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Note

Gas chromatographic determination of *trans*-3′-hydroxycotinine, a major metabolite of nicotine in smokers

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Hydroxycotinine was first described as a metabolite of nicotine in the urine of several mammalians and in smokers urine by McKennis et al. [1], but neither the position of the substituent nor the amount of the compound could be given by the authors at that time. The structure of the metabolite was identified as *trans*-3′-hydroxycotinine (Fig. 1) by Dagne and Castagnoli [2], who also described a synthesis for the compound.

A study of the steady-state plasma levels of nicotine and cotinine and the total urinary excretions of several metabolites (cotinine, nicotine-N-oxide, nornicotine, and N-methylnicotinium ions) in nine male smokers showed that they excreted no more than 18.7%, 18.0%, or 13.3%, respectively, of the estimated maximum of nicotine uptake. The study consisted of three runs of seven days of smoking nineteen cigarettes per day, and elimination periods of five further days.

This marked shortfall prompted a search for further metabolites in the urine. *trans*-3′-Hydroxycotinine accounts for a large part of the deficiency, bringing the estimated balances to 39.8%, 38.2%, and 31.5% in the respective studies. It is the most prominent metabolite so far detected in smokers' urine [4]. Its mean plasma concentration, 69 ng/ml on the sixth day of smoking, is second only to that of cotinine.

A gas chromatographic (GC) method for the determination of this compound in plasma and urine of smokers is described in this paper.

EXPERIMENTAL

Apparatus

GC was performed on a Hewlett-Packard HP 5713 gas chromatograph equipped with an electron-capture detector (^{63}Ni) and a Hewlett-Packard HP 3380 inte-

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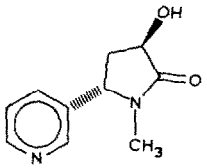

Fig. 1. Structure of *trans*-3′-hydroxycotinine.

grator. The mass spectrum of the trifluoroacetate of metabolic *trans*-3′-hydroxycotinine was measured using a Hewlett-Packard HP 5992 A computer controlled GC–MS system.

Materials

cis-3′-Hydroxycotinine was synthesized in this laboratory as described in ref. 2 (m.p., 148–149°C; lit., 148–149°C). *trans*-3′-Hydroxycotinine was prepared by epimerization of the *cis*-epimer [2] (m.p., 108–110°C; lit., 110–111°C). Heptafluorobutyric anhydride was supplied by Fluka (Neu-Ulm, F.R.G.). All solvents used for extraction were specified nanograde or purissimum and purchased from Merck (Darmstadt, F.R.G.) or Promochem (Wesel, F.R.G.). Blank samples of plasma and urine were obtained from healthy non-smoking volunteers.

Extraction

To 1 ml of plasma or urine, 2 ml of dichloromethane and 3 ml of saturated, aqueous potassium carbonate solution were added in a 10-ml centrifuge tube with a screw-cap (Sovinel, France). The mixture was shaken for 30 min on a rotary mixer (Cenco, Breda, The Netherlands) at 40 rpm, and centrifuged for 5 min at 5760 *g* (Labofuge III, Heraeus-Christ, Osterode, F.R.G.). Then 500 μl of the dichloromethane were placed in a 1-ml Reactivial (Pierce-Eurochemie, Rotterdam, The Netherlands) and treated with 25 μl of heptafluorobutyric anhydride for 30 min. The solution was evaporated under a flow of nitrogen, and the residue was dissolved in 50 μl of benzene.

Gas chromatography

Aliquots of 10 μl of the benzene–ethyl acetate solution were directly injected for GC. A glass column (540 cm×2 mm I.D.) packed with 20% Silicon QF 1 (WGA, Düsseldorf, F.R.G.) on Chromosorb W-HP 100–120 mesh (Macherey-Nagel, Düren, F.R.G.) was used for the separation. A temperature programme from 240°C to 340°C was run at 4°C/min, followed by a hold for 8 min at 350°C. The injector temperature was 250°C, the detector temperature 300°C, and the flow-rate of argon–methane (1:1) 50 ml/min. The retention time of *trans*-3′-hydroxycotinine under those conditions was $t_R = 7.1$ min, and that of the *cis*-epimer was $t_R = 7.8$ min.

Sensitivity, accuracy, and precision of GC

A standard curve for *trans*-3′-hydroxycotinine was prepared by carrying out the analysis with test solutions and twelve repeated injections ranging from 20 to

200 ng of *trans*-3′-hydroxycotinine per millilitre of plasma. For this, a 1-ml plasma blank was spiked with 2–20 μl of a solution of *trans*-3′-hydroxycotinine in 1,2-dimethoxyethane (10 μg/ml). The peak area was found to be 2.5 mm^2/ng of *trans*-3′-hydroxycotinine. Linear regression was obtained over the entire range: $y = 2.46x + 0.27$; $r^2 = 1.00$.

The recovery rate was determined by comparing the stated analyses on plasma samples with solutions of *trans*-3′-hydroxycotinine with different concentrations ranging from 20 to 200 ng/ml in benzene–ethyl acetate ($y = 3.27x + 0.46$; $r^2 = 1.00$). Finally, the recovery rate was calculated from the average peak areas of the linear regression curves (2.46 and 3.27 mm^2/ng, respectively), corresponding to a recovery of 75.2% (S.D. ± 2.8%).

Corresponding figures were found for urine samples: spiked blanks ranging in concentration from 500 ng/ml to 10 μg/ml in benzene–ethyl acetate ($y = 0.183x - 12.4$; $r^2 = 0.986$ and $y = 0.206x + 2.7$; $r^2 = 1.00$, respectively), corresponding to a recovery rate of 88.8% (S.D. = ± 3.7%).

Because of the stated recovery rates, the use of an internal standard was considered unnecessary.

The limits of quantitation and of detection are 5 ng/ml and 1 ng/ml plasma, respectively.

Fig. 2 shows presence and absence of the analyte in typical chromatograms of plasma samples of a smoker and a non-smoker and of spiked plasma samples. The derivatization rules out interference by nicotine and its known metabolites. None of them can undergo acylation reactions.

RESULTS

This GC assay of *trans*-3′-hydroxycotinine gives precise and reproducible results. Table I shows the steady-state plasma levels of the compound of nine volunteer smokers on the sixth day of controlled smoking. The values are compared with nicotine and cotinine levels in the same samples. Table II gives the total urinary excretion of the nine smokers in three runs of seven days of smoking of three brands differing in mainstream nicotine: 0.5 mg, 0.9 mg, and 1.35 mg of nicotine per cigarette. Nineteen cigarettes per day were smoked over seven consecutive days followed by five days of elimination. The total urine was collected by the subjects, and analysed (in daily fractions) for nicotine, cotinine, nicotine-N-oxide, N-methylnicotinium ions [3], and nornicotine, and subsequently in combined aliquot samples for *trans*-3′-hydroxycotinine. The table gives the accumulated values in nicotine equivalents.

The nicotine uptake was monitored during smoking of two of the brands of cigarettes on different days of every smoking period by means of a puff-parameter analyser. Additonally the nicotine uptake was calculated from filter nicotine of the individually collected filter tips. Both methods of assay give an estimate for the maximum of uptake of the alkaloid. For the final estimation preference was given to the latter values because of their better linear regressions with steady-state plasma levels for nicotine and cotinine.

The identity of the *trans*-3′-hydroxycotinine from urine with the synthetic

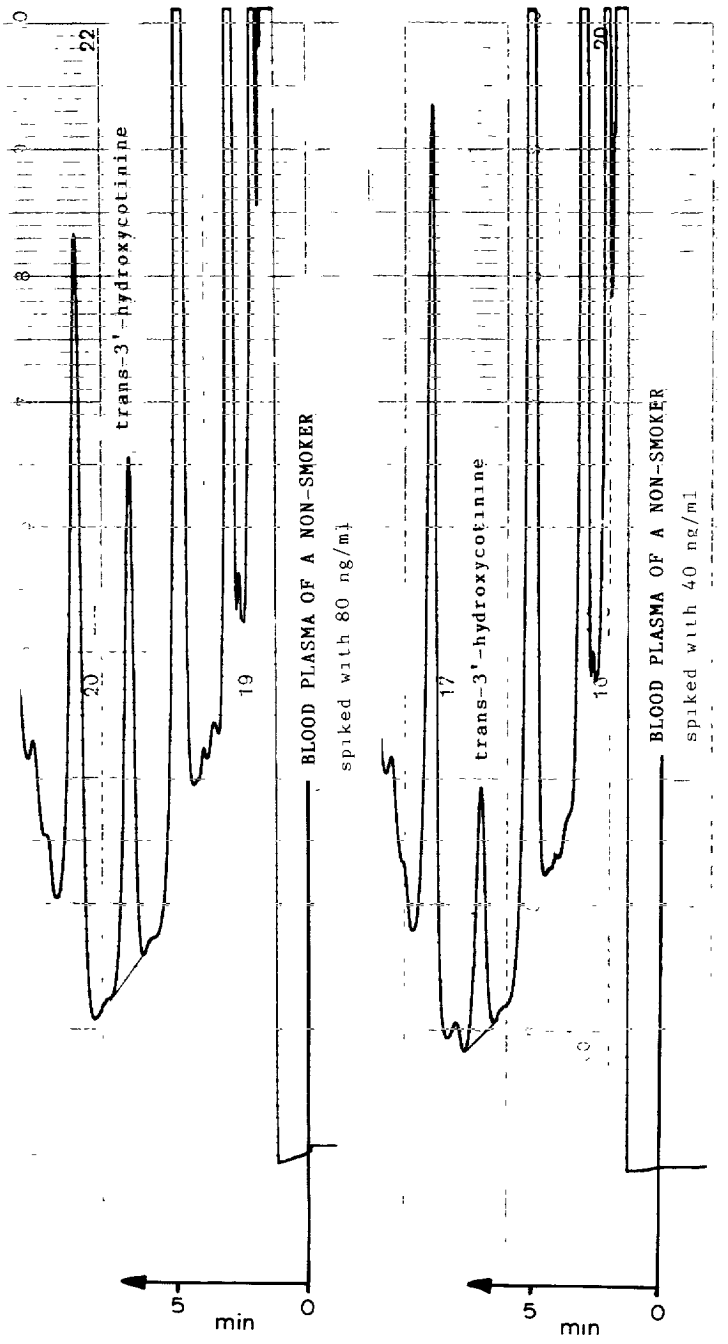

Fig. 2.

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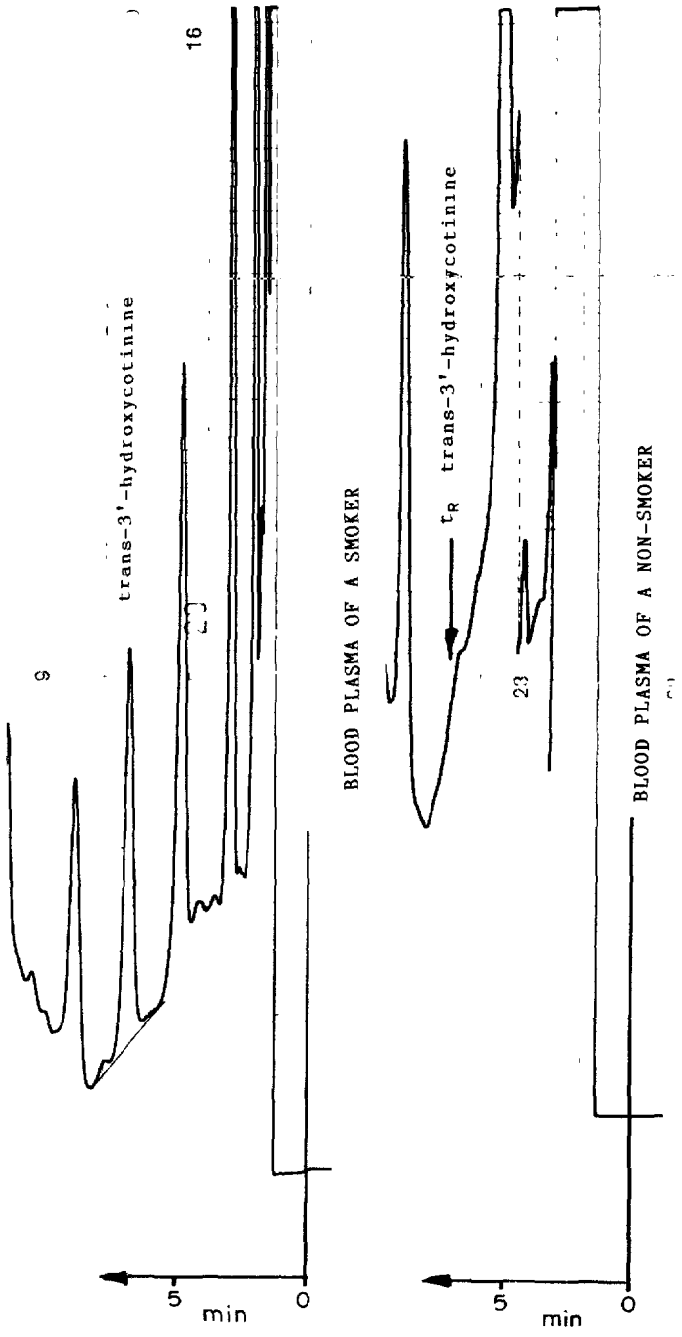

Fig. 2. Gas chromatograms of plasma samples of smokers and non-smokers and of spiked plasma samples.

TABLE I

STEADY-STATE PLASMA CONCENTRATIONS ON SIXTH DAY OF SMOKING

Brand with 1.35 mg of mainstream nicotine. Subjects smoked nineteen cigarettes per day; blood samples were taken at 20:00 on the sixth day of a smoking period of seven days. Samples were taken at the last puff of a cigarette, i.e. at one short time maximum of the nicotine concentration ($t_{1/2}=52$ min). Abbreviations: NIC = nicotine; COT = cotinine; HCO = *trans*-3′-hydroxycotinine. Values are ng/ml blood plasma.

Subject	NIC	COT	HCO
1	26	334	109
2	18	129	115
3	28	242	112
4	15	74	47
5	16	144	56
6	7	41	24
7	44	108	45
8	28	117	57
9	41	206	160
$\bar{x}$	25	155	69
±S.D.	12	91	34

TABLE II

URINARY EXCRETION OF NICOTINE METABOLITES

Three brands of cigarettes, means of nine subjects. Urine collected over smoking periods of seven days (133 cigarettes), and five days of elimination. Values are mg of nicotine equivalent.

	Brand 1	Brand 2	Brand 3
Mainstream nicotine	0.5	0.9	1.35
Nicotine uptake	103.9	156.4	231.7
Urinary excretion			
Nicotine	8.4	13.5	14.0
Cotinine	7.4	10.8	12.2
Nicotine-N′-oxide	2.4	2.8	3.4
Nornicotine	0.8	1.1	1.1
N-Methylnicotinium ions	1.0	1.5	1.0
trans-3′-Hydroxycotinine	22.6	33.2	49.5
Total	42.6	62.9	81.2
Proportion of uptake (%)	39.8	38.2	31.5
±S.D.	15.4	19.7	21.1

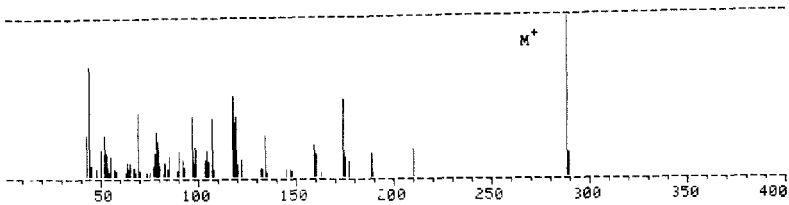

Fig. 3. Mass spectrum of trifluoroacetyl *trans*-3′-hydroxycotinine.

sample was proved by mass spectral comparison of the trifluoroacetyl derivatives: $m/e = 288$, 174, 117, 210, 134, 96, in order of intensity and retention times. The mass spectrum of *trans*-3′-hydroxycotinine as its trifluoroacetyl derivative is given in Fig. 3.

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