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High-performance liquid chromatographic determination of nicotine and its urinary metabolites via their 1,3-diethyl-2-thiobarbituric acid derivatives

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ABSTRACT

The 1,3-diethyl-2-thiobarbituric acid (DETBA) assay for nicotine metabolites has been improved so that it can be used to determine the concentrations of nicotine and up to 12 metabolites in the urine of humans and laboratory animals, including phase 2 metabolites. The products of β-glucuronidase cleavage found in human urine were mainly *trans*-3′-hydroxycotinine, cotinine, and a small amount of nicotine. Following isolation, spectroscopic analyses showed the structure of the nicotine DETBA derivative to be the one-to-one ring-opening product of DEBTA and the cyanopyridinium salt of nicotine.

INTRODUCTION

In recent years the metabolism of nicotine has been found to be very complex. More than 20 urinary nicotine metabolites have been described [1]. For their determination in the urine of humans and laboratory animals, an appropriate analytical method is required. However, only a few methods that can quantify more than two metabolites simultaneously have been reported, and these generally require the use of radioactively labeled nicotine [2–4]. Barlow *et al.* [5] described a pre-column derivatization method yielding 1,3-diethyl-2-thiobarbituric acid (DETBA) derivatives, which can be separated by high-performance liquid chromatography (HPLC). They were able to determine nicotine and cotinine. In addition, they observed five metabolites that they could not identify. Parviainen and Barlow [6] reported that, by using an extraction procedure, the sensitivity of the assay was enhanced. O'Doherty *et al.* [7], in addition to determining nicotine and cotinine, were able to determine three identified metabolites using modified chromatographic conditions. Barlow *et al.* [5] and O'Doherty *et al.* [7] proposed that the mechanism of the derivatization reaction is based, analogous to the König reaction [8], on a ring-opening reaction of the pyridine substructure, starting with an electrophilic attack on the pyridine nitrogen. If this is true, only those metabolites that contain an intact pyridine ring and bear no substituent at the nitrogen atom can undergo this reaction. This is the case for the majority of the known nicotine metabolites with few exception, *e.g.* cotinine-N-oxide and the previously reported N-glucuronides [9].

This paper describes the extension of the HPLC assay to detect and quantify all those metabolites in the urine of humans and laboratory animals that form DETBA derivatives. As the

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nicotine metabolites vary to a high degree in their polarity and basicity, we developed suitable chromatographic conditions and in particular an appropriate solvent gradient. As the final product of the derivatization reaction was not known, the structure of an exemplary DETBA derivative was determined.

EXPERIMENTAL

Materials

1,3-Diethyl-2-thiobarbituric acid (DETBA) was obtained from Aldrich (Steinheim, Germany), chloramine-T, 1-pentanesulphonic acid sodium salt, and β-glucuronidase (*Helix pomatia*, type HP-2) from Sigma (Deisenhofen, Germany), and silica gel 60 from E. Merck (Darmstadt, Germany). Elution solvents were prepared from HPLC-grade actonitrile, methanol, and tetrahydrofuran and ultrapure water from a Milli-Q-apparatus (Millipore, Eschborn, Germany). They were filtered and degassed before use. All other chemicals were of reagent grade.

(*S*)-Nicotine (NIC) was obtained from Sigma, cotinine (COT) from Roth (Karlsruhe, Germany), 3-pyridylacetic acid (PAAc) and 3-pyridylcarbinol (PC) from Aldrich, dihydrometanicotine (DMNIC), *cis*-3′-hydroxycotinine (CHOC), *trans*-3′-hydroxycotinine (THOC), nicotine-N′-oxide (NN′O), norcotinine (NCOT), nornicotine (NNIC), 4-(3-pyridyl)-4-oxobutyric acid (POBAc), and N′-ethylnorcotinine (internal standard) from Dr. Neurath (Institut für Biopharmazeutische Microanalytik, Hamburg, Germany). N′-Methylnicotinium iodide (N′MNIC) was a gift of Prof. Crooks (University of Kentucky, Lexington, USA).

Standard stock solutions were prepared in water and added to blank urine of the respective species to give the calibration solutions used for quantification.

The human urine samples were randomly collected from a smoker (approximate daily consumption 50 cigarettes, nicotine delivery 1 mg per cigarette) and from a non-smoker not exposed to environmental tobacco smoke. Rat urine samples were collected on ice over 24 h from male Sprague–Dawley rats treated i.v. with 0.86 mg of (*S*)-nicotine per kg body weight and from untreated rats. Hamster urine samples were collected on ice over 24 h from male Golden Syrian hamsters treated i.p. with 1.2 mg (*S*)-nicotine per kg body weight and from untreated hamsters. The samples were stored at −70°C.

Instrumentation

HPLC was performed on a Hewlett-Packard liquid chromatograph 1090 L (Waldbronn, Germany) equipped with a ternary solvent-delivery system and a diode-array detector. The sample wavelength was set to 529 nm with a bandwidth of 12 nm, and the reference wavelength to 590 nm with a bandwidth of 10 nm. The signal output was evaluated using a chromatography data system (Multichrom, VG Instruments, Wiesbaden, Germany). Alternatively, a Hewlett-Packard 1090 M combined with a HP 9000 workstation was used to record the UV–Vis spectra in the range 230–600 nm during the chromatographic run. The analytical column was a Nova Pak RP18 (150 mm × 3.9 mm I.D., 4 μm) from Millipore connected to a precolumn Nucleosil 100 C_{18} (25 mm × 4.0 mm I.D., 5 μm) from Knauer (Berlin, Germany). The injection volume ranged from 50 to 250 μl.

Additional spectroscopic analyses were performed for identification purposes by Spectral Service (Cologne, Germany). The FT-IR spectrum was recorded using a Bruker IFS 85. Mass spectra were taken on a Finnigan Mat 8230 using the positive and negative fast atom bombardment (FAB); ion mode. ^{1}H NMR spectra were taken on a Bruker AM 300.

Derivatization

The following reagents were prepared: internal standard (I.S.) solution (6 *M* sodium acetate adjusted to pH 4.7 with 6 *M* trichloroacetic acid containing the I.S., N′-ethylnorcotinine, at a concentration of 5 mg/l), 1.5 *M* potassium cyanide solution, 1 *M* chloramine-T solution (kept at 40°C, and 0.075 *M* DETBA in water–acetone (50:50, v/v) (kept at 40°C).

The urine samples were centrifuged at 10 000 *g*

for 5 min, and the supernatants were ultrafiltered (Ultrafree-MC, 30.000 NMWL, Millipore). Samples ranging from 10 to 150 μl were diluted with water to a final volume of 150 μl, and 60 μl of I.S. solution, 30 μl of potassium cyanide solution, 15 μl of chloramine-T solution and finally 75 μl of DETBA solution were added. The vial contents were mixed vigorously for 30 s, followed by centrifugation for 2 min. The supernatant was transferred to an autosampler vial and injected into the HPLC column after a time delay resulting in a total reaction time of 13–14 min (measured from the addition of the last reagent to the time of injection).

HPLC conditions

The HPLC separations were performed at ambient temperature or, for the separation of COT and NN'O, at 50°C. A ternary solvent programme (solvents A, B, and C) and the flow programme shown in Fig. 1 were used. Solvent A was 0.06 *M* 1-pentanesulphonic acid sodium salt adjusted to pH 3.8 using 1% solution of orthophosphoric acid; solvent B was methanol–tetrahydrofuran (95:5, v/v); and solvent C was acetonitrile–tetrahydrofuran (97:3, v/v).

Isolation of the nicotine DETBA derivative

A 40-ml volume of NIC solution (0.01 *M*) was mixed with 16 ml of 4 *M* acetate buffer (pH 4.7), 8 ml of 1.5 *M* potassium cyanide solution, 8 ml of 0.8 *M* chloramine-T solution, and 20 ml of 0.1 *M* DETBA solution in water–acetone (50:50, v/v). After 15 min, the yellow precipitate was spun down. The supernatant was discarded. The residue was washed twice with 12 ml of water and resolved in 6 ml of ethyl acetate. The organic phase was washed with 6 ml of water, dried using sodium sulphate, and evaporated under vacuum. Further purification was achieved by liquid chromatography, using a 300 mm × 20 mm glass column filled with silica gel and toluene–acetone (5:1, v/v) as eluent. The yellow fraction was collected and the solvents were evaporated. The solid red residue was checked for purity by HPLC (the final product contained *ca.* 5% of a by-product) and used for spectroscopic analysis.

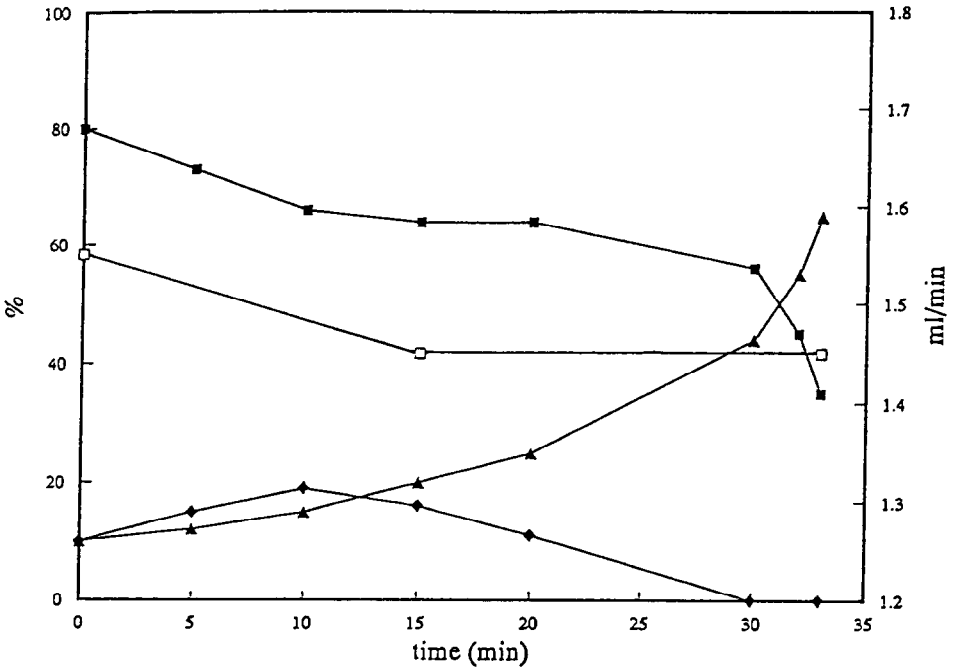


Fig. 1. Solvent and flow programmes used for the separation of DETBA derivatives. ■ = Solvent A; ◆ = solvent B; ▲ = solvent C; □ = flow-rate.

Enzymic cleavage of conjugated metabolites

A urine sample from a smoker, concentrated up to 15-fold, was saturated with potassium carbonate and washed twice with dichloromethane to extract the non-polar metabolites. After neutralization with phosphoric acid, *ca.* 100 μl of the aqueous phase was used for derivatization and HPLC analysis.

To the remaining aqueous phase, equal volumes of a 0.05 *M* sodium acetate buffer (pH 4.7) and β-glucuronidase were added, resulting in a enzyme concentration in the incubation mixture of 5000 units/ml. After incubation at 37°C for 18 h, the mixture was centrifuged and the supernatant was used for derivatization and HPLC analysis.

RESULTS AND DISCUSSION

Separation of DETBA derivatives of nicotine and its metabolites

HPLC analysis of the DETBA derivatives yielded two peaks for each metabolite. The area of the by-product with the shorter retention time was *ca.* 25% of the area of the main product peak with the longer retention time. Two main product peaks and two by-product peaks were found only for DMNIC. We assume that in previous reports the by-products were sometimes misinterpreted as unknown metabolites. Our optimization of the chromatographic procedure focused on separating the main product peaks, which were also used to quantify metabolites. The separation was achieved by gradually replacing the ion-pairing buffer with organic solvent. The best peak shapes were found when methanol was used to elute the polar metabolites in the first part of the run and acetonitrile to elute the non-polar basic metabolites in the second part of the run. The addition of a small amount of tetrahydrofuran improved the shapes of all the peaks. The run had to be maintained for *ca.* 34 min to elute nicotinamide (NICAm), an endogenous DETBA reactive compound.

Fig. 2a shows the separation of NIC, ten reference metabolites, and the I.S. added to rat blank urine. The first part of the chromatogram, up to *ca.* 13 min, contains the by-product peaks, and the second part contains the main product peaks. All main product peaks are baseline-separated with the exception of COT and NN'O, and NIC and the second peak of DMNIC (DMNIC2). The separation of COT and NN'O could be achieved at a higher temperature (50°C). The partial overlapping of the DMNIC2 and NIC peak was not a problem as DMNIC can easily be identified by its first peak, DMNIC1, and the NIC concentration in most urine samples is much higher than the DMNIC concentration.

The assay was performed using the urine of

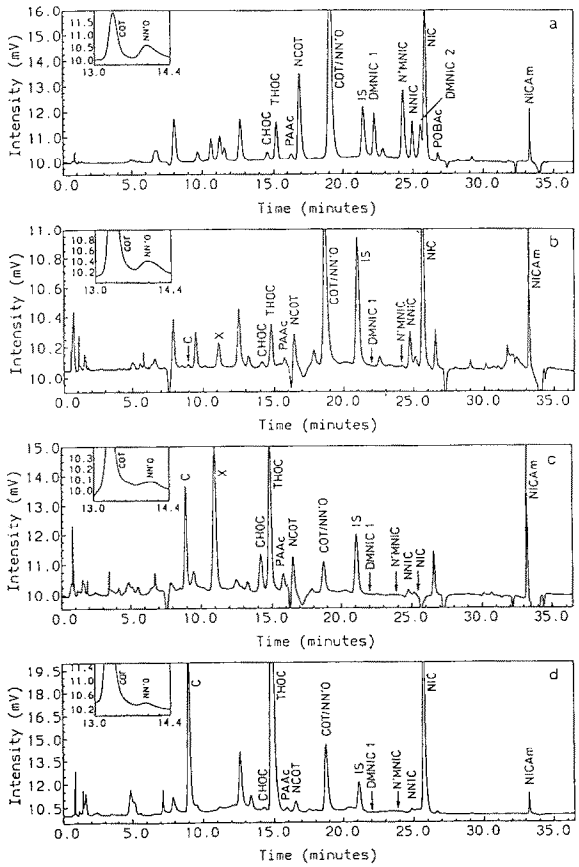


Fig. 2. Typical chromatograms obtained at ambient temperature for: (a) a metabolite standard solution added to rat blank urine [amounts injected (pmol): CHOC, 40; THOC, 260; PAAc, 23; NCOT, 494; COT, 546; NN'O, 2250; DMNIC, 976; N'MNIC, 564; NNIC, 162; NIC, 246; POBAc, 2200]; (b) a urine sample of a nicotine-treated rat; (c) a urine sample of a nicotine-treated hamster; (d) a urine sample of a smoker. The inserts show the separation of COT and NN'O at 50°C.

rats and hamsters (treated with NIC) and the urine of a human smoker (Fig. 2b, c and d) and respective blank urine samples (see Fig. 3a and b for examples). In the nearly identical chromatograms of blank rat and hamster urine samples, some negative peaks and a positive peak overlapping with the POBAc peak were seen. In spite of these interferences, it was possible to detect NIC, nine nicotine metabolites, and additional metabolites (C and X) not included in our calibration mixture. The human blank urine sample contained no interfering substances with the exception of a small peak at the POBAc position, so NIC and nine metabolites could be quantified. Among these was CHOC. As previously reported [10], the determination of the CHOC/THOC ratio by quantification of the DETBA derivatives was carried out to verify gas chromatographic results.

PC, suggested by Parviainen *et al.* [11] to be a human urinary nicotine metabolite, had the same retention time as THOC. GC–MS of several urine samples, however, revealed no PC, or only negligible amounts. These results were supported by the UV–Vis spectrum of the peak found at *ca.* 15 min in the chromatogram obtained for human urine. The spectrum recorded was in agreement with that of a THOC standard peak and differed distinctly from that of a PC standard peak.

The large peak C occurring in the by-product region of the chromatogram did not have a counterpart in the main product region. This peak was also one of the largest peaks in the hamster urine chromatogram and could be detected as a small peak in the rat urine. Its identification is described below.

Establishment of the structure of the nicotine DETBA derivative

The structure of the DETBA derivatives has not been investigated to date. A reaction mechanism analogous to the König reaction [8] has been proposed by O'Doherty *et al.* [7], leading to a compound (**1**) containing two DETBA groups and an opened pyridine ring (Fig. 4).

We investigated the HPLC fractions containing the main derivatives of NIC, COT, and THOC but no underivatized metabolites by GC–MS. No indication of the proposed reaction product was found in the chromatograms, but each one showed a peak with a retention time and a mass spectrum of the respective underivatized metabolite. Obviously, a recyclization of the pyridine ring had taken place during GC. This reac-

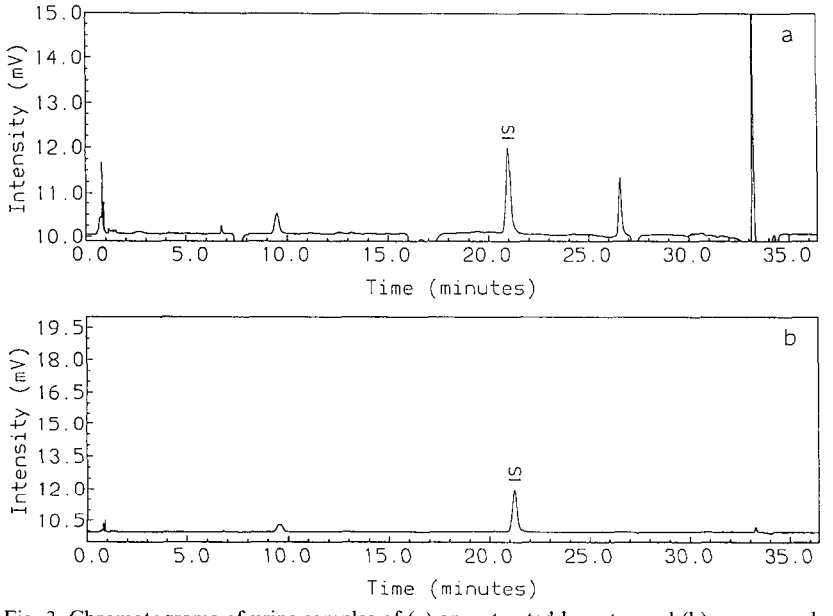
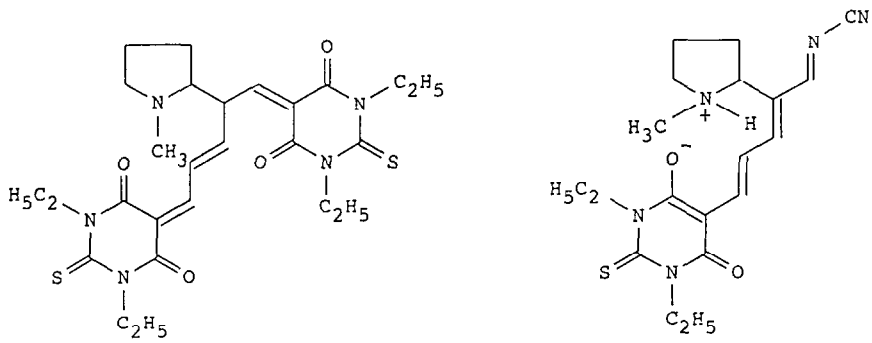


Fig. 3. Chromatograms of urine samples of (a) an untreated hamster and (b) a non-smoker.



(1) (2)

Fig. 4. Molecular structure of the DETBA derivative of nicotine: (**1**) as proposed by O'Doherty *et al.* [7]; (**2**) as proposed from our investigations.

tion is not possible with the proposed reaction product (**1**), because it does not contain the pyridine nitrogen.

In order to establish the structure of an exemplary derivative, the DETBA derivative of NIC was isolated and its structure investigated by FAB-MS, FT-IR, ^{1}H NMR, and UV–Vis spectroscopy. From the results obtained, we propose the compound (**2**) to be the main reaction product.

Both positive and negative FAB mass spectra exhibit a molecular peak at *m/z* 387. In the IR spectrum, a strong band at 2175 cm^{-1} indicates a CN triple bond.

A comparison of the ^{1}H NMR spectrum of NIC and its reaction product shows that remarkable changes occur for the protons of the double bond region (δ between 7.2 and 8.2). The coupling constants increase by up to *ca.* 17 Hz. This can be explained by the opening of the pyridine ring leading to a *trans* configuration. This opening of the aromatic ring also reduces the long-range coupling constants so that they are no longer observable. The suggestion that a positive charge is located at the pyrrolidine nitrogen is supported by a shift of +0.6 in the signals of the N-methyl protons compared with NIC.

UV–Vis spectra of the main products of all metabolites were recorded during the HPLC run using the diode-array detector. Their spectra were similar to the spectra of either the nicotine or cotinine derivative (Fig. 5a and b), the maximum absorbance being between 528 and 536 nm.

The UV–Vis spectra and the recyclization results found for three derivatives in the GC experiments lead us to the assumption that the derivatization of all the various metabolites results in the formation of products with the same chromophore as found for the DETBA derivative of NIC.

The by-products eluting in the first part of the chromatogram exhibited a wavelength shift of *ca.* −15 nm compared with the main products in their UV–Vis spectra. Their structures were not investigated.

Identification of unknown peak C in human urine

The short retention time of peak C compared with the other main product peaks indicates the occurrence of a metabolite distinctly more polar than the others. For this reason, and because the occurrence of phase 2 metabolites of NIC was recently reported [9,12–14], we assumed that peak C represents DETBA-derivatized phase 2 metabolites. To investigate this assumption, human urine was incubated with and without β-glucuronidase and subsequently analysed using the DETBA assay. As expected, peak C disappeared

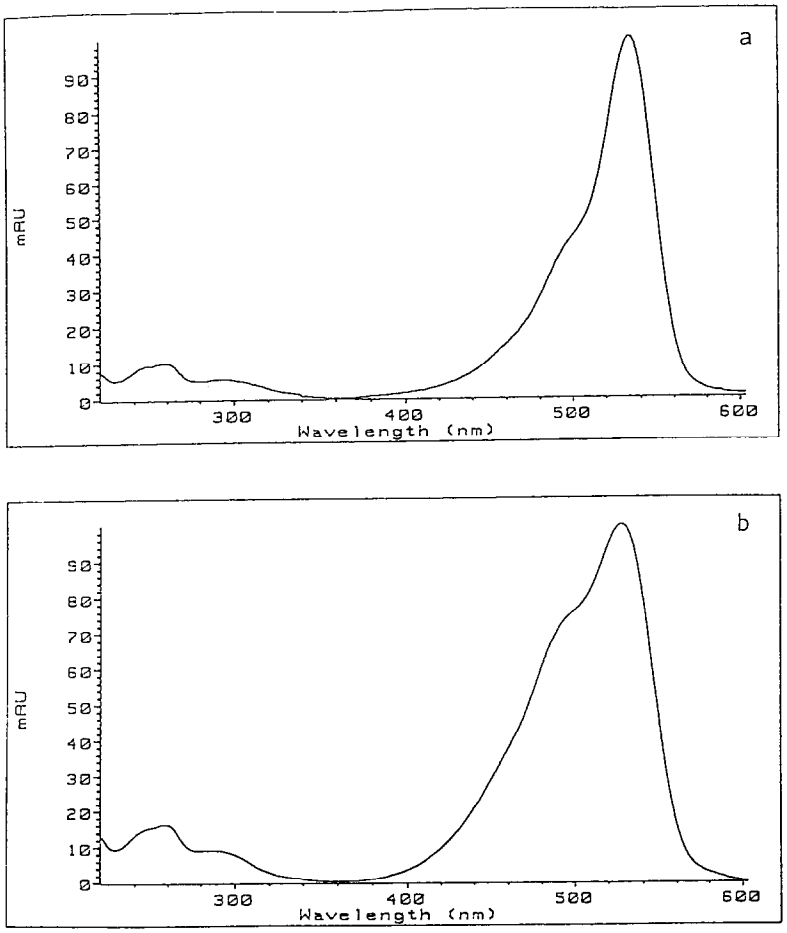


Fig. 5. UV–Vis spectra of the DETBA derivatives of (a) nicotine and (b) cotinine.

after enzyme treatment. The peak areas of NIC, COT, and THOC increased, indicating the predominance of cleavable conjugates of these three compounds. To improve their quantification precision, less polar compounds were removed by washing the urine with dichloromethane prior to the enzyme treatment. In the residual aqueous phase, all other metabolite peaks except peak C disappeared or were considerably reduced, as shown in Fig. 6a. After incubation with β-glucuronidase, peak C completely disappeared, whereas THOC, COT, and NIC peaks reappeared (see Fig. 6b). The quantitative evaluation of this chromatogram showed that the enzymic cleavage of DETBA-positive and DETBA-negative conjugates (present in this exemplary urine sample) produces 67% THOC, 31% COT, and 2% NIC. Treatment of the respective reference compounds with β-glucuronidase under the same conditions showed no effects.

The UV–Vis spectrum of peak C was identical with that of the DETBA derivative of THOC. Therefore, that part of the conjugates positive in the DETBA reaction is assumed to form DETBA products with structures containing the same chromophore as the nicotine DETBA derivative. This is only possible if these conjugates still contain an intact pyridine ring. As an example for a conjugation that did not take place at the pyridine nitrogen, the O-glucuronide of THOC was recently characterized [14].

Quantitative results

The use of calibration solutions of ten stan-

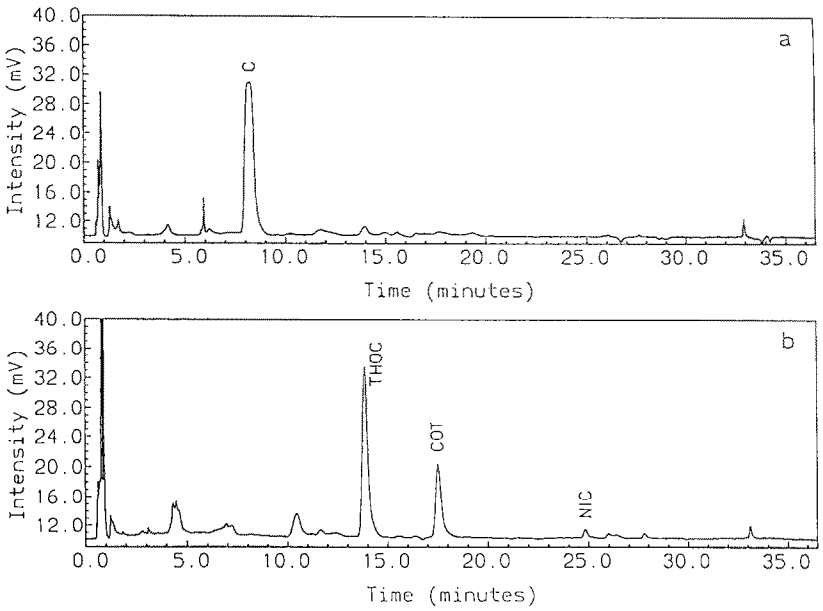


Fig. 6. Chromatograms obtained (a) from a dichloromethane-washed urine sample of a smoker, and (b) after additional treatment with β-glucuronidase.

dard compounds led to multilevel calibration curves that were linear over a wide concentration range (Table I). Quantitative results for a smoker's urine are given in Table II.

Parviainen and Barlow [6] described an extraction procedure that was reported to enhance the sensitivity of the assay. Using their procedure, we found a poor recovery for several metabolites. Thus, the development of a method tolerating large injection volumes seemed to be a more efficient way to enhance the sensitivity of our assay. Under the optimized conditions, volumes up to 250 μl could be injected without any deterioration of the peak shapes. The detection limits for this injection volume were between 0.5 and 1.5 n*M* for most of the metabolites. For NN'O it was

TABLE I

LINEAR REGRESSION OF METABOLITE/INTERNAL STANDARD PEAK-AREA RATIOS AGAINST CONCENTRATION OF METABOLITE CALIBRATION SOLUTIONS IN HUMAN URINE

Metabolite	Calibration range (μ*M*)	Regression coefficient ($1/\mu M$)	Intercept on *y*-axis	Correlation coefficient (r^2)
CHOC	0.1–1.7	0.18	0.0000	0.9981
THOC	0.7–120	0.13	0.010	0.9981
PAAc	0.1–2.4	0.092	0.0033	0.9891
NCOT	3.0–50	0.071	−0.14	0.9975
COT	4.0–54	0.16	0.0003	0.9990
NN'O	14.0–225	0.014	−0.068	0.9990
DMNIC	3.0–100	0.036	−0.019	0.9977
N'MNIC	3.0–60	0.038	−0.0093	0.9993
NNIC	1.0–16	0.059	−0.0037	0.9996
NIC	1.5–60	0.18	0.17	0.9922

TABLE II

CONCENTRATION OF NICOTINE METABOLITES IN THE URINE OF A SMOKER

Metabolite	Concentration (μM)
THOC conj.[a]	22.7
COT conj.[a]	10.5
NIC conj.[a]	0.7[b]
CHOC	0.6
THOC	119.7
PAAc	1.3
NCOT	7.1
COT	13.8
NN'O	15.5
DMNIC	n.d.
N'MNIC	n.d.
NNIC	1.6
NIC	34.4

[a] Conjugates of the respective metabolites determined after enzymic cleavage.

[b] Extrapolated from the calibration curve.

found to be distinctly higher, *ca.* 5 nM. These limits are below those reported for the above-mentioned extraction procedure as well as below those known for most GC procedures.

The intra-assay coefficients of variation for the assay were calculated from five determinations of a blank rat urine sample spiked with standard solutions of NIC and nine metabolites (Table III).

CONCLUSION

The DETBA assay described can be used to determine NIC and twelve metabolites, including three phase 2 metabolites, in human urine. The structure of the nicotine derivative has been identified. Compared with published methods using non-radiolabelled material, the assay provides the most complete profile of nicotine metabolites in humans. This highly selective and sensitive assay can also be applied to the analysis of urine samples of laboratory animals.

TABLE III

INTRA-ASSAY VARIATION COEFFICIENTS

Metabolite	Concentration (μM)	R.S.D. (%)
CHOC	0.83	1.9
THOC	5.21	0.2
PAAc	1.15	0.7
NCOT	24.60	3.0
COT	27.30	3.5
NN'O	11.20	3.6
DMNIC	18.30	6.2
N'MNIC	4.10	3.2
NNIC	8.00	4.2
NIC	12.30	2.6

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