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# A rapid gas-liquid chromatographic method for the determination of cotinine and nicotine in biological fluids

C. FEYERABEND, M. A. H. RUSSELL*, *Nicotine Laboratory, Poisons Unit, New Cross Hospital, London SE14 5ER, *Health Behaviour Unit, Institute of Psychiatry, Maudsley Hospital, London SE5, UK*

**Abstract**—A rapid method is described for the simultaneous measurement of nicotine and cotinine in biological fluids using capillary column gas-liquid chromatography. Using 100 μL sample volume the lower limit of detection for both nicotine and cotinine was 100 pg $mL^{-1}$, allowing the method to be used for the measurement of these compounds in both smokers and non-smokers. The extraction time is 3 min per sample, and by using multi-pipetting and vortexing systems 250 samples can be extracted per day. The average coefficient of variation over the nicotine range 1·0 to 100 ng $mL^{-1}$ was 3·9% and for cotinine over the range 1·0 to 1000 ng $mL^{-1}$ was 2·2%. Saliva cotinine concentrations were quantitatively related to passive exposure to parental smoking in a population study of 1118 non-smoking schoolchildren.

In the last few years there has been a dramatic increase in the demand for nicotine and cotinine estimations. Measurements of plasma nicotine concentration have become an important component of behavioural studies on the role of nicotine in smoking (e.g. Pomerleau et al 1987). They are also essential in the development, testing, clinical evaluation and monitoring of nicotine replacement treatments for smoking, such as nicotine skin patches (Rose et al 1984), nasal sprays (Russell et al 1983) and nicotine polacrilex chewing gum (Pomerleau & Pomerleau 1988). Cotinine, a major nicotine metabolite, has a longer elimination half-life (about 20 h compared with 2 h for nicotine) and is therefore an ideal measure of average nicotine intake during the previous few days (Benowitz 1983). Saliva cotinine concentrations closely reflect those in plasma with a mean saliva to plasma ratio of 1·13 or less (Jarvis et al 1984) and provide a non-invasive quantitative measure of smoke intake from active and passive smoking. Cotinine is the most sensitive and specific biochemical marker of smoke intake for use in epidemiological studies and clinical trials. It provides an objective validation of reported smoking habits and a quantitative measure of exposure which is more reliable than self-reports of inhalation, daily cigarette consumption or the tar and nicotine yields of the cigarettes (Jarvis et al 1985, 1987).

With such widespread use and demand an ideal assay method should be able to determine nicotine and cotinine simultaneously. It should also be rapid, reproducible and suitable for use at levels found in smokers. At the same time the method should be sensitive enough to examine levels from passive exposure found in non-smokers.

Previously published methods using a wide range of techniques including immunoassays, high-performance liquid chromatography, gas-chromatography and GC-mass spectrometry either lack sensitivity, require large sample volumes or cannot measure cotinine and nicotine simultaneously. We report here a method which is both rapid and sensitive and measures cotinine and nicotine simultaneously in 100 μL urine, plasma, or saliva.

## Materials and methods

*Reagents.* All reagents used were of analytical reagent grade: dichloroethane, sodium hydroxide, nicotine (+)-tartrate, Dow Corning antifoam RD emulsion (BDH Chemicals Ltd); 5-methylcotinine (kindly donated by Dr P. Jacob, San Fransisco, USA); cotinine base and phenol red (Sigma Chemicals Ltd).

Correspondence to: C. Feyerabend, Nicotine Laboratory, Poisons Unit, New Cross Hospital, London SE14 5ER, UK.

*Apparatus.* A Hewlett Packard model HP5890A gas chromatograph equipped with a nitrogen detector, model HP7673A auto-injector and a model HP3392A reporting integrator was used. Dreyer tubes (Samco Ltd), micro centrifuge Hermle model Z230M (Anderman Ltd) was adapted for use with Dreyer tubes.

*Chromatographic conditions.* An HP-FFAP capillary column (crosslinked, 7 m × 0·32 mm i.d. × 0·53 μm film thickness, Hewlett Packard Ltd) was used. The detector gas flow rates were: air 110 mL $min^{-1}$, hydrogen 3·3 mL $min^{-1}$ and make up gas (helium) 13 mL $min^{-1}$. Carrier gas (helium) column head pressure was 15 psi. The temperatures of the injection port and detector were 170°C and 300°C, respectively. The oven was temperature programmed from 70°C to 110°C at 40°C $min^{-1}$ for 1·5 min then to 200°C at 40°C $min^{-1}$ for 3 min. The retention times for nicotine, cotinine and 5-methyl cotinine were 2·2, 6·4 and 6·7 min, respectively. An inlet insert purge operates in between sample injection, but is turned off automatically at the time of injection for 24 s to allow the sample to enter the column.

*Procedure.* To 100 μL of biological fluid in a Dreyer tube were added an aqueous solution of 5-methylcotinine (100 μL, 117 ng $mL^{-1}$) as internal standard, sodium hydroxide solution (300 μL, 5M), antifoam/phenol red mixture (20 μL, 5% antifoam, 200 mg $L^{-1}$ phenol red) and dichloroethane (50 μL). The solution was vortex mixed (1 min) and centrifuged (2 min). The organic phase was then transferred to a glass auto-injector microvial using a glass pasteur pipette. A 2 μL aliquot was injected automatically onto the chromatographic column.

*Calibration.* A calibration graph for plasma was constructed by adding cotinine, nicotine and the internal standard to blank bovine plasma to give a concentration range of 1·0 to 1000 ng $mL^{-1}$ for cotinine and 1·0 to 100 ng $mL^{-1}$ for nicotine. These solutions were then carried through the extraction procedure. The calibration curve was linear and passed through the origin.

For the analysis of saliva and urine, water was used to construct the calibration graph. This gave a line with the same slope as those derived by analysing samples of urine and saliva with added cotinine and nicotine. The calibration curves were again linear and passed through the origin.

*Reproducibility.* The reproducibility over the concentration ranges 1·0 to 100 ng $mL^{-1}$ for nicotine and 1·0 to 1000 ng $mL^{-1}$ for cotinine in bovine plasma are shown in Table 1. The average coefficient of variation over the nicotine and cotinine range was 3·9% and 2·1%, respectively.

## Results and discussion

Typical chromatograms of extracts from human plasma (A), urine (B) and saliva (C) are shown (Fig. 1). The extracts represent a nicotine and cotinine concentration of 23 ng $mL^{-1}$

Table 1. Reproducibility of results of ten determinations at various cotinine and nicotine concentrations in bovine plasma.

| Cotinine | | | Nicotine | | |
|---|---|---|---|---|---|
| Added ng $mL^{-1}$ | Found ng $mL^{-1}$ (s.d.) | CV% | Added ng $mL^{-1}$ | Found ng $mL^{-1}$ (s.d.) | CV% |
| 1·0 | 1·06 (0·08) | 7·7 | 1·0 | 1·0 (0·13) | 13·0 |
| 2·5 | 2·3 (0·13) | 5·6 | 2·5 | 2·4 (0·13) | 5·4 |
| 5·0 | 4·9 (0·20) | 4·1 | 5·0 | 5·2 (0·18) | 3·4 |
| 10·0 | 10·3 (0·12) | 1·2 | 10·0 | 9·9 (0·32) | 3·2 |
| 25·0 | 24·9 (0·23) | 0·9 | 20·0 | 19·4 (0·48) | 2·5 |
| 50·0 | 49·8 (0·35) | 0·7 | 40·0 | 40·9 (0·79) | 1·9 |
| 100·0 | 100·3 (0·87) | 0·9 | 60·0 | 59·9 (1·10) | 1·8 |
| 200·0 | 199·6 (1·39) | 0·7 | 80·0 | 79·6 (1·52) | 1·9 |
| 400·0 | 398·9 (2·80) | 0·7 | 100·0 | 100·1 (1·87) | 1·9 |
| 500·0 | 501·1 (5·44) | 1·1 | | | |
| 600·0 | 600·1 (8·21) | 1·4 | | | |
| 800·0 | 800·5 (13·79) | 1·7 | | | |
| 1000·0 | 1005·9 (18·80) | 1·9 | | | |

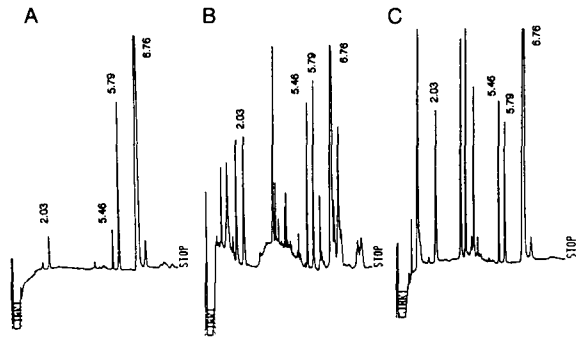


FIG. 1. Chromatograms from plasma (A), urine (B) and saliva (C) extracts. Numbers above peaks are retention times in min: nicotine, 2·03; cotinine, 5·46; internal standard, 5·79; caffeine, 6·76. Nicotine and cotinine concentrations were 23 and 27 ng $mL^{-1}$ for plasma, 73 and 100 ng $mL^{-1}$ for urine and 115 and 130 ng $mL^{-1}$ for saliva, respectively.

and 27 ng $mL^{-1}$ for plasma, 73 ng $mL^{-1}$ and 100 ng $mL^{-1}$ for urine and 115 ng $mL^{-1}$ and 130 ng $mL^{-1}$ for saliva.

A more suitable internal standard for nicotine than 5-methylcotinine would be *N*-ethylnornicotine which is a close analogue. However, for ease of operation, and maximum through-put using an integrator, a single internal standard is a major advantage. On evaluation, the use of 5-methylcotinine for nicotine did not significantly reduce the accuracy and reproducibility when measuring this compound. This single internal standard has therefore been adopted for both cotinine and nicotine measurements.

Table 2. Saliva cotinine concentrations (ng $mL^{-1}$) in non-smoking schoolchildren by reported parental smoking.

| | Neither parent smokes | Father only smokes | Mother only smokes | Both parents smoke |
|---|---|---|---|---|
| *n* | 507 | 236 | 160 | 215 |
| Median | 0·50 | 1·35 | 2·15 | 3·70 |
| Mean | 0·79 | 1·98 | 2·72 | 4·46 |
| 95% CI | ±0·10 | ±0·27 | ±0·34 | ±0·43 |

Using a single calibration curve the limit of detection for nicotine and cotinine is 0·1 ng $mL^{-1}$ and, providing stringent care is taken to avoid contamination with cigarette smoke, there are no positive blank values. Correlation between the described micro method and our previously reported macro methods for cotinine (Feyerabend & Russell 1980a) and nicotine (Feyerabend & Russell 1980b) in urine, saliva, and plasma was >0·998 for both compounds.

Calibration curves have been stable over the year during which the method has been in use. No slope changes were

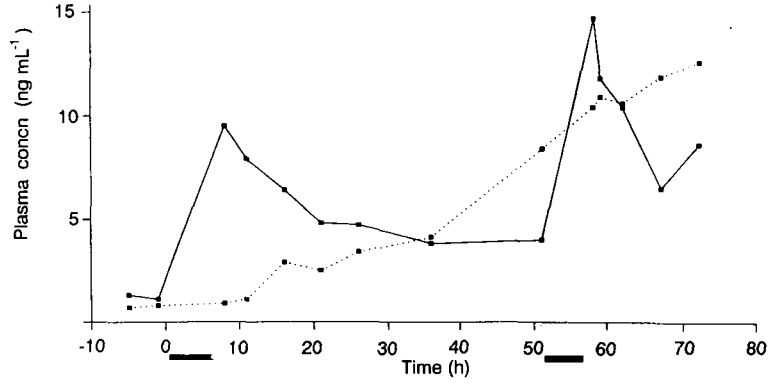


FIG. 2. Plasma nicotine and cotinine concentrations before and after inhalation of nicotine from a cigarette-sized cylindrical plastic vapourizer. On each of two occasions (shown by the horizontal bars) the subject took 13 inhalations at 30 s intervals in 6 min. Blood samples were taken at 2, 5, 10, 15, 20, 30 and 45 min after inhaling on the first, and at 1, 2, 5, 10 and 15 min on the second occasion. The subject was a 75 kg male ex-smoker aged 49 years. ●—● nicotine, ● · · · · · ● cotinine.

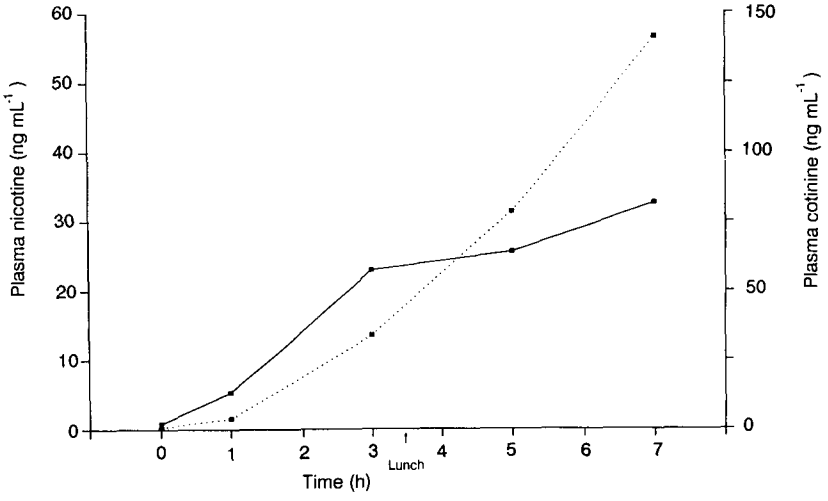


FIG. 3. Plasma nicotine and cotinine concentrations before and at 1, 3, 5 and 7 h after application of a 16 cm$^2$ nicotine-containing skin patch to the left side of the chest. The subject was an 80 kg non-smoker aged 55 years. The relative dip in the nicotine curve at 5 h is attributed to enhanced liver blood flow and metabolism following lunch. ●—● nicotine, ● · · · · · · ● cotinine.

detected during analysis for cotinine of a batch of 2000 saliva samples. The reproducibility data for pooled saliva assayed during this time was $194{\cdot}4 \pm 1{\cdot}8$ ng mL$^{-1}$ for cotinine, giving a coefficient of variation of 0·9%. No deterioration in the column or excessive deterioration in the performance of the nitrogen detector has been seen after the analysis of over 5000 biological samples.

This method has significant advantages over previously published methods in that it uses a single rapid extraction step (1 min) without any further concentration, purification, or evaporation steps thus allowing 150 samples per day to be extracted for both nicotine and cotinine by a relatively inexperienced technical assistant. Moreover, as only one calibration curve is used for both high and low levels of nicotine and cotinine, and only 100 μL sample volumes are required, the method is ideal for epidemiological studies. In such studies it is important to have a method with sufficient sensitivity to measure levels found in non-smokers preferably without having to alter the procedure in any way. For example, cotinine concentrations were determined in a national sample of 1118 non-smoking schoolchildren, aged 11 to 15 years (Goddard 1989). Table 2 shows the saliva cotinine concentrations of the children according to the smoking status of their parents and demonstrates a clear and significant dose relationship to passive exposure from their parents' smoke. These values are similar to those found in a different group of schoolchildren using our previously published method (Jarvis et al 1985).

The method is also sufficiently precise for use in experimental studies where both nicotine and cotinine concentrations are required. For example, Figs 2 and 3 show the plasma nicotine and cotinine concentrations produced by prototypes of two different forms of nicotine administration being developed for use in the treatment of smoking.

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