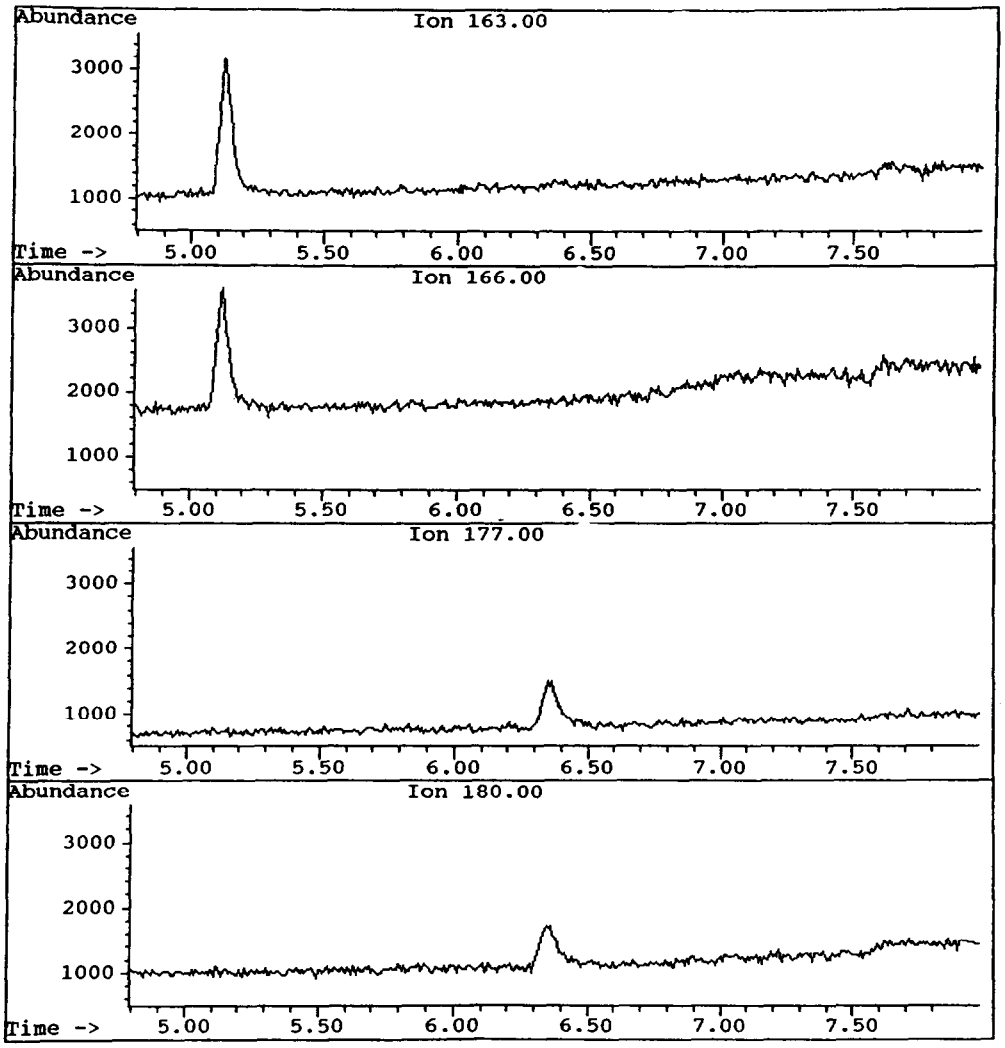

Fig. 4. CI SIM profiles obtained from 1.0 g of brain spiked with nicotine, cotinine and the deuterium-labeled (N-methyl-d_3) analogues (50 ng each). Upper is due to nicotine (*m/z* 163), whereas lower profiles are due to deuterium-labeled nicotine (*m/z* 166), cotinine (*m/z* 177), and deuterium-labeled cotinine (*m/z* 180).

Detection in brain

The estimated limits of detection in brain (signal-to-noise = 2:1) using EI or CI were 5 ng/g for nicotine and 15 ng/g for cotinine. The estimated limits of quantitation (defined above) in brain using EI or CI were 10 ng/g for nicotine and 50 ng/g for cotinine. Two standard curves were constructed by spiking 1.0-g samples of brain from untreated rats: (a) 10–50 ng of nicotine and deuterium-labeled nicotine (50 ng) and (b) 50–300 ng of nicotine and cotinine and deuterium-labeled nicotine and cotinine (100 ng). The linear regression coefficient of each standard curve was 0.99. The coefficient of variation for five replicate measurements of nicotine (100 ng/g) was 5.4% and for cotinine (100 ng/g) was less than 10%.

Animal pharmacokinetics

A single dose of nicotine hydrogen(+)tartrate was administered intraperitoneally to rats, and plasma and brain concentrations were measured at times between 5 and 120 min thereafter. Time-

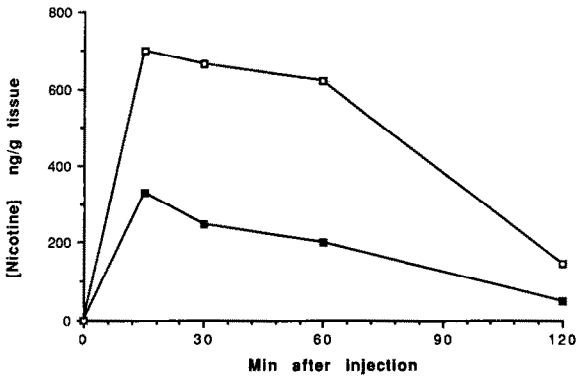

Fig. 5. Plasma (■) and brain (□) concentration *versus* time profiles of nicotine following intraperitoneal administration to rats of 1.0 mg/kg (mean of two samples per time point).

dependent plasma and brain concentration profiles are shown in Fig. 5. Plasma levels declined from a peak level of 340 ng/ml at 15 min to 40 ng/ml at 120 min. Concentrations in brain reached 700 ng/g at 15 min and fell to 200 ng/g by 120 min. The brain concentration was greater than the plasma concentration at all time points. No cotinine was detected.

DISCUSSION

This report describes a sensitive GC–MS assay for nicotine and its metabolite, cotinine, that is suitable for plasma and brain pharmacokinetic studies. Nicotine and cotinine were extracted together from plasma and brain, chromatographed, and the resulting GC peaks were monitored with a mass-selective detector either in EI or CI mode. For nicotine, in the EI mode, the most abundant peak was at m/z 84. This peak was shifted to m/z 87 in the deuterium-labeled (N-methyl-d_3) nicotine mass spectrum, indicating that the fragment ion contains the pyrrolidine moiety. Cotinine (Fig. 1b), the oxidation product of nicotine in which two hydrogen atoms are replaced by an oxygen atom, showed in its spectrum a corresponding peak at m/z 98, that was shifted by three units for the deuterium-labeled cotinine. The CI method, compared to EI ionization, gave lower ion yields, but the molecular ions appeared at a higher mass range, resulting in greater selectivity. Although others [14] have speculated that CI might be superior to EI for detection of these compounds, we did not find it to be so.

The present detection procedure for nicotine in plasma is similar or superior in sensitivity (1.0 ng/ml) to that of previous assays [3,5,7–16]. Most prior methods were developed for analysis of biological fluids and are not readily adaptable to tissue homogenates [14]. The present assay is more fully described than a previously reported assay for nicotine in brain tissue [3]. The use of *n*-butyl chloride combined with the back-extraction procedure yielded comparable sensitivity for nicotine (10 ng/g) in rat brain extracts as for plasma extracts.

Of note are the poor stabilities of nicotine and cotinine in untreated frozen brain and plasma, which have not been previously reported. By acidification of plasma and brain, the stabilities of nicotine and cotinine can be improved but, in our hands, cotinine remains unstable and is lost within one week at −70°C. Hence the primary problem in quantitation of these compounds in biological fluids and tissues may be their limited stability, rather than the sensitivity of the assay method.

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