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Improved gas-chromatographic method and micro-extraction technique for the measurement of nicotine in biological fluids

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A rapid and sensitive method for the measurement of nicotine in plasma, urine, saliva and breast milk is described. An internal standard (quinoline) is added to the samples and these are made alkaline and extracted with diethyl ether. The solvent is evaporated to small bulk and extracted with dilute acid which is then made alkaline. The nicotine is finally extracted into butyl acetate and an aliquot of this extract is injected onto a gas-chromatograph fitted with a nitrogen detector. Quantitation relies on comparison of peak areas and the calibration curve is linear over the concentration range 0·5 to 100 ng ml^{-1}. Nicotine concentrations as low as 0·1 ng ml^{-1} can be measured. In addition, a micro-method is described which requires only 100 μl of sample and yields an accurate result in 5 min.

Previously published methods for measuring nicotine in nanogram quantities have been difficult to apply (Turner 1969; Horning et al 1973) or have a lengthy extraction procedure (Schievelbein & Grundke 1969; Burrows et al 1971; Falkman et al 1975; Hengen & Hengen 1978). The Isaac & Rand (1972) direct extraction procedure was improved by Feyerabend et al (1975) and has now been modified further. This has simplified the extraction and at the same time improved the reproducibility. In addition, a micro-method is described which requires only 100 μl sample and yields an accurate result in 5 min.

MATERIALS AND METHODS

To samples (3 ml) in 12·5 ml glass stoppered tapered centrifuge tubes were added an aqueous solution of quinoline (0·75 μg ml^{-1}; 100 μl) as internal standard, sodium hydroxide (5M; 2 ml) and diethyl ether (3 ml). After agitation on a Vortex mixer (2 min) the tube was centrifuged (10 min) and the organic layer removed to a second tube. The extract was evaporated gently (room temperature 20 °C) under a stream of nitrogen to approximately 200 μl. Hydrochloric acid (2M; 100 μl) was added and the mixture vortexed (2 min). After centrifugation the ether layer was discarded and the aqueous layer was washed with ether (ca 0·5 ml) which was subsequently discarded. Any ether remaining above the aqueous layer was evaporated under a stream of nitrogen and the aqueous phase was then centrifuged.

After transfer of the solution to a Dreyer tube, sodium hydroxide (5M; 400 μl) and butyl acetate (50 μl) were added. The tube was vortexed for 1 min and then centrifuged. The butyl acetate layer (5 μl) was injected onto the gas chromatograph.

Chromatography

A Hewlett-Packard model 5730A gas chromatograph equipped with a nitrogen detector was used. The column was a 6′ × 6 mm glass tube which had been silanized over 24 h with a 5% (v/v) solution of dimethyldichlorosilane in toluene. This was packed with 80–100 mesh Chromosorb W (Perkin-Elmer Ltd) coated with 10% (w/w) Apiezon L and 10% KOH. The column is stable, one having been in constant use for over 5 years. The instrument settings were as follows: column temperature 220 °C; injection port temperature 250 °C; detector temperature 300 °C; carrier gas (helium) flow rate 60 ml min^{-1}; air flow rate 50 ml min^{-1}; hydrogen flow rate 30 ml min^{-1}. The gas chromatograph was coupled to an Hewlett-Packard model 3380A integrator which had attached to it an external time delay relay.

Calibration

Calibration curves were constructed by adding nicotine and the internal standard quinoline to blank solutions of the sample type to be analysed to give concentrations of 5, 10, 20, 60, and 100 ng ml^{-1}. These were then carried through the extraction procedure. The calibration curve of the peak height response was linear over the working range 0·5 to 100 ng ml^{-1} in plasma, urine, water,

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saliva and breast milk. The retention times for quinoline and nicotine were 2·5 and 3·0 min respectively.

Reproducibility

Reproducibility over the working range was s.d. 0·5 ng ml^{-1}. To test the reproducibility at varying sample volumes nicotine was added to bovine plasma to give a stock solution containing 30 ng ml^{-1} nicotine. Ten determinations were carried out at varying plasma volumes. In each case the appropriate volume of water was added to give a final tube volume of 3 ml. The results with means and standard deviations are:

Sample volume (ml)	3·0	2·0	1·0	0·8	0·4	0·2	0·1
Mean (s.d.)	29·8 (0·5)	29·9 (0·7)	30·4 (0·7)	30·4 (0·7)	29·8 (1·0)	29·6 (1·5)	29·7 (2·4)

Sensitivity

Using an appropriate sample volume, this method is sufficiently sensitive to detect nicotine concentrations of 0·1 ng ml^{-1}. No common basic drugs have been found to interfere with the analysis. If used in excess, preparations of heparin containing chlorocresol as a preservative give rise to a peak with a similar retention time to quinoline. Heparin containing chlorbutol as a preservative produces no interference.

DISCUSSION

This modified method has been in constant use in this laboratory for over two years and has proved extremely reliable. The extraction procedure is much shorter than that recently reported by Hengen & Hengen (1978) and, unlike these authors, we have not experienced interference from impurities in tap-water. Nicotine is still encountered in aqueous and plasma blank samples, but with careful checking of each batch of sodium hydroxide before use and adhering to analytical grade solvents, the blank values can be kept to between 0·3 and 0·5 ng ml^{-1}. In addition, all glass-ware is machine-washed and the analyses are carried out in an isolated, smoke-free laboratory.

Fig. 1 illustrates a normal chromatogram trace from the start of an injection and shows an initial negative peak. This caused integration problems in that the integrator used the bottom of this peak as the base line and thus calculated fallacious values for the nicotine and quinoline peak areas. Use of the start-delay facility provided with the integrator failed to eliminate this problem since the device

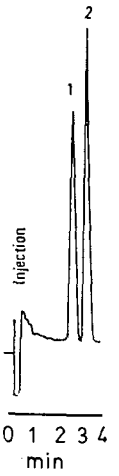

FIG. 1. Gas chromatogram of an extract of plasma from a smoker without the external time delay relay in operation. 1, Quinoline. 2, Nicotine.

still took the apex of the negative peak as the baseline. This difficulty was finally overcome by fitting an external start-delay relay. Following an injection, this device is activated and, after the negative peak has passed, it triggers the integrator which then draws traces such as those shown in Figs 2 and 3. Fig. 2 illustrates the chromatogram from extracts of (A) water, (B) a true blank plasma from a non-smoker and (C) plasma from a non-smoker who passively absorbed nicotine from the atmosphere which is equivalent to a nicotine concentration of 3·4 ng ml^{-1}. This underlines the dangers of using so-called blank human plasma to prepare standards and consequently we have preferred to derive

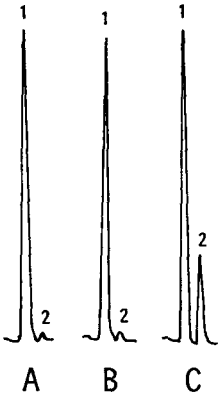

FIG. 2. Gas chromatograms of extracts from tap water (A) non-smokers blank plasma (B) non-smokers plasma with passively absorbed nicotine (C). 1, Quinoline. 2, Nicotine.

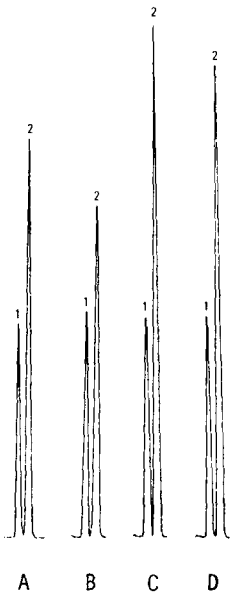

FIG. 3. Gas chromatograms of extracts from aqueous standard (A) plasma (B) urine (C) saliva (D). 1, Quinoline. 2, Nicotine.

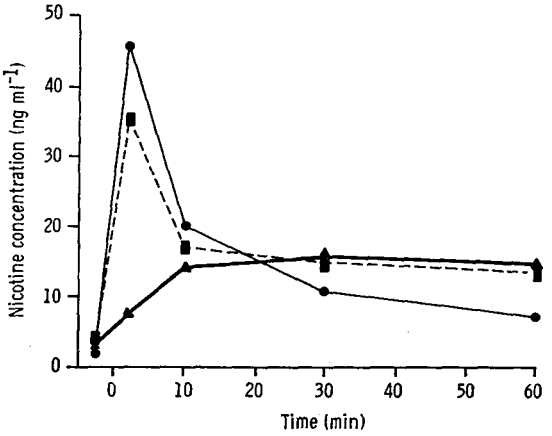

FIG. 4. Plasma nicotine concentrations (ng ml^{-1}: ordinate) after intravenous nicotine injection (●—●), smoking one cigarette (■- -■) and chewing a 4 mg nicotine gum (▲—▲). Abscissa: time (min).

plasma from fresh bovine blood and store it at −20°C until required. Calibration curves obtained from bovine plasma standards or true blank human plasma are identical. Typical chromatograms of extracts of an aqueous standard, plasma, urine and saliva are shown in Fig. 3, The urine sample was diluted to 1 in 30 before extraction. It can be seen that none of these samples gave rise to interfering peaks and that the base-line is stable and 'noise-free'. The nicotine concentrations derived from those gas chromatograms are A, 26·9, B, 21·0, C, 34·0, D, 31·1 ng ml^{-1}.

Nicotine standards made up in tap-water are only stable for 2 to 3 days and after 5 days no nicotine can be detected. The decay time can be extended to approximately 1 month by using distilled-water and can be further increased by (a) storage in the dark (slight effect only), (b) storage at 4°C (doubles the decay time), (c) adding a metal (e.g. powdered zinc) or (d) making the solutions acidic or basic. A free radical reaction is thought to be the cause of this instability. In practice, it is recommended that nicotine standards be prepared fresh every week.

Some of the results obtained using the modified method described above are illustrated in Fig. 4. Each time the subject abstained from smoking for 12 h and on different days he was either injected intravenously with nicotine base (1·775 mg), smoked one cigarette (1·3 mg nicotine) or chewed nicotine gum (4 mg nicotine), the slower release and absorption of nicotine from the gum compared to the rapid absorption via injection or smoking is clearly illustrated.

Approximately 93% of the nicotine in a sample is removed by the single diethyl ether extraction and this simplification allows one worker to process up to 50 samples a day. This method has the added advantage of being able to use varying sample volumes without the need to recalibrate at each volume.

Subsequently, we have found that a direct micro-extraction technique applied to only 100 μl of sample can yield an accurate result in approximately 5 min. Although slightly less reproducible than the method described above, this micro-method is essential when the volume of sample available is extremely small or when quick results are required, for example when checking solutions for nicotine contamination.

To samples (100 μl) in a Dreyer tube were added an aqueous solution of quinoline (0·075 μg ml^{-1}; 100 μl) as internal standard, sodium hydroxide (5M; 400 μl) and di-isopropyl ether (5 μl). After agitation on a Vortex mixer (1 min) the tube was centrifuged (1 min) and 5 μl of the organic layer was injected onto the gas chromatograph.

Calibration curves were constructed by adding nicotine to blank plasma and analysing 100 μl aliquots.

The means and standard deviations for ten

assays from a range of nicotine concentrations using blank plasma are:

Added nicotine ng ml^{-1}	2	5	10	20	40	80
Mean (s.d.)	2·07 (0·14)	4·93 (0·36)	9·91 (0·38)	19·81 (0·54)	40·1 (0·60)	80·0 (0·98)

Fig. 5 shows a typical chromatogram obtained when using the external time delay relay. The

FIG. 5. Gas chromatogram of an extract of plasma from a smoker. 1, Quinoline. 2, Nicotine.

extract was from a smoker's plasma and represents a concentration of 33 ng ml^{-1}. Using this method it is possible to measure nicotine concentrations down to 1 ng ml^{-1} and the sensitivity at these lower concentrations can be increased by using 200 μl sample volumes.

To maintain the stable blank value it is essential to use analytical grade chemicals and to re-distill solvents. The sodium hydroxide solution should first be checked for contamination before use and any impurities in the Dreyer tubes should be removed by leaving them in a gas-chromatograph oven overnight at 450 °C.

The good sensitivity and reproducibility of this method enables its use in both routine and research work.

Acknowledgements

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