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Assay of nicotine in biological materials: sources of contamination and their elimination

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A previous method for the measurement of nicotine in biological samples was subject to occasional interference from a contaminant which eluted simultaneously with the internal standard (quinoline). This has been rectified by substituting isoquinoline as the internal standard and modifying the operating conditions of gas chromatography. Other sources of contamination in nicotine assays such as the analytical reagents, the atmosphere and the sample itself are discussed in detail.

Contamination has been a major source of error in previous methods designed to measure nicotine in biological fluids (Burrows et al 1971; Isaac & Rand 1972; Falkman et al 1975; Feyerabend et al 1975; Hengen & Hengen 1978). Nicotine itself is the prevalent contaminant, although a compound eluting simultaneously with the internal standard (quinoline) has occasionally been found (Feyerbend & Russell 1979). This paper describes how this can be avoided and suggests means by which interference from other substances can be minimized.

MATERIALS AND METHODS

To samples (3 ml) in 12·5 ml glass-stoppered tapered centrifuge tubes were added sodium hydroxide (5 M; 2 ml), diethyl ether (3 ml) and an aqueous solution of isoquinoline (0·7 μg ml^{-1}; 100 μl) as internal standard. After agitation on a vortex mixer (2 min) the tube was centrifuged (10 min) and the organic layer removed to a second tube. Hydrochloric acid (2 M; 100 μl) was added and the extract was evaporated gently (room temperature 20 °C) under a stream of nitrogen to approximately 200 μl and then 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 solvent 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 (5 M; 400 μl) and butyl acetate (50 μl) were added. The tube was vortexed for 1 min and then centrifuged. An aliquot of the butyl acetate layer (3 μl) was injected on to the gas chromatograph.

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Chromatography

A Hewlett-Packard mode 5730A gas chromatograph equipped with a nitrogen detector was used. The column was a 2·4 m × 2 mm i.d. glass tube packed with 80–100 mesh Chromosorb W (Perkin-Elmer Ltd.) coated with 10% (w/w) Apiezon L and 10% KOH. The instrument settings were as follows: column temperature 180 °C; injection port temperature 250 °C; detector temperature 300 °C; carrier gas (helium) flow rate 20 ml min^{-1}; air flow rate 50 ml min^{-1}; hydrogen flow rate 30 ml min^{-1}. The gas chromatograph was coupled to a 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 isoquinoline to blank solutions of the sample type to be analysed to give concentrations of 5, 10, 20, 40, 80 and 100 ng ml^{-1}. These were then carried through the extraction procedure. The calibration curve of the peak areas was linear over the working range 0·5 to 100 ng ml^{-1}. The retention times for isoquinoline and nicotine were 5·7 min and 6·6 min respectively.

Reproducibility

Ten determinations were carried out at each of the following concentrations. The results with means and standard deviations were:

ng ml^{-1}	5	10	20	40	80	100
Mean (s.d.)	5·0 (0·1)	9·9 (0·3)	20·1 (0·4)	40·3 (0·4)	79·9 (0·7)	100·0 (1·5)

Reproducibility at varying sample volumes was similar to that reported in our previously published

method (Feyerabend & Russell 1979). The evaporation stage, where the diethyl ether is evaporated down to approximately 200 μl, was not critical since the extract could be evaporated to dryness without loss of reproducibility. However, leaving a small volume ensured that a cleaner extract was injected on to the gas chromatograph.

Sensitivity

This modified method is sufficiently sensitive to measure nicotine concentrations of 0·1 ng ml^{-1}. No common basic drugs have been found to interfere with the analysis and the use of heparin preparations containing chlorbutol or chlorocresol as a preservative has presented no problems.

DISCUSSION

Contamination can be a hazard with such a sensitive method, although with care and rigid control the problem can be minimized. Sources of contamination can be categorized as follows:

(1) Contamination from the sample.
(2) Contamination from the reagents.
(3) Contamination from the atmosphere.
(4) Contamination from the apparatus.

1. *Contamination from the sample*

We have encountered a contaminant in certain samples which has a retention time similar to that of quinoline, the internal standard used in our previous methods. Although this contaminant is normally insignificant, occasionally it has been found to give rise to a peak one-fifth the size of that of the internal standard. We have avoided this problem by changing to a different internal standard, isoquinoline. This has a longer retention time than quinoline and therefore the column temperature had to be lowered to give adequate separation from nicotine. Further separation was achieved by reducing the column internal diameter and increasing the column length. Since isoquinoline decays slightly when left in samples for long periods, the compound was added after the diethyl ether and the mixture vortexed immediately to standardize the time. These modifications achieved good separation of the contaminant (Fig. 1A) and, despite the increase in retention times, it was still possible to process 50 samples per day. The micro-technique previously reported (Feyerabend & Russell 1979) has been similarly modified, and a typical chromatogram from a contaminated plasma sample is shown in Fig. 1B. Some samples were found to contain contaminants which eluted very slowly and gave rise to peaks that interfered with consecutive analyses. This was avoided by raising the column temperature to 250 °C after injecting six extracts and maintaining this temperature for at least 10 min. By this means, the contaminants were flushed from the column and further analyses could proceed. Finally, with such a sensitive method the possibility of contaminating the sample with nicotine during or after removal from the subject should be guarded against at all costs. Thus, although the micro-technique (Feyerabend & Russell 1979) is applicable in theory to finger-prick blood samples, the contamination which occurs even after thorough washing of the puncture site can lead to a 20-fold increase in plasma nicotine concentration compared with that in a sample taken simultaneously by venepuncture. Similarly, if a smoker touches the rim of a specimen tube containing 10 ml of blood, the plasma nicotine content can be increased by as much as 10 ng ml^{-1}.

2. *Contamination from the reagents*

Sodium hydroxide solutions. These are readily contaminated by nicotine derived either from glassware which has been handled by smokers or by storage in a nicotine-contaminated atmosphere. The purity of the reagent was easily checked by extracting 400 μl with 50 μl of butyl acetate in a Dreyer tube and injecting 3 μl of the solvent on to the gas chromatograph.

Butyl acetate. This solvent was found to be free from impurities if the analytical grade was redistilled before use.

Diethyl ether. Batches of this solvent supplied by various manufacturers and described as 'analytical grade' were tested for purity. These were taken through the extraction procedure using water as the sample and excluding the addition of the internal standard. The chromatogram obtained from four different brands analysed at the normal range and attenuation showed one batch to be highly contaminated and this was still unacceptable even after re-distillation or acid washing (Fig. 2). Batches of diethyl ether derived from the same manufacturer also varied in the degree of contamination. Thus, in practice, we have found it necessary to test the purity of each batch before use in the assay.

Distilled water. This can be contaminated by nicotine derived from the atmosphere or by smokers handling apparatus. Tap water is therefore always used when samples are diluted.

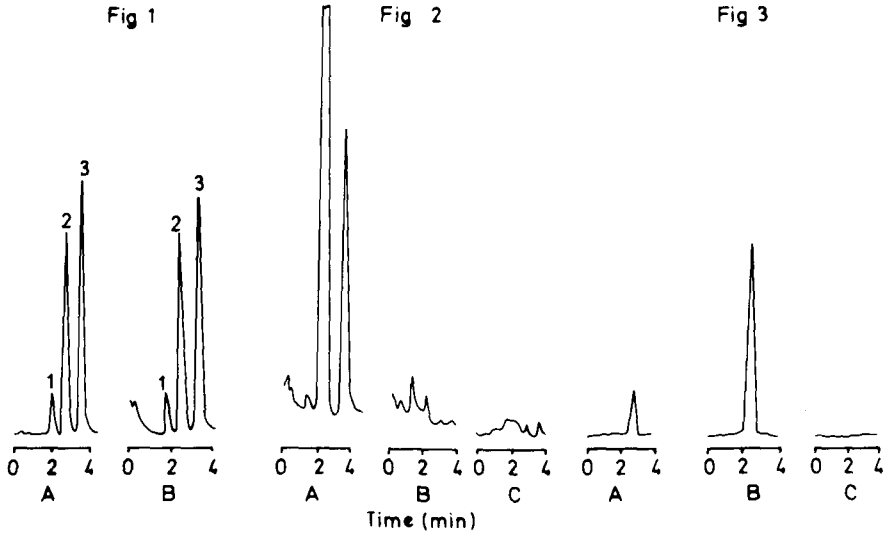

FIG. 1. Gas chromatograms of extracts from plasma (A) macro method (B) micro method. 1. contaminant. 2. Isoquinoline. 3. Nicotine.
FIG. 2. Gas chromatograms of extracts from tap water using contaminated diethyl ether (A), after acid washing (B), after redistilling (C).
FIG. 3. Gas chromatograms of extracts of tap water (A) contaminated Dreyer tubes, (B) contaminated centrifuge tubes, (C) using new Dreyer and centrifuge tubes.

3. *Contamination from the atmosphere*

The need to carry out nicotine analyses in a smoke-free area cannot be overstressed since the atmosphere is by far the most important source of contamination. This entails ensuring that the smoke-free laboratory is not connected by corridors or a ventilation system to areas where smoking is permitted. Contamination derived from the atmosphere was strikingly illustrated in our laboratory when a 50 ml bottle of pure frozen isoquinoline fell out of a refrigerator and broke on the floor. Although the contents were cleaned up immediately and the floor thoroughly washed, all the extraction tubes in the laboratory were affected (Fig. 3). Both Dreyer tubes (Fig. 3A) and centrifuge tubes (Fig. 3B) were polluted and clean chromatograms were obtained only after careful washing (Fig. 3C). Clean tubes stored for 48 h in the laboratory several weeks after the incident adsorbed sufficient isoquinoline to give rise to peaks which were equivalent to 25% of the normal internal standard peak. This one short exposure, therefore, resulted in atmospheric contamination which persisted for many months.

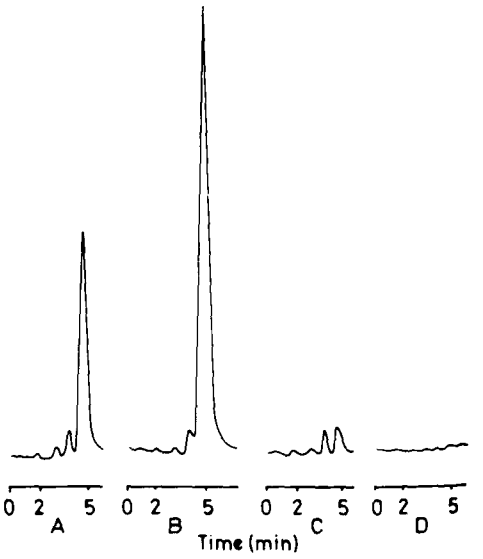

FIG. 4. Gas chromatograms of extracts from tap water using different batches of untreated Dreyer tubes (A–C) and after heat purification (D).

4. *Contamination from apparatus*

Dreyer tubes, even when new, could yield impure chromatograms (Fig. 4). As a precaution, all Dreyer tubes were kept in a fan-assisted oven at 450 °C for at least 24 h before use.

All new centrifuge tubes were found to be contaminated with nicotine and required machine-washing before they could be used.

Spurious chromatograph peaks were obtained if rubber tubing was used in the evaporation device,

but this was easily avoided by substituting plastic tubing. Needless to say, all purified tubes had to be stored in a smoke-free atmosphere to prevent further uptake of nicotine.

CONCLUSION

Nicotine is so ubiquitous a compound that it is hardly surprising that several workers have experienced difficulty in reproducibly measuring the small concentrations present in plasma samples. Although a laboratory may be designed as smoke-free in the sense that smoke cannot be perceived by sight or smell, sufficient contamination may be present to render any attempts to measure low concentrations of nicotine futile. Even in an isolated laboratory, painstaking precautions are essential to guard against external contamination. Nevertheless, it is feasible, in our experience, to achieve an acceptable working situation and, thereby, reproducible and meaningful results.

Acknowledgements

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