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Note

Assessment of exposure to environmental tobacco smoke using a high-performance liquid chromatographic method for the simultaneous determination of nicotine and two of its metabolites in urine

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The determination of nicotine, or one of its metabolites (cotinine), in biological fluids provides a useful objective assessment of exposure to environmental tobacco smoke (ETS) in studies to estimate the risks associated with passive smoking [1,2]. Cotinine levels are generally more informative than nicotine levels because cotinine has a longer half-life, and several studies have demonstrated a quantitative relationship between cotinine levels and exposure to ETS [3–5].

Whilst it is well known that the metabolism of nicotine yields many metabolites [6–8] there is no concise information on either the number or relative concentrations of these metabolites in the urine, serum or saliva of smokers. One reason for this is the lack of analytical methods, for the known nicotine metabolites, which are suitable for epidemiological studies. A radiometric high-performance liquid chromatographic (HPLC) method has been used to determine the pharmacokinetics of twelve nicotine metabolites in the rat [8], but the corresponding information is not available for humans.

We have recently described a simple HPLC method for the simultaneous determination of nicotine, cotinine and five additional nicotine metabolites in the urine of smokers [9]. One of the unidentified metabolites detected by this method, hereafter called metabolite 5 (see Fig. 1 of ref. 9), was present in significantly greater concentration than cotinine and we concluded that it might be a more sensitive measure of exposure to ETS than cotinine. However, the method as

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described was not sufficiently sensitive to detect nicotine or cotinine in the concentrations observed in passive smokers.

We describe here a modification of that method, which allows the simultaneous determination of nicotine, cotinine and metabolite 5 with sufficient sensitivity to provide a measure of exposure to ETS. We have evaluated the relative sensitivity of nicotine, cotinine and metabolite 5 as markers of exposure to ETS using samples collected from six non-smokers for three days after a period of exposure to ETS.

EXPERIMENTAL

Materials

Nicotine, cotinine and 1,3-diethylthiobarbituric acid were purchased from Sigma (Poole, U.K.). *trans*-3′-Hydroxycotinine was a gift from Professor Adlkofer (Hamburg, F.R.G.) and 3-pyridylcarbinol was obtained from Aldrich (Gillingham, U.K.). All other chemicals and HPLC solvents were from Fisons (Loughborough, U.K.).

Chromatography

We used a Waters (London, U.K.) 590 pump, a WISP 710B autosampler, a Model 440 detector and a Nova-Pak C_{18} column (15 cm×3.9 mm I.D.). Chromatography was performed at ambient temperature using a flow-rate of 1.0 ml/min and a mobile phase of water–methanol (3.5:5.5, v/v) containing 20 m*M* pentanesulphonic acid. Peak detection was at 546 nm, with detector sensitivity at 0.005 a.u.f.s. and a chart speed of 5 mm/min.

Assay procedure

The method employed the same precolumn derivatization of the urinary nicotine metabolites as previously described [9], with the following modifications to increase assay sensitivity: the volume of urine treated was increased, and a butanol extraction step was employed to concentrate the metabolites. The extraction step had the additional advantage of making the nicotine metabolite derivatives stable for several hours.

A 1.0-ml sample of urine was added to a Pyrex test-tube (7.5 cm×10 mm I.D.) followed by the sequential addition of 0.4 ml of 4 *M* sodium acetate buffer (pH 4.7), 0.2 ml of 1.5 *M* potassium cyanide in water, 0.2 ml of 0.4 *M* chloramine-T in water and 0.5 ml of 50 m*M* diethylthiobarbituric acid in water–acetone (1:1, v/v). The tube was capped, and the contents were mixed thoroughly and incubated for 15 min. This was followed by addition of ca. 0.5 g of sodium sulphate and 125 μl of butanol. The tube was recapped and the contents were again mixed thoroughly. A further 50-μl aliquot of butanol was added, and the tube was inverted cautiously twice to allow the two phases to separate. The phase separation was completed by centrifuging for 5 min at 2000 rpm (ca. 1000 *g*). A 25-μl aliquot of the upper butanol phase was transferred to a WISP autosampler microvial, and 5 μl were injected onto the column. Each sample required a total run-time of 8 min, and ten samples could be handled simultaneously.

Standards and samples

Standard curves were obtained using pure nicotine or cotinine in water, with concentrations from 0 to 570 nmol/l and the peak height was plotted versus concentration. Metabolite 5 concentrations were estimated by comparing the peak height obtained with the cotinine standard and expressing the results as nmol/l cotinine equivalents.

A urine sample from a moderate smoker was diluted 1:100 and 1:25 in non-smoker's urine for use as a quality control sample.

The following experiment was conducted to monitor the appearance and disappearance of nicotine, cotinine and metabolite 5; this was similar to that described by Jarvis et al. [10] and involved short-term acute exposure to ETS in a public house. Six non-smoking volunteers, three men and three women, participated in the study. They provided samples of urine immediately before a 2-h period of exposure to ETS in a public house. Further samples of urine were collected at the end of the exposure period (2 h) and at 4, 6, 8, 18, 24, 28, 48 and 72 h. All the subjects were asked to avoid ETS during the three-day period. All the urine samples collected were analysed for nicotine, cotinine and metabolite 5 by the HPLC method described.

RESULTS

Fig. 1 shows typical chromatographic results obtained with the cotinine standard, and with two urine samples collected from a non-smoker before and 4 h after exposure to ETS. The retention times of pure nicotine, cotinine and 3-pyr-

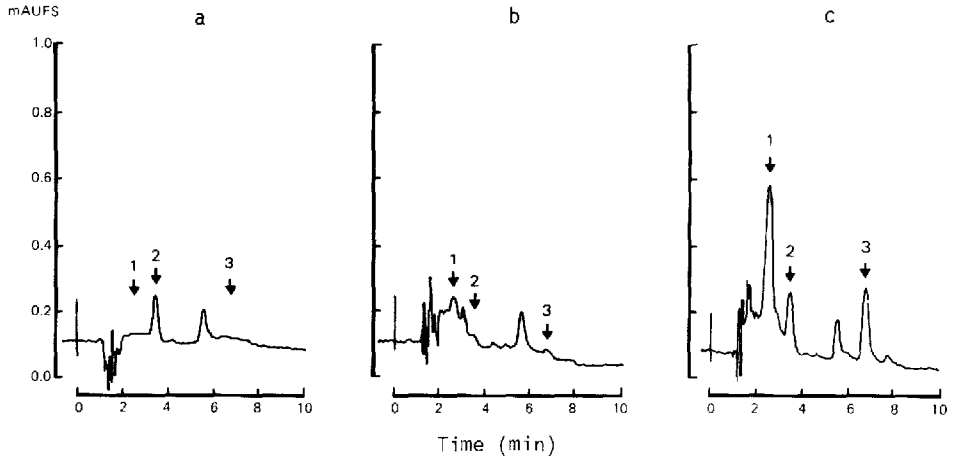

Fig. 1. Typical chromatograms obtained with a cotinine standard, and with urine sample from non-smokers before and after exposure to ETS. (a) Cotinine standard (56.7 nmol/l) in water; (b) urine sample from a non-smoker before exposure to ETS in a public house; (c) urine sample from a non-smoker 4 h after exposure to ETS in a public house. Retention times: metabolite 5, 2.5 min; cotinine, 3.5 min; nicotine, 6.7 min. The minor peak at 5.5 min represents a reagent trace seen in all chromatograms.

TABLE I

ESTIMATED VARIATION OF THE HPLC METHOD FOR URINARY NICOTINE, COTININE AND METABOLITE 5

Typical smokers levels for cotinine are 2000–20 000 nmol/l [6].

Compound	Within-assay variation ($n=5$)			Between-assay variation ($n=5$)					
	Mean (nmol/l)	S.D. (nmol/l)	C.V. (%)	Mean (nmol/l)	S.D. (nmol/l)	C.V. (%)	Mean (nmol/l)	S.D. (nmol/l)	C.V. (%)
Nicotine	99.3	5.9	5.9	154	9.4	6.2	39.7	5.7	14.3
Cotinine	90.8	7.2	7.9	121	16.2	13.4	31.8	4.2	13.1
Metabolite 5	247	11.1	4.5	300	27.6	9.2	111	18.6	16.8

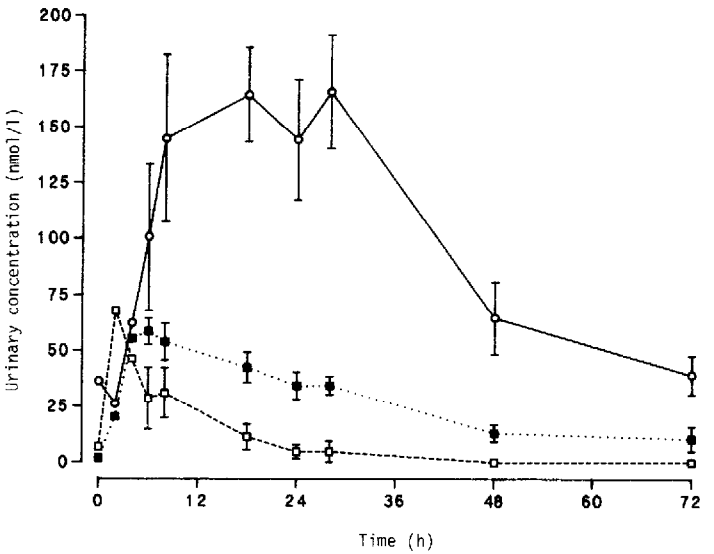

Fig. 2. Mean urinary nicotine concentrations in the six non-smokers for three days after a 2-h exposure to ETS in a public house. (□) Nicotine; (■) cotinine; (○) metabolite 5. All points are ± 1 S.E.

idylcarbinol were identical with those of the three peaks observed in the urine samples tested (Fig. 1). Standard curves were linear over at least 0–570 nmol/l.

Recoveries of 73–115 and 75–94% were obtained for nicotine and cotinine, respectively, when added to a urine sample from a non-smoker in concentrations in the range 57–570 nmol/l, and the results were compared with the respective standard curves. The absolute recoveries, i.e. the extent to which the butanol extracted the substances, were estimated by comparing the results with the original HPLC method [9], which does not employ an extraction step. The absolute recoveries of pure nicotine and cotinine were 79 and 107%, respectively. The absolute recoveries of nicotine, cotinine and metabolite 5 from a smoker's urine sample were 89–104, 83–84 and 90–93%, respectively.

The sensitivity of the method was estimated to be 5 nmol/l (at a signal-to-

noise ratio of 3) with an injection volume of 5 μl. Increasing the injection volume did not increase sensitivity since the peaks tend to tail as a result of the higher proportion of butanol.

The within-assay and between-day variations for nicotine, cotinine and metabolite 5 are shown in Table I.

The mean values of urinary nicotine, cotinine and metabolite 5 for the six non-smokers before and after exposure to ETS are shown in Fig. 2. After the period of exposure there were significant increases in urinary nicotine, cotinine and metabolite 5 concentrations. The nicotine concentration peaked after ca. 2 h, cotinine after ca. 6 h and metabolite 5 after ca. 20 h. Urinary cotinine and metabolite 5 concentrations increased at approximately the same rate, but the metabolite 5 concentrations continued to increase as cotinine levels were generally falling, to reach a peak concentration about three times higher than cotinine.

DISCUSSION

The HPLC method described allows the simultaneous determination of nicotine, cotinine and metabolite 5 in urine with sufficient sensitivity to estimate exposure to ETS. The sensitivity of the method was ca. 5 nmol/l and its performance in terms of accuracy and precision was satisfactory. About 30 samples could be analysed each day.

The results obtained with the six volunteers exposed to ETS in a public house demonstrate that metabolite 5 is a useful marker of exposure to ETS, and is likely to be a more sensitive measure than cotinine since it is produced in greater amounts and appears to have a longer urinary half-life (Fig. 2).

However, the identity of metabolite 5 remains uncertain. A recent report by Neurath et al. [11] concluded that *trans*-3′-hydroxycotinine was a major nicotine metabolite in the urine of smokers, accounting for ca. 40% of the urinary nicotine metabolites, similar to our own observations for metabolite 5 [9]. However, they provide no independent evidence that the metabolite measured by their gas chromatographic method is hydroxycotinine. They have also reported that the serum half-life of hydroxycotinine is ca. 6 h [12], which suggests that hydroxycotinine is unlikely to be a major component of metabolite 5 since the latter appears to have a long urinary half-life (Fig. 2). A recent study by Kyerematen et al. [8] has shown that hydroxycotinine is a minor metabolite in the urine of rats, accounting for only 4.5% of a [^{14}C]nicotine dose, but the applicability of these results to human is uncertain.

We have partially purified metabolite 5 from several litres of smoker's urine, and preliminary results suggest that it comprises 3-pyridylcarbinol and hydroxycotinine [13]. However, until we develop analytical methods that can satisfactorily discriminate between these two substances in urine we do not know their relative proportions in the urine of smokers.

The general conclusions are that the HPLC method is suitable for the simultaneous determination of nicotine, cotinine and metabolite 5 in passive smokers and that metabolite 5 estimations are a useful addition to cotinine in the assessment of exposure to ETS.

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