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Note

Simple high-performance liquid chromatographic method for rapid determination of nicotine and cotinine in urine

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Nicotine is a major component of tobacco smoke and plays a significant role in maintaining the smoking habit [1, 2]. Monitoring individual nicotine exposure provides a helpful biochemical measure for the control of active smoking behaviour or for validation of abstinence from smoking. The additional determination of cotinine (a metabolite of nicotine) offers advantages over that of nicotine alone, because the biological half-life of cotinine is as long as 10.9—37.0 h [3]. Thus cotinine can be taken as an indicator of chronic nicotine exposure whilst the excretion of nicotine, which is rapidly detoxified, provides information about recent exposure.

Several methods for the determination of nicotine and/or cotinine in biological fluids have been reported employing UV spectroscopy [4], gas chromatography (GC) [5—9], radioimmunoassays [10—13], and high-performance liquid chromatography (HPLC) [14—16].

UV spectroscopy has some disadvantages as to sensitivity and specifity, but GC is a well established method even for the low urinary levels of non-smokers [17]. However, it is difficult to determine nicotine and cotinine by GC in the same run, and low-nicotine samples are subject to considerable contamination problems [18].

Radioimmunoassays are very sensitive and cross-reactivity problems appear to have been solved [11], but they require frequent checking of standard curves and are in general more expensive than chromatographic methods.

Watson [14] has presented a HPLC method with UV detection for the simultaneous determination of nicotine and cotinine in the urine of smokers, but the chromatograms shown are not satisfactory. Owing to the use of ethyl acetate in the mobile phase there is also the possibility of UV cut-off problems leading to impaired sensitivity.

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Another technique has been introduced by Maskarinec et al. [15]. Sample preparation is accomplished here by adsorption on an ion-exchange resin column, subsequent elution with a solvent mixture and evaporation to dryness. This procedure leads to well purified extracts, but large amounts of solvent are necessary. The evaporation step lengthens the analysis time and adversely affects the reproducibility for the volatile alkaloid nicotine.

Only for special research purposes, HPLC is also capable of the determination of nicotine and some of its metabolites in human plasma after the administration of ^{14}C-labelled nicotine [16].

The present work provides a convenient HPLC method for the determination of nicotine and cotinine in human urine. Optimized HPLC conditions, i.e. selection of mobile phases and columns, lead to a good and fast resolution of peaks. A simple, reproducible and time-saving solvent extraction technique is presented. By avoiding solvent evaporation steps, sufficient concentration and prepurification is achieved with small amounts of solvent (only 1 ml of dichloromethane per sample).

EXPERIMENTAL

Chemicals

(−)-Nicotine-(+)-tartrate (mol. wt. 498.4 g/mol) was purchased from B.D.H. (Poole, U.K.), (−)-cotinine from Roth (Karlsruhe, F.R.G.) and (+)-amphetamine sulphate from E. Merck (Darmstadt, F.R.G.). Solvents and chemicals (E. Merck) of at least analytical-reagent grade were used. Solutions were prepared with twice-distilled water.

Preparation of standards

A standard solution contained 12.30 mg of nicotine tartrate (corresponding to 4.00 mg of nicotine base) and 4.00 mg of cotinine in 200 ml of water.

The internal standard solution was prepared by dissolving 25 mg of amphetamine sulphate in 50.0 ml of water.

Extraction procedure

If determinations could not be performed within 24 h, urine samples were stored at −20°C.

A 4.00-ml volume of urine, 200 μl of internal standard solution, 1.0 ml of dichloromethane and 0.5 ml of 3 *M* sodium hydroxide were mixed in a 10-ml screw-capped glass vial (Sovirel, France). In every run, an additional extraction of nicotine- and cotinine-free urine was made in the same way after the addition of 200 μl of standard solution (calibration standard). The tubes were closed and shaken for 10 min on a rotating device (ca. 40 r.p.m.). After centrifugation for 10 min at 3000 *g*, 500 μl of the organic layer were added to 500 μl of 0.5 *M* sodium hydroxide in a conical 1.5-ml polypropylene tube (Sarstedt, Nümbrecht, F.R.G.) and shaken vigorously using a vortex stirrer three times for 10 sec. Finally, the tubes were centrifuged for 1 min (Eppendorf centrifuge 5412). For each HPLC determination, 40 μl of the dichloromethane layer were used.

HPLC conditions

The HPLC system consisted of a Waters Model 510 pump (Waters Assoc., Milford, MA, U.S.A.), a Pye-Unicam LC-3 variable-wavelength UV detector (Pye-Unicam, Cambridge, U.K.), operated at 260 nm, and a Pye-Unicam PM 8252 recorder.

Separations were achieved using a Nucleosil Si 50 column (particle size 5 μm; 125 × 4 mm I.D.) obtained from Gynkotek (Munich, F.R.G.).

The mobile phase was dichloromethane—diisopropyl ether—methanol—conc. ammonia solution (25%) (62:30:7.9:0.1). Usually, a flow-rate of 2.0 ml/min was maintained and the pressure of the system did not exceed 7 MPa.

Peak heights of nicotine, cotinine and amphetamine were measured, and the drug to internal standard ratios compared with those of the calibration standard, which corresponds to a urinary concentration of 1 μg/ml as well of nicotine as of cotinine.

For assessment of selectivity and identity of peaks, an extract of a smoker's urine was injected and the eluates of the nicotine and cotinine peaks were collected separately. Both samples were evaporated from originally 1—2 ml to dryness under vacuum at 30°C, taken up in 2-propanol and then analysed by gas chromatography—mass spectrometry (GC—MS). The evaporative loss was ca. 15% for nicotine and 25% for cotinine. The corresponding recoveries seemed to be adequate, because no quantitative measurements were intended here.

Precision

A single urine sample was analysed on three separate occasions in order to determine the between-day coefficient of variation. For elucidation of the within-day variability, five specimens of the same sample were analysed on each day.

Human urine samples

The 24-h urine samples of 56 persons, mainly students (smokers and non-smokers) were taken, and nicotine and cotinine were determined as described above; 31 of the subjects were males, 25 were females, and the median age was 24 (range 13—36) years. For comparison with previous work, 24-h urine volumes were measured and concentrations of creatinine were determined using a colorimetric method (Test-Combination Creatinin, Boehringer Mannheim, F.R.G.). Afterwards, urinary data were compared with the respective smoking status based on the subject's self-report.

RESULTS

Nicotine, cotinine and internal standard peaks are well resolved as shown in Fig. 1. The washing step within 0.5 *M* sodium hydroxide affords sufficient chromatographic purity, as can be seen especially from Fig. 1B (non-smoker's urine). Retention times were 2.9 min for cotinine, 4.4 min for nicotine, and 8.1 min for amphetamine. Nicotine and cotinine were identified as described above in HPLC eluates by GC—MS and, apart from certain amounts of solvent stabilizers, revealed to be pure.

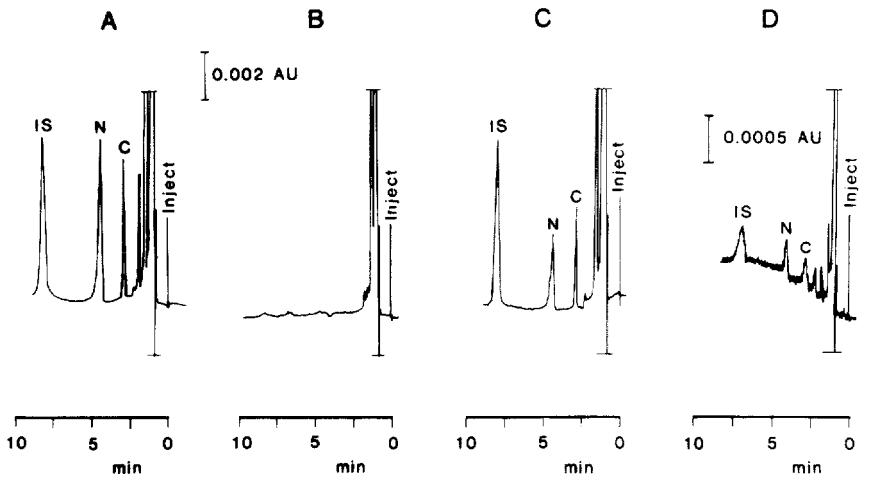

Fig. 1. Chromatograms of extracts of (A) a smoker's urine, (B) a non-smoker's urine (blank urine, exceptionally not spiked with internal standard) and (C) blank urine spiked with 1 μg/ml nicotine and 1 μg/ml cotinine (internal standard added). (D) For illustration of the signal-to-noise ratio, a ten-fold dilution of (C) in dichloromethane was injected and detected at four-fold sensitivity as indicated. UV detection at 260 nm. Peaks: C = cotinine; N = nicotine; IS = internal standard (amphetamine).

No interferences were seen in the 56 human urine samples studied. However, haloperidol, chlorpromazine, amitryptiline and cyproheptadine appear also (disturbingly) in the chromatogram, and this may also happen with some other lipophilic drugs containing a basic nitrogen. Although even in these cases peak overlap is usually slight enough to accomplish estimation of peak heights, it is advisable to exclude subjects under neuroleptic or antidepressant therapy. Because amphetamine is less frequently used medically than most other psychoactive drugs, its application as an internal standard is advantageous in respect to interference problems.

Other internal standards employed in HPLC of nicotine and cotinine, such as desipramine [14] and acetanilide [16], are less suitable in this respect. The basic idea of the internal standard here is only to check for equal extraction conditions and the possibility to calculate unrespectively of the injection volume. The recoveries were found to be 94% for nicotine and 51% for cotinine.

At a signal-to-noise ratio of 5, the minimum detectable amounts of nicotine and of cotinine were found to be ca. 15 ng direct on column (see Fig. 1D). Thus, quantitations were performed only for peaks corresponding to more than 50 ng/ml of each alkaloid in order to assure sufficient accuracy.

Calibration curves for nicotine and cotinine showed linearity over the range 0.1—10 μg/ml in urine. Within-day and between-day coefficients of variation for cotinine and nicotine were ca. 5% or less (Table I). This is an acceptable range for precision as well as reproducibility.

Fig. 2 gives an impression of nicotine and cotinine levels in the urine of smokers and non-smokers. In the majority of cases, non-smokers have nicotine and cotinine concentrations below the detection limit. Urine samples from smokers contain (depending on their individual smoking dose) increasing

TABLE I

ANALYTICAL INTRA- AND INTER-DAY VARIABILITY OF NICOTINE AND COTININE CONCENTRATIONS IN A URINE SAMPLE OF A SMOKER

Compound	Concentration (mean ± S.D.) (μg/ml)	Coefficient of variation (%)
Within-day (n = 5)		
Nicotine	1.63 ± 0.08	4.6
Cotinine	1.37 ± 0.08	5.9
Between-day (n = 3)		
Nicotine	1.55 ± 0.07	4.5
Cotinine	1.37 ± 0.07	5.2

amounts of these alkaloids. For comparison, the mean cotinine levels in the heavy smoking group (more than five cigarettes per day) can also be expressed as 2.86 (range 1.26—5.71) μg/mg of creatinine or as 2.50 (1.21—3.86) mg per day (n=7). These findings are consistent with literature data concerning both nicotine [12, 17, 19—21] and cotinine [12, 13, 20—25] values.

Creatinine excretion rates were 1.11 ± 0.68 g per day (mean ± S.D.) in all 56 subjects. This great inter-individual variation may introduce some more spread into urinary cotinine data when based on creatinine concentrations. Hence I prefer the units μg/ml and mg per day for cotinine.

Whereas renal cotinine clearance is only slightly affected by urinary pH fluctuations [3], nicotine excretion is markedly increased in acidified urine [3, 26—29]. From this point of view, interpretation of urinary nicotine levels is difficult and necessarily requires consideration of pH values.

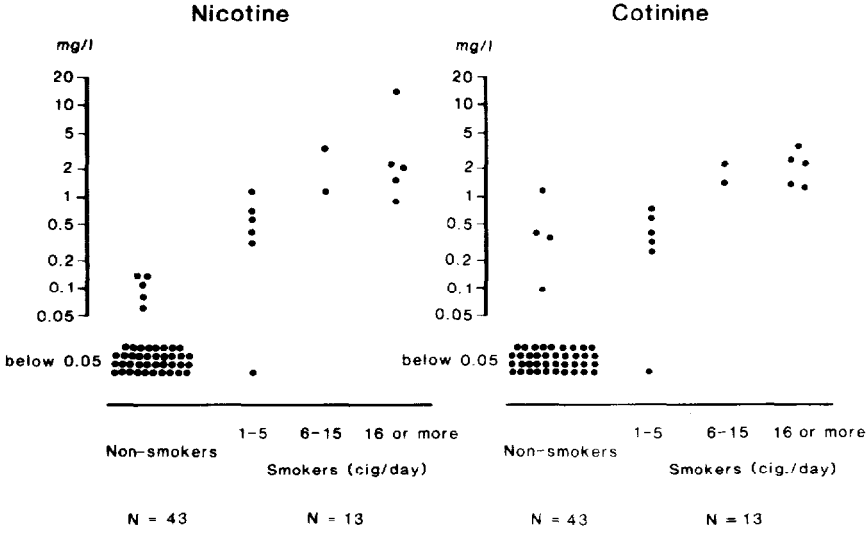

Fig. 2. Urinary nicotine and cotinine levels of smokers and non-smokers. Notice the large number of non-smokers with levels below the limit of quantification.

The present method makes is possible to differentiate between smokers and non-smokers. Commonly, non-smoking status is accepted for urinary cotinine concentrations below 100 ng/ml [25], but passive smoking exceptionally can lead to levels of up to 250 ng/ml nicotine [19] and 1885 ng of cotinine per mg creatinine [12]. All nicotine and cotinine levels of non-smokers presented here are below these limits. Nevertheless, considering the three highest cotinine concentrations for non-smokers in Fig. 2, it is more probable that these subjects (only 7% of the self-reported non-smokers) are indeed smokers.

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REFERENCES

1 M.A.H. Russell and C. Feyerabend, Drug Metabol. Rev., 8 (1978) 29.
2 L.T. Kozlowski, Drug Merchandising, 1 (1980) 36.
3 N.L. Benowitz, F. Kuyt, P. Jacob, III, R.T. Jones and A.L. Osman, Clin. Pharmacol. Ther., 34 (1983) 604.
4 H.B. Hucker, J.R. Gillette and B.B. Brodie, J. Pharmacol. Exp. Ther., 129 (1960) 94.
5 A.H. Beckett and E.J. Triggs, Nature (London), 211 (1966) 1415.
6 P.F. Isaac and M.J. Rand, Nature (London), 236 (1972) 308.
7 C. Feyerabend and M.A.H. Russell, J. Pharm. Pharmacol., 31 (1979) 73.
8 C. Carruthers and A. Neilson, Mikrochim. Acta, 1980 II (1980) 59.
9 G.E. Hardee, T. Stewart and A.C. Capomacchia, Toxicol. Lett., 15 (1983) 109.
10 J.J. Langone, H.B. Gjika and H. Van Vunakis, Biochemistry, 12 (1973) 5025.
11 A. Castro, N. Monji, H. Malkus, W. Eisenhart, H. McKennis, Jr. and E.R. Bowman, Clin. Chim. Acta, 95 (1979) 473.
12 R.A. Greenberg, N.J. Haley, R.A. Etzel and F.A. Loda, N. Engl. J. Med., 310 (1984) 1075.
13 N.J. Wald, J. Boreham, A. Bailey, C. Ritchie, J.E. Haddow and G. Knight, Lancet, i (1984) 230.
14 I.D. Watson, J. Chromatogr., 143 (1977) 203.
15 M.P. Maskarinec, R.W. Harvey and J.E. Caton, J. Anal. Toxicol., 2 (1978) 124.
16 G.A. Kyerematen, M.D. Damiano, B.H. Dvorchik and E.S. Vesell, Clin. Pharmacol. Ther., 32 (1982) 769.
17 C. Feyerabend, T. Higenbottam and M.A.H. Russell, Brit. Med. J., 284 (1982) 1002.
18 C. Feyerabend and M.A.H. Russell, J. Pharm. Pharmacol., 32 (1980) 178.
19 M.A.H. Russell and C. Feyerabend, Lancet, i (1975) 179.
20 R.G. Wilcox, J. Hughes and J. Roland, Brit. Med. J., 2 (1979) 1026.
21 S.K. Ghosh, J.R. Parikh, V.N. Gokani, M.N. Rao, S.K. Kashyap and S.K. Chatterjee, J. Soc. Occup. Med., 29 (1980) 113.
22 W. Luck and H. Nau, N. Engl. J. Med., 311 (1984) 672.
23 S. Matsukura, T. Taminato, N. Kitano, Y. Seino, H. Hamada, M. Uchihashi, H. Nakajima and Y. Hirata, N. Engl. J. Med., 311 (1984) 828.
24 A. Woodward, H. Miles and N. Grgurinovich, Lancet, i (1984) 935.
25 D. Hoffmann and K.D. Brunnemann, Cancer Res., 43 (1983) 5570.
26 H.B. Haag and P.S. Larson, J. Pharmacol. Exp. Ther., 76 (1942) 235.
27 A.H. Beckett, J.W. Gorrod and P. Jenner, J. Pharm. Pharmacol., 24 (1972) 115.
28 C. Feyerabend and M.A.H. Russell, Brit. J. Clin. Pharmacol., 5 (1978) 293.
29 S. Matsukura, N. Sakamoto, K. Takahashi, H. Masuyama and H. Muranaka, Clin. Pharmacol. Ther., 25 (1979) 549.