Journal of Chromatography, 419 (1987) 375–380
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3711

Note

Simultaneous determination of nicotine, cotinine and five additional nicotine metabolites in the urine of smokers using pre-column derivatisation and high-performance liquid chromatography

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(First received January 15th, 1987; revised manuscript received March 18th, 1987)

The determination of cotinine, a nicotine metabolite, in serum, urine and saliva has become widely used as a marker of cigarette smoking, in studies of smoking-related diseases, on account of its sensitivity and specificity [1]. It is usually analysed in specialist laboratories using gas chromatography (GC) [2–4], high-performance liquid chromatography (HPLC) [5] or radioimmunoassay (RIA) methods [6, 7].

We have previously described a simple colorimetric assay for urine nicotine metabolites which is a suitable inexpensive alternative to cotinine assays for the assessment of smoking status [8]. The method is based upon the production of coloured derivatives of nicotine metabolites using a chemical reaction similar to that originally described by Konig in 1904 [9]. The results obtained, using urine samples from 128 pregnant smokers, were highly correlated with a cotinine RIA method in our laboratory ($r=0.85$) but demonstrated that cotinine accounted for only 2–30% of the nicotine metabolites in urine. This result was unexpected since cotinine is often referred to as the major nicotine metabolite [10–12]. We decided to determine the number of metabolites detected by the colorimetric assay by separating the derivatives using isocratic reversed-phase HPLC. We describe here the results of this study.

EXPERIMENTAL

Nicotine, cotinine and 1,3-diethyl-2-thiobarbituric acid were obtained from Sigma (Poole, U.K.). Pyridyl acetic acid, 3-pyridinecarboxaldehyde, 3-pyridyl-

0378-4347/87/$03.50

acetonitrile, 3-pyridylcarbinol, 3-pyridylcarbinol N-oxide, pyrrole and pyrrolidin were obtained from Aldrich (Gillingham, U.K.). Nicorette was obtained from Lundbeck (Luton, U.K.) and all other chemicals including HPLC solvents were obtained from Fisons (Loughborough, U.K.).

Chromatography conditions

We used a Waters (London, U.K.) 590 pump, WISP 710B autosampler, Model 440 detector and a Waters μBondapak C_{18} column (30 cm×3.9 cm). The operating conditions were as follows: temperature, ambient; pressure, 260 bar; flow-rate, 2 ml/min; chart speed, 1 cm/min; wavelength, 546 nm; solvent, water–methanol (1:2) containing 0.02 *M* pentanesulphonic acid.

Preparation of nicotine metabolite derivatives

Urine samples were subjected to the colorimetric assay, reported previously [8] except that we used diethylthiobarbituric acid, rather than barbituric acid, as the final colour reagent. This gave derivatives with absorbance maxima around 530 nm rather than 505 nm.

A 0.5-ml sample of urine was added to a 3-ml WISP vial followed by the sequential addition of 0.2 ml of 4 *M* acetate buffer (pH 4.7), 0.1 ml of 1.5 *M* potassium cyanide in water, 0.1 ml of 0.4 *M* chloramine-T in water and 0.5 ml of 50 mmol/l diethylthiobarbituric acid in water–acetone (50:50, v/v). The vial contents were then mixed, incubated for 15 min, transferred to the WISP autosampler and 5 μl injected onto the column.

The timing of the injection was carefully controlled since the derivatives are unstable, decaying at a rate of about 10% in 15 min. The coloured derivatives are stable when extracted into organic solvents, such as butanol, ethyl acetate and chloroform, but the extraction is incomplete and therefore not suitable for the purposes of this study.

Standards

Standard curves were obtained using pure nicotine or cotinine in water, with concentrations from 0 to 20 μg/ml, and plotting peak height versus concentration. The unknown metabolites were quantified by comparing the peak height obtained with the cotinine standard and expressing the results as μg/ml cotinine equivalents.

Samples

Urine samples from twenty male cigarette smokers were retrieved from storage at −40°C and analysed for nicotine metabolites. To confirm that the coloured derivatives were nicotine metabolites we also assayed urine samples from two non-smokers after they had chewed a single piece of Nicorette containing 4 mg nicotine.

RESULTS

Separation

An example chromatogram obtained with a smoker's urine sample is shown in Fig. 1. Under the chromatographic conditions described seven distinct peaks were observed. The samples obtained after chewing Nicorette also demonstrated seven peaks with the same retention times as those obtained with the smokers (Fig. 2). No peaks were observed with urine samples from non-smokers.

Solutions of pure nicotine and cotinine yielded single peaks with the same retention times as peaks 6 and 7 (Fig. 1). 3-Pyridylcarbinol had the same retention time as metabolite 5, 3-pyridylacetic acid and 3-pyridylcarboxaldehyde had the same retention time as metabolite 4. None of the substances tested had retention times equivalent to metabolites 1, 2 and 3.

Each sample required a run time of 6 min which allowed the analysis of about 50 samples per day.

Analytical data

Calibration curves for nicotine and cotinine were linear over at least 0–20 μg/ml.

Recoveries of 91–96% and 84–91% were obtained for nicotine and cotinine, respectively, when added to urine samples in known amounts and the results compared with the nicotine or cotinine standard curves.

The minimum detectable levels of nicotine and cotinine (defined as a peak height of 2 mm at 0.005 a.u.f.s. or a signal-to-noise ratio of about 3) were found to be 0.01 μg/ml, using known dilutions of a smokers urine, with an injection volume of 20 μl. If the injection volume was increased beyond 50 μl the metabolites were less well separated.

Repeat analyses of a smokers urine pool, assayed once each day for ten days, yielded a coefficient of variation of 9.0% for nicotine and 8.4% for cotinine.

Cotinine results obtained with the HPLC method were highly correlated with a cotinine GC method [4]; $r=0.99$ using ten smokers' urine samples, HPLC = 0.93(GC) − 0.11 μg/ml.

The results obtained for twenty cigarette smokers are shown in Fig. 3. The nicotine results ranged from 0.03 to 1.4 μg/ml and the cotinine results from 0.4 to 3.5 μg/ml. The average contribution of each of the individual metabolites to the total concentration of metabolites is shown in Table I. The mean contribution of nicotine and cotinine for the twenty smokers was 5% and 15%, respectively.

DISCUSSION

The results of this study demonstrate that the colorimetric assay reacts primarily with seven substances (Fig. 1) in the urine of smokers to produce derivatives with absorbances around 530 nm. These derivatives can be separated using the HPLC method described and we conclude that they are nicotine, cotinine and five unidentified nicotine metabolites since they are present in the urine of smokers and Nicorette users (Fig. 2) but not non-smokers.

The results obtained with the pyridyl chemicals suggest that metabolite 5 may

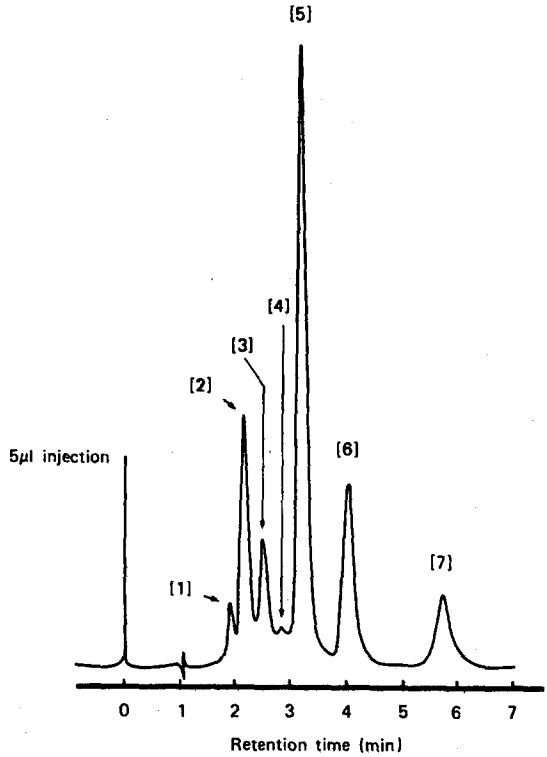

Fig. 1. Example chromatogram observed with a 5-μl smoker's urine sample after having subjected the sample to a chemical reaction producing coloured nicotine metabolite derivatives. Column, μBondapak C_{18}; mobile phase, water–methanol (2:1) containing 0.02 *M* pentanesulphonic acid; flow-rate, 2 ml/min; detection, 546 nm. Peaks: 1-5 = unidentified nicotine metabolites; 6 = cotinine; 7 = nicotine.

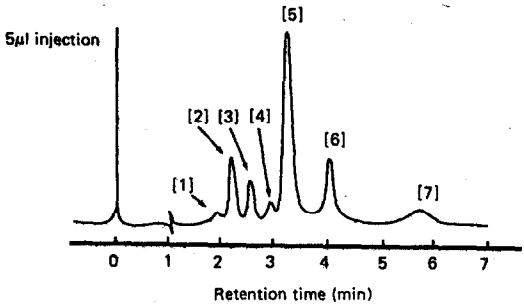

Fig. 2. Chromatogram obtained with a 5-μl urine sample from a non-smoker after chewing nicotine gum. Pre-column derivatisation, chromatography conditions and peaks are identical to those in Fig. 1.

be 3-pyridylcarbinol and metabolite 4 may be 3-pyridylacetic acid and/or 3-pyridylcarboxaldehyde. None of the other chemicals tested gave retention times equivalent to metabolites 1, 2 and 3. Only 3-pyridylacetic acid has been previously identified in the urine of smokers [13] but further experiments using mass spectrometry will be necessary to identify the structures of metabolites 1–5.

TABLE I

PERCENTAGE CONTRIBUTION OF EACH INDIVIDUAL NICOTINE METABOLITE TO THE TOTAL METABOLITE CONCENTRATION OBSERVED IN TWENTY MALE CIGARETTE SMOKERS

Metabolite★	Mean percentage of the total metabolite concentration	Standard error of the mean	Maximum	Minimum
1	4.9	0.2	6.1	3.5
2	20.0	0.9	28.4	14.1
3	11.5	0.4	16.5	8.9
4	3.6	0.1	4.7	2.7
5	40.4	1.9	51.6	25.9
6 (Cotinine)	14.9	1.4	30.5	8.6
7 (Nicotine)	4.6	0.8	16.5	1.0

★See Fig. 1.

We have used peak heights, rather than areas, to quantify the unknown metabolites, as we do not have an integrator. Since the peaks become broader with increasing retention times the estimates of relative concentrations will, therefore, only be approximate.

The metabolite concentrations obtained for each of the twenty smokers (Fig. 3) demonstrate that metabolite 5 is proportionately the most important, accounting for 40%, on average, of the total metabolite concentration (Table I). Nicotine and cotinine accounted for 5% and 15% of the total, respectively.

Cotinine is, therefore, not the major urinary nicotine metabolite but just one in the sequence of nicotine degradation to pyridylacetic acid [13, 14, 16]. This

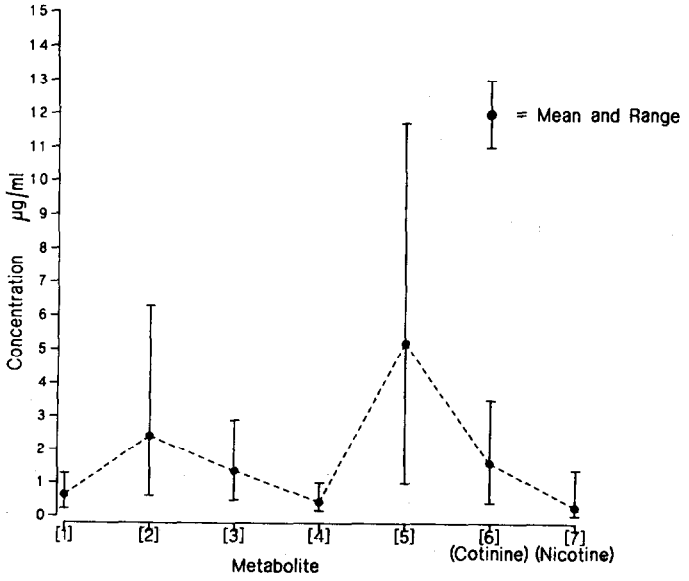

Fig. 3. Nicotine metabolite concentrations (μg/ml) in urine samples from twenty male cigarette smokers.

has implications for future research on smoking-related diseases since researchers use cotinine as a quantitative marker of smoke intake [17] and, more recently, have started to use such data to estimate the health risks of passive smoking [18]. The plasma half-life of cotinine has been shown to vary from 11 to 37 h, in a study involving twelve subjects [11], which suggests that between-person differences in nicotine metabolism and excretion may account for much of the variability of urine nicotine metabolites observed here (Table I and Fig. 3). This will limit the value of cotinine as a quantitative marker of smoke intake and the HPLC method described will be useful in investigating the potential of other nicotine metabolites as markers of smoke intake. In particular, metabolite 5 may be a more sensitive marker of passive smoking than cotinine since it is present in higher concentration than cotinine (Fig. 3).

This HPLC method allows the rapid simultaneous determination of urine nicotine and cotinine. The results are highly correlated ($r=0.99$) with a GC method and the range of values obtained with smokers samples are in agreement with other studies [2, 4, 5]. The method provides an estimate of the concentration of five other metabolites and one of these, i.e. metabolite 5, may be particularly useful in estimating passive exposure to tobacco smoke.

ACKNOWLEDGEMENTS

We would like to thank the Independent Scientific Committee on Smoking and Health, and the Tobacco Products Research Trust for their financial support. We also thank Dr. C. Feyerabend for the GC comparison used here.

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