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DETERMINATION OF COTININE IN BIOLOGICAL FLUIDS BY CAPILLARY GAS CHROMATOGRAPHY—MASS SPECTROMETRY—SELECTED-ION MONITORING

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SUMMARY

A sensitive and selective method for the determination of cotinine in plasma and urine is presented. Quantitation is effected by capillary gas chromatography—mass spectrometry after liquid—liquid extraction of 0.25—1 ml of biological specimens with a trideuterated cotinine internal standard. The procedure is linear and has acceptable precision over the range of concentrations encountered in pharmacokinetic studies of nicotine or cotinine. The suitability of the assay is shown by a number of plasma concentration—time curves after a single oral or intravenous administration of cotinine to a human volunteer and after multiple-dose intravenous administration of nicotine

INTRODUCTION

Cotinine [5-(3′-pyridyl)-1-methylpyrrolidone-2] is a major phase I metabolite of nicotine and has been used as an indicator of overall cumulative cigarette smoke intake [1]. In order to undertake a collaborative pharma-

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cokinetic study necessitating the analysis of cotinine in a large number of biological specimens, a gas chromatographic—mass spectrometric (GC—MS) method with selected ion monitoring (SIM) has been developed. Apart from a radioimmunoassay [2] and a few high-performance liquid chromatographic techniques [3—5], a variety of GC methods have been described for the determination of cotinine in biological samples. A summary of these methods is given in Table I. Most techniques involve the simultaneous measurement of cotinine and nicotine. GC is usually combined with nitrogen-selective detection [7—14], which minimizes interference from extraneous compounds in the organic extracts. More recently, capillary GC has been introduced [12—15]. Capillary columns provide a much higher separation efficiency than standard packed columns, allowing time-consuming clean-up procedures and back-extractions and hence losses of analyte to be avoided. Thompson et al.

TABLE I

COMPARISON OF QUANTITATIVE GC METHODS FOR THE DETERMINATION OF COTININE IN BIOLOGICAL SAMPLES

Detection*	Sensitivity (sample size, final dilution)	Internal standard	Ref.
FID	N.M.** (5 ml urine, 50 μl dichloromethane)	Lignocaine	6
NPD	1 6 ng/ml of blood (3 ml blood or 0 5 ml urine, 10 μl ethyl acetate)	Diphenylamine	7
NPD	0.1 ng/ml of plasma (1 ml plasma, 10 μl acetone)	Lidocaine	8
NPD	N.M. (1 ml plasma, 50 μl acetone)	Pheniramine	9
NPD	<5 ng/ml of plasma (1 ml blood, plasma or urine, 50 μl ethyl acetate)	N-(2-Methoxyethyl)-norcotinine	10
NPD	5 ng/ml of plasma (1 ml plasma, 30 μl methanol)	Ketamine	11
NPD	0.4 ng/ml of plasma*** (1 ml plasma, 20 μl ethanol)	N-Ethylnorcotinine	12
GC—MS§	2—3 ng/g of tissue (1 g tissue, ca. 30 μl ethyl acetate)	[$^3H_3$]Cotinine	13
NPD§§	2—3 ng/g of tissue (1 g tissue, ca. 30 μl ethyl acetate)	1-Methyl-6-(3-Pyridyl)-2-piperidone	13
NPD	5 ng/ml of plasma (0.5 ml plasma, 60 μl isoamyl alcohol)	Ketamine	14
FID	30 ng/ml of urine; 15 ng/ml of plasma [2 ml plasma, 100 μl diethyl ether—dichloromethane (1 4)] (4 ml urine, 1 ml same mixture)	Lidocaine	15

*FID = flame-ionization detection; NPD = nitrogen—phosphorus detection.
**N.M. = Not mentioned.
***A micromethod for cotinine in 0.1 ml of plasma is also described
§On a packed column, in the chemical ionization mode.
§§On a capillary column.

[13] examined the GC—MS analysis of cotinine in tissue homogenates using a column packed with 3% OV-22. They showed that the use of electron-impact conditions was not sufficiently selective for quantitating cotinine in tissue extracts. In the chemical ionization mode, however, good sensitivity and selectivity were achieved. The SIM technique is a superior method of detection and identification, particularly when it is combined with capillary GC. This paper describes a technique that allows a simple and rapid work-up procedure and yet results in excellent sensitivity and specificity.

EXPERIMENTAL

Chemicals and reagents

Cotinine of high purity (>99.7%) used in the development of this method was donated by Dr. G. Stungis of the Research Department of Brown and Williamson Tobacco Corp. (Louisville, KY, U.S.A.). Residue-quality dichloromethane was purchased from E. Merck (Darmstadt, F.R.G.). All other solvents were of analytical-reagent grade. 3-Pyridinecarboxaldehyde, ethyl acrylate and [$^3H_3$]methylamine hydrochloride (>98% ^3H) were obtained from Janssen Chimica (Beerse, Belgium). Sodium cyanoborohydride was purchased from Fluka (Buchs, Switzerland).

Glassware

All glassware was thoroughly cleaned by normal laboratory procedures, rinsed with ethanol and silylated prior to use.

Synthesis of the internal standard

3-[$^3H_3$]Methylcotinine was synthetized by the method described [16] for 2-[$^3H_3$]cotinine. A mixture of 0.2 mol of sodium cyanide in 200 ml of dry dimethylformamide was allowed to react at 15°C with freshly distilled 3-pyridinecarboxaldehyde (0.48 mol), followed by slow addition of 0.4 mol of ethyl acrylate below 40°C. The reaction product was diluted with distilled water and extracted with chloroform. The organic extract was concentrated and the remaining oil distilled twice in vacuo to yield pure ethyl 3-(3-pyridoyl)-propionate (purity by GLC >95%) (yield 10%). The latter compound (4 mmol) was stirred for four days with 13.8 mmol of [$^3H_3$]methylamine hydrochloride and 6 mmol of sodium cyanoborohydride in methanol. The reaction mixture was treated with 10% hydrochloric acid for 2 h and the methanol distilled off. The residue was stirred for 16 h with 10 ml of 10% sodium hydroxide at room temperature and then continuously extracted with diethyl ether The organic extract was finally concentrated and purified using preparative silica gel thin-layer chromatographic plates (E. Merck; art. 5717) with the solvent system chloroform—methanol—acetic acid (60:10:1). The yield was 12% The purified internal standard was analysed by GC—MS and found to be chromatographically pure (>99%).

Instrumentation

All analysis were performed on a Hewlett-Packard 5995A GC—MS system consisting of a quadrupole spectrometer, a Model 5710A gas chromatograph,

an HP 18964A capillary injector and an HP 9825B data system. The mass spectrometer was operated with an electron-ionization voltage of 70 eV and a source temperature of 150°C. The source pressure was 10^{-5} Torr and the accelerating voltage 2 kV. The temperature of the GC—MS transfer line was set at 225°C. A fused-silica chemically bounded Carbowax 20M column was inserted in the GC—MS transfer line about 0.5 cm from the ion source block. A volume of 1 μl was injected in the splitless mode at a column flow-rate of helium of 0.5 ml/min and an oven temperature of 40°C for 1 min; the oven temperature was then raised ballistically to 150°C and further programmed at 15°C/min to 224°C, where it was held for a further 15 min. The temperature of the injection port was 260°C.

Prior to the beginning of any series of SIM measurements, the ion source and mass analyser voltage settings were optimized to monitor the centres of each of the selected ions. Therefore, 1 ng each of cotinine and internal standard were injected into the GC—MS system and six channels monitored at 0.1 units difference around the theoretical mass value. The mass of the most intense signal to the nearest 0.1 unit was determined by integration of the different peak areas.

Extraction procedure

A flow diagram of the extraction procedure for reference and unknown plasma and urine samples is shown in Fig. 1. After thawing the samples, 1 ml of plasma or 1 ml of 1:4 diluted urine was transferred into a 10-ml conical glass tube and 0.1 ml (100 ng) of internal standard in isopropanol prepared from a stock solution (0.2 mg/ml) was added. The mixture was vortex-mixed, allowed to equilibrate for 15 min, made alkaline with 0.1 ml of 10 *M* potassium hydroxide solution and extracted with 4 ml of dichloromethane by shaking vigorously on a vortex mixer for 30 sec. The phases were separated by centrifugation at 6000 *g* for 10 min. The upper, aqueous phase was removed by means of a disposable pasteur pipette and the remaining bottom organic layer dried with approximately 0.3 g of anhydrous sodium sulphate.

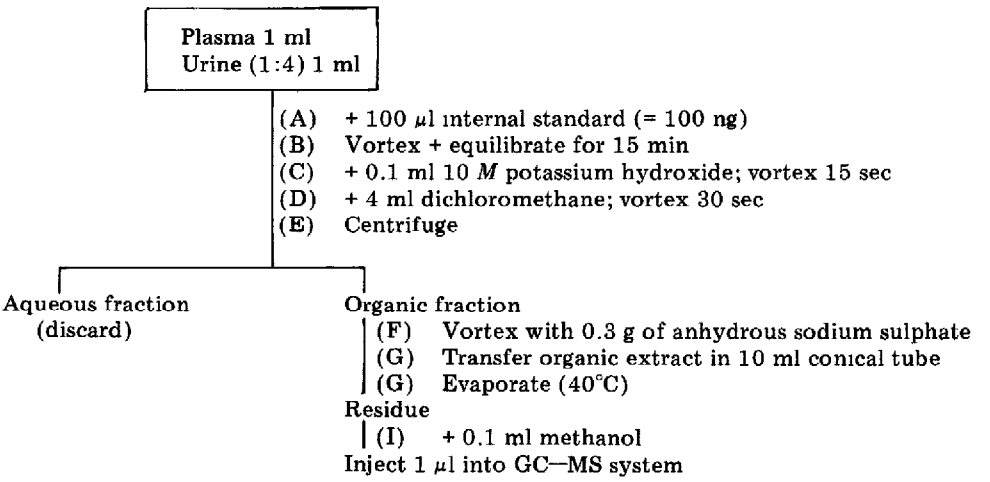

Fig. 1. Flow diagram of plasma and urine processing.

The organic layer was transferred by decantation into a 10-ml conical evaporation tube and subsequently evaporated on a heating block at 40°C. The top of the tube was covered with Parafilm and the residue stored below 0°C until the GC—MS analysis was ready to be performed. Prior to the injection, the residue was reconstituted with 100 μl of methanol and a 1-μl portion injected on to the GC—MS system.

Calibration and calculation

Made-up standards containing known amounts of cotinine in the range 50—500 ng/ml in blank plasma or urine samples from non-smokers were used to construct the calibration graphs. These samples, together with a plasma blank, were taken through the extraction procedure and analysed in the same way as the unknowns. The peak area ratios of unlabelled to labelled compound (*m/z* 176 and 179) were plotted against the known concentration of unlabelled compound. Linear calibration graphs of concentration versus peak-area ratio were obtained, from which unknown samples were quantified via interpolation from the regression line.

Validation and feasibility study

To test the precision and accuracy of the assay, a three-day validation study was carried out. A five-point calibration graph in duplicate together with nine or ten plasma samples from a plasma pool containing 85 ng/ml cotinine were analysed each day. A few more samples ($n = 6$) with a higher cotinine concentration (200 ng/ml) were also examined.

To investigate the sensitivity and specificity, the assay was applied to human clinical samples. In one experiment a 10-mg cotinine dose was administered to a healthy non-smoker volunteer by intravenous infusion over 30 min. Subsequently, the same volunteer received 20 mg of cotinine over 30 min. Plasma cotinine concentrations and the urinary levels were followed for 48 h (see Fig. 2). In another experiment, 5 μg of nicotine per kg body weight were infused by a volunteer over 10 min every 30 min for 12 h on four consecutive days, i.e. until a steady-state level of cotinine was reached. On each day and at the end of the fourth day the concentration of cotinine was determined in sequential plasma samples collected during and over a 24-h period after the end of the infusions (see Fig. 3).

RESULTS AND DISCUSSION

Standards

The base peak of cotinine is located at *m/z* 98 (100%) and the parent ion is at *m/z* 176 (25.5%). The base peak and the molecular ion of 3-[$^3H_3$]methylcotinine are conveniently three mass units greater than those of cotinine and are located at *m/z* 101 (100%) and *m/z* 179 (28.6%), respectively. Although both base peak and parent peak ions allow SIM analysis, the protonated molecular ions were chosen for monitoring in order to minimize non-specific interferences at lower *m/z* values from co-extracted endogenous material.

GC—MS considerations

Under the GC conditions described above, the Kováts retention indices of cotinine and deuterated cotinine are 2894 and 2902, respectively. Samples can be injected every 22 min, taking into account the time necessary for cooling the GC oven after each run. The use of a high-resolution capillary column allowed laborious clean-up procedures and back-extractions in the sample preparation step to be avoided whilst still providing adequate separation of cotinine from compounds that could interfere with the quantitation. To confirm the identity of cotinine, extracts of plasma samples, selected at random, were also chromatographed by monitoring at other diagnostic ions, such as *m/z* 177 and 98.

Extraction

In order to minimize the loss of cotinine due to absorption on glass surface, which could be a problem with low nanogram levels, all glassware was previously thoroughly cleaned and silylated. Using the one-step extraction procedure described above with dichloromethane in excess, the overall extraction efficiency of cotinine from plasma is above 95%. Several workers have shown dichloromethane to be one of the most efficient solvents for the removal of cotinine from biological fluids [9—11].

Sensitivity, linearity and precision

Calibration graphs were prepared daily for each batch of unknown samples. The calibration graphs for spiked plasma specimens, using the previously described extraction procedure, were linear from the limit of detection up to at least 500 ng. The least-squares linear regression line that describes a typical cotinine curve can be expressed by the equation $y = 0.0013x - 0.006$ ($r = 0.999$). Calibration graphs were also constructed for pure standards. No difference was seen between the slopes of the two calibration graphs, indicating that no isotopic effects occur between cotinine and its trideuterated variant.

Under the operating conditions of the mass spectrometer, the limit of detection of cotinine was below 1 ng/ml using 1 ml of plasma or 0.25 ml of urine. The sensitivity is comparable to that reported for other methods (Table I).

Table II shows the results of a three-day validation study for replicate analyses of aliquots of a pooled plasma sample containing 85 ng/ml of co-

TABLE II

DAY-TO-DAY VARIATION OF IDENTICAL PLASMA SAMPLES

Day No.	n^*	Mean	$S.D._{n-1}$	C.V. (%)
1	9	83	2.96	3.57
2	10	90	4 88	5.41
3	10	87	3.88	4 47

*n = Number of samples.

tinine. The slopes of the calibration graphs for the three series remained virtually constant. The coefficient of variation (C.V.) varied between 3.6 and 5.4%. For pooled plasma samples containing 200 ng/ml of cotinine, the C.V. was 2.9% (S.D.$_{n-1}$, 5.6; number of aliquots, 6). The proposed method is relatively rapid and simple, yet it results in excellent sensitivity and specificity and is therefore suitable for routine analysis.

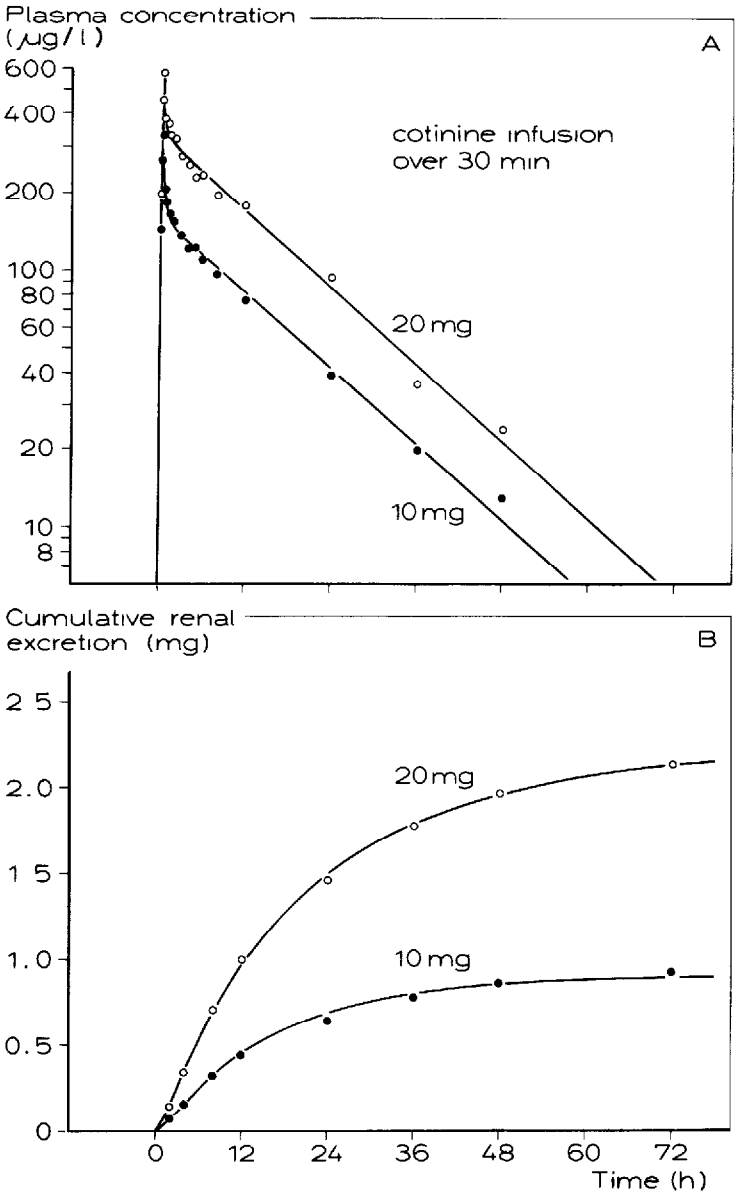

Fig. 2. (A) Plasma concentration curve (logarithmic scale) for 10 and 20 mg of cotinine infused over 30 min to a volunteer. The biexponential plasma concentration profiles are identical for the two doses. (B) Cumulative renal excretion curve following i.v. infusion of 10 and 20 mg of cotinine. In both instances about 10% of the dose is excreted by the kidney.

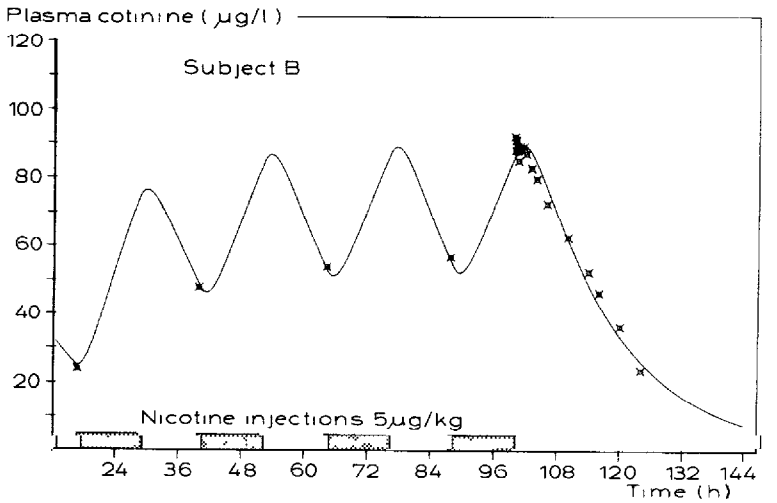

Fig. 3. Plasma concentration of cotinine following daily injections of 5 µg/kg nicotine every 30 min over 12 h, four days in succession. The solid line is a computer fit of the plasma data over five days The data show that within 4 days a steady-state situation is reached. The steady-state level of cotinine can therefore be used as a measure of the daily intake of nicotine in a smoker.

Application

The method was used to follow cotinine levels in plasma and urine samples from volunteers to whom nicotine or cotinine had been administered at different dose levels by either the intravenous or the oral route. The results of an extensive study will be reported elsewhere; some of the following partial results must be viewed as preliminary data. Fig. 2 shows cotinine plasma levels versus time for a volunteer to whom 10 and 20 mg of cotinine had been given as an intravenous infusion. The terminal plasma half-life of cotinine was found to be about 10 h. Oral administration of cotinine demonstrated that its absorption from the gastrointestinal tract is fast and virtually complete.

Fig. 3 shows the results of the determination of cotinine in plasma samples collected from a volunteer after multiple-dose intravenous administration of nicotine, as the free base, in sterile 0.9% sodium chloride solution. Every 30 min during 12 h the amount of 5 µg of cotinine per kg body weight was given by continuous infusion over 10 min with a precision pump. This administration was applied for four consecutive days, thereby producing close to steady-state levels of cotinine. During and for 24 h after the last administration of nicotine on day 4, plasma cotinine levels were followed. The peak level of cotinine in plasma after the last infusion of nicotine showed a long-lasting plateau for about 2 h. This was followed by slow elimination of cotinine with a half-life of approximately 11 h.

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