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Determination of cotinine in biological fluids of non-smokers by packed column gas-liquid chromatography

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A method is described for the analysis of cotinine in plasma, saliva and urine using packed-column gas-liquid chromatography, which is sufficiently sensitive and reproducible for quantitative study of the low levels resulting from exposure of non-smokers to other people's smoke. The lower limit of detection of cotinine in these fluids was 100 pg ml^{-1}. The coefficient of variation over the range 0·25 to 2·0 ng ml^{-1} averaged 7·7%. In a sample of 85 non-smokers the concentrations of cotinine in plasma correlated 0·82 with those in urine and saliva, while the correlation between the saliva and urine concentrations was 0·91. Saliva cotinine concentrations were quantitatively related to passive exposure to parental smoking in a population study of 569 non-smoking schoolchildren.

Over the past ten years, measurements of nicotine concentrations (Langone et al 1973; Feyerabend et al 1975; Jacob et al 1981), and those of its major metabolite cotinine (Langone et al 1973; Jacob et al 1981; Feyerabend & Russell 1980) in blood, urine or saliva have been used to study the role of nicotine in smoking behaviour (Russell et al 1975; Ashton et al 1979; Benowitz & Jacob 1985) and as markers for comparing the smoke intake of smokers of high and low-yield cigarettes (Russell et al 1980; Benowitz et al 1983; Hill et al 1983; Gori & Lynch 1985). More recently, epidemiologists have recognized the potential value of such measures in predicting mortality and morbidity from lower yield cigarettes without having to wait two or three decades for the full pathological effects (Benowitz et al 1985). Due to its rapid absorption and relatively short elimination half-life (about 2 h), nicotine is less suitable than cotinine (half-life about 20 h) as a measure of average daily intake. Other markers such as carbon monoxide and thiocyanate levels are not specific to tobacco smoke. It is therefore generally agreed that cotinine is the best single measure of daily exposure to tobacco smoke (Benowitz 1983).

With the increasing concern being shown about the risk to non-smokers of exposure to other people's smoke (passive smoking) it has become important to adapt analytical methods to the low concentrations found in non-smokers. On average, the concentrations of cotinine in the biological fluids of non-smokers are about 0·5 to 1·0% of the levels found in smokers (Jarvis et al 1984; Greenberg et al 1984; Wald et al 1984; Jarvis et al 1985). Although capillary gas-liquid chromatography is likely to be the method of choice for the measurement of such low concentrations, this technique is not yet available in many laboratories. We therefore report here a procedure for the determination of cotinine at concentrations down to 100 pg ml^{-1} using packed column GLC and its use as a quantitative measure of exposure in non-smokers.

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Materials and methods

Reagents. All reagents used were of analytical reagent grade: acetone and dichloromethane, (both glass-distilled), (BDH Chemicals Ltd), lignocaine hydrochloride monohydrate (internal standard), *N*-(2-methoxyethyl)-norcotinine succinate (internal standard), cotinine base (Sigma Chemicals Ltd), hydrochloric acid (1 M), sodium carbonate (1 M), sodium hydrogen carbonate (1 M), sodium hydroxide (5 M). Buffer solution: sodium hydrogen carbonate (20 ml, 1 M) plus sodium carbonate (325 ml, 1 M) made up to 1 litre with distilled water. This gives a buffer solution of pH 11·0 with a buffer value of 2·2.

Apparatus

A Hewlett-Packard, Model 5890A, gas-chromatograph fitted with a nitrogen-phosphorus flame ionization detector and a Model 3392A reporting integrator was used.

Chromatographic conditions

A glass column (6′ × 2 mm i.d.) was packed with 2% Carbowax 20M-terephthalic acid/5% KOH on Chromosorb W-HP/80–100 mesh. The gas flow rates were: helium (carrier gas) 25 ml min^{-1}, air 50 ml min^{-1} and hydrogen 3 ml min^{-1}. The temperatures of the oven, detector and injection port were 205, 300, and 250 °C, respectively. The retention times for lignocaine and cotinine were 4·0 and 5·4 min, respectively.

Procedure

Plasma. To 1·0 ml of plasma in a 12 ml centrifuge tube were added sodium hydroxide solution (2·0 ml, 5 M), an aqueous solution of lignocaine hydrochloride monohydrate (100 µl, 150 ng ml^{-1}) as the internal standard and dichloromethane (3·0 ml). The solution was vortex-mixed for 2 min and then centrifuged for 5 min. The aqueous layer was discarded and the tube vortex-mixed to break up the emulsion and centrifuged for 2 min. The organic layer was transferred to a second tube and evaporated to dryness under a stream of nitrogen at

room temperature (20 °C). Acetone (50 µl) was added and the tube vortex-mixed for 1 min and centrifuged for 1 min. A 3 µl sample of this solution was injected on to the chromatographic column.

Saliva. This was processed as for plasma but the sodium hydroxide was replaced by 2·0 ml of the sodium carbonate/sodium hydrogen carbonate buffer solution.

Urine. To 1·0 ml of urine in a 12 ml centrifuge tube were added sodium hydroxide (2·0 ml, 5 M), an aqueous solution of *N*-(2-methoxyethyl)-norcotinine succinate (100 µl, 640 ng ml^{-1}) as internal standard and dichloromethane (3·0 ml). The solution was vortex-mixed for 2 min and then centrifuged for 5 min. The organic layer was transferred to a second tube and evaporated to dryness under a stream of nitrogen at room temperature. Hydrochloric acid (100 µl, 1 M) was added and the tube vortex-mixed for 1 min and centrifuged for 1 min. The solution was then transferred to a Dreyer tube and to this was added sodium hydroxide (400 µl, 5 M) and dichloromethane (50 µl). The tube was then vortex-mixed for 1 min and centrifuged for 1 min. A 3 µl sample of the dichloromethane layer was injected on to the chromatographic column.

Calibration

A calibration graph for plasma samples was constructed by adding cotinine and the internal standard to blank bovine plasma to give a concentration range of 0·25 to 10 ng ml^{-1}. These solutions were then carried through the extraction procedure. The calibration curve was linear and passed through the origin.

For the analysis of saliva samples (range 0·25 to 10 ng ml^{-1}) and urine samples (range 0·25 to 100 ng ml^{-1}) 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 spiked with cotinine. The calibration curve was again linear and passed through the origin.

Blanks. No cotinine was detected in blank solutions but substances which interfered with the assay were present in plastic microvials which were used with an autosampler. This could be avoided by the use of glass microvials or glass inserts.

Reproducibility. The reproducibility over the concentration range 0·25 to 2 ng ml^{-1} is shown in Table 1. The average coefficient of variation over this range was 7·7%.

Results and discussion

A typical chromatogram of an extract from human saliva is shown in Fig. 1. This extract represents a cotinine concentration of 0·4 ng ml^{-1}. At 7·7%, the average coefficient of variation was greater over these low concentration ranges than the 1·8% reported previously for higher concentrations (Feyerabend & Russell 1980). The reproducible lower limit of determination of cotinine was 100 pg ml^{-1}, when applying the calibration curve used to measure cotinine concentrations up to 10 ng ml^{-1}.

For practical use in epidemiological studies, especially those involving children, non-invasive sampling of urine or saliva is more convenient than the collection of plasma samples. Eighty-five adult non-smokers provided samples for the comparison of cotinine concentrations in these three body fluids (Jarvis et al 1984). The average concentrations were 1·48 ng ml^{-1} (s.e.m. 0·25) in plasma, 1·69 ng ml^{-1} (s.e.m. 0·25) in saliva and 4·84 ng ml^{-1} (s.e.m. 0·93) in urine. The correlations between the three body fluids (n = 85) were: plasma vs saliva cotinine: r = 0·82, y = 0·09 + 0·82 x; plasma vs urine cotinine r = 0·82, y = 0·41 + 0·22 x; saliva vs urine cotinine r = 0·91, y = 0·49 + 0·25 x. Plasma and saliva concentrations were similar, the minor elevation in saliva being attributable to pH partition. The high correlation of the cotinine concentrations in urine with

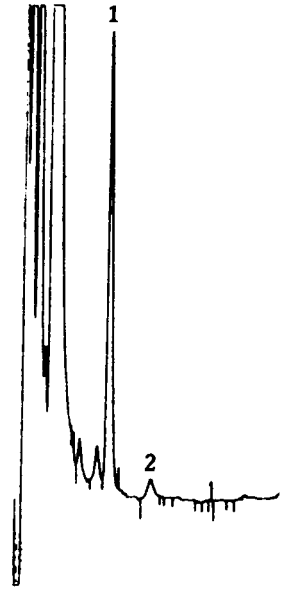

FIG. 1. Gas chromatogram of an extract from human saliva: (1) lignocaine and (2) cotinine. The retention times of lignocaine and cotinine were 4·0 and 5·4 min, respectively.

those in the other two body fluids suggests that for practical purposes it is unnecessary to correct for urine flow by use of the cotinine/creatinine ratio, especially when large samples of subjects are being compared.

An example of the practical application of the cotinine assay method is provided by its use to quantify the smoke intake of 569 non-smoking schoolchildren resulting from exposure to their parents' cigarette smoke (Jarvis et al 1985). Table 2 shows the saliva cotinine concentrations of the non-smoking children according to the smoking habits of their parents. The

Table 1. Reproducibility of results of ten determinations at various cotinine concentrations.

	Cotinine added (ng ml^{-1})			
	0·25	0·5	1·0	2·0
	Cotinine found (ng ml^{-1})			
Mean	0·26	0·52	0·99	2·01
s.d.	0·03	0·04	0·07	0·09
Coefficient of variation (%)	11·5	7·7	7·1	4·5

Table 2. Saliva cotinine concentrations (ng ml^{-1}) of non-smoking children by parental cigarette smoking habits.

	Neither parent smokes (n = 269)	Only father smokes (n = 96)	Only mother smokes (n = 76)	Both parents smoke (n = 128)
Mean	0·44	1·31	1·95	3·38
S.e. mean	0·04	0·12	0·20	0·22
Median	0·20	1·00	1·35	2·70

Note: The children's non-smoking status and parental smoking habits were based on questionnaires completed by the children so that the variance would include that due to error of these self-reports. The differences between each of the four passive exposure categories is statistically significant, that between father only and mother only at the 0·05 level, all the others at the 0·01 level.

differences between each of the passive exposure categories are statistically significant and show a clear relationship to dose with the means of father only and mother only summing approximately to the mean for both parents smoking.

In conclusion, we have described a method for measuring cotinine concentrations in biological fluids which is sufficiently sensitive and reproducible for quantitative study of the low levels resulting from exposure of non-smokers to other people's smoke.

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