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Short Communication

Simultaneous determination of cotinine and *trans*-3′-hydroxycotinine in human serum by high-performance liquid chromatography

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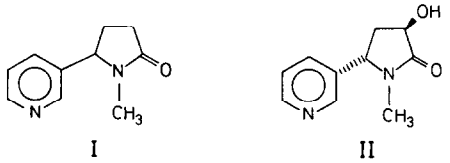
ABSTRACT

A high-performance liquid chromatographic method with ultraviolet photometric detection has been developed for the quantitation of cotinine and *trans*-3′-hydroxycotinine in human serum. A solid-phase extraction procedure was performed for the analytes and the internal standard, N-ethylnorcotinine, before chromatography. The use of a 30-cm reversed-phase column and a mobile phase of water–methanol–0.1 *M* sodium acetate–acetonitrile (67:24.5:6.5:2, v/v), pH 4.3, prevented the co-elution of caffeine with cotinine. The limit of quantitation observed with this method was 5 ng/ml for both cotinine and *trans*-3′-hydroxycotinine. The present method proved useful for the determination of serum levels of these metabolites, correlating with nicotine daily intake.

INTRODUCTION

The major metabolic pathways of nicotine [1] in the mammalian species involve a series of oxidations of the pyrrolidine ring to produce more polar and pharmacologically less active compounds. The γ-lactam cotinine, a major metabolite, has been reported to be further metabolized to *trans*-3′-hydroxycotinine (Fig. 1) [2,3]. The latter is the most abundant nicotine metabolite in the urine of habitual smokers, and its steady-state plasma concentration is second only to that of cotinine [4].



I II

Fig. 1. Structures of cotinine (I) and *trans*-3′-hydroxycotinine (II).

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Several chromatographic methods have been developed to determine nicotine metabolites in biological samples [5–7]. However, only a few articles describe the simultaneous quantitation of cotinine and *trans*-3′-hydroxycotinine [5,8,9]. The principal difficulty is the extraction of *trans*-3′-hydroxycotinine from aqueous media, because of its solubility in water [8,10]. In addition, determinations based on gas chromatography (GC) are usually preceded by derivatization [11] and, if not, poor sensitivity is observed [12]. High-performance liquid chromatographic (HPLC) methods usually require the formation of a coloured complex [5,13,14]. Furthermore, assays have mostly been performed on urine samples, but rarely on blood samples.

Nevertheless, plasma steady-state levels of nicotine metabolites could be used to estimate nicotine intake after both active and passive smoking [15,16], or to indicate compliance to a non-smoking clinical trial [17].

A radiometric HPLC assay for nicotine metabolites has been reported by Kyerematen *et al.* [9] in plasma samples from rats, but it appears to be of low applicability for routine determinations in humans [7]. To improve the investigation of serum concentrations of cotinine and *trans*-3′-hydroxycotinine in smokers, we have developed a simple, reliable isocratic method for both metabolites by HPLC with UV detection. A solid-phase extraction was performed for serum samples, using N-ethylnorcotinine as an internal standard.

EXPERIMENTAL

Materials

Cotinine was purchased from Sigma (St. Louis, MO, USA). *trans*-3′-Hydroxycotinine and N-ethylnorcotinine were obtained from Dr. Georg B. Neurath (Hamburg, Germany). Extrelut-1 extraction columns were from Merck (Bracco, Milan, Italy). All solvents were analytical grade.

Blood samples (5 ml) from ten smokers were obtained by venipuncture with silicone-coated vacutainers. The samples were collected *ca.* 8 h after the last cigarette. Blank samples were obtained from non-smokers who were not exposed to environmental tobacco smoke for at least seven days before the samples were taken. All samples were immediately centrifuged at 1000 *g* for 5 min. Serum was collected and stored at −20°C until analysis.

High-performance liquid chromatography

The HPLC system consisted of a Merck-Hitachi L6200 intelligent pump, a Merck-Hitachi L4200 UV–VIS detector set to 254 nm and a Merck-Hitachi D2000 chromato-integrator (Bracco).

The column was a reversed-phase μBondapak C_{18} steel column (10 μm particle size, 30 cm × 3.9 mm I.D.; Waters, Milford, MA, USA). The column was equilibrated with the mobile phase for 30 min prior to analysis of samples.

The mobile phase was water–methanol–0.1 *M* sodium acetate–acetonitrile (67:24.5:6.5:2, v/v), adjusted to pH 4.3 with acetic acid and used at a flow-rate of 1.5 ml/min.

Standards and controls

Solutions of stock reference standards (1 mg/ml, 10 μg/ml, 1 μg/ml) were prepared in methanol and stored below 0°C. Dilutions were made fresh daily for each analysis.

Serum standards were prepared daily by adding known amounts of the stock standards to drug-free human serum; these standards were used to create calibration curves as a control.

Sample preparation

A 0.5-ml aliquot of serum, with 100 μl of N-ethylnorcotinine (3 μg/ml) and 0.4 ml of 0.5 *M* sodium hydroxide added, was placed in a glass tube, shaken on a vortex-mixer, and transferred to an Extrelut 1 glass column, which was preconditioned with 5 ml of dichloromethane the day before. After 5 min, the analytes were eluted under the force of gravity with 5 ml of dichloromethane. The organic phase was evaporated to dryness under nitrogen and redissolved in 100 μl of mobile phase. A 20-μl volume was injected into the HPLC column.

RESULTS AND DISCUSSION

Extraction procedure

Solid-phase extraction was used in preference to classical liquid–liquid extraction under alkaline conditions. Using the procedures described in literature [7,11,12], we did not obtain a good recovery of *trans*-3′-hydroxycotinine. Furthermore, the reproducibility of the extraction was not satisfactory.

Extrelut pre-packed columns, filled with special diatomaceous earth, are able to absorb and retain hydrophilic fluids, and release low-molecular-mass compounds on elution with organic solvents. We modified an extraction procedure with Extrelut columns for serum cotinine [18,19] by using serum alkalization with 0.5 *M* sodium hydroxide and dichloromethane for the extraction of the analytes. The day before sample extraction, we conditioned the columns with dichloromethane because we noticed that, without this procedure, the elution was too fast and the recovery of the analytes decreased. Furthermore, serum was not retained.

Other concentrations of sodium hydroxide led to a decrease in the percentage recovery of *trans*-3′-hydroxycotinine and the appearance of an interfering peak. Dichloromethane was found to be the best extraction solvent.

High-performance liquid chromatography

A typical chromatogram of *trans*-3′-hydroxycotinine, cotinine and N-ethylnorcotinine in a spiked serum sample is presented in Fig. 2: the retention times were 3.3, 6.0 and 9.1 min, respectively.

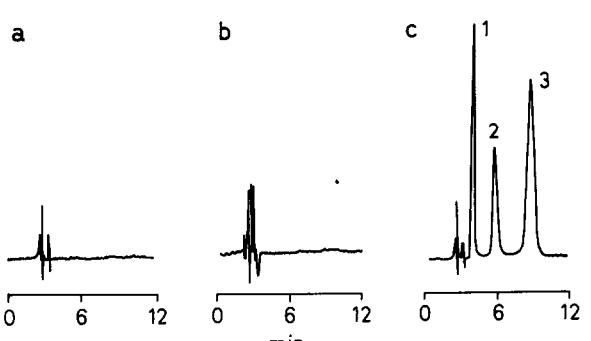

Fig. 2. Chromatograms of (a) reagent blank, (b) extract of 0.5-ml serum blank sample from a non-smoker, and (c) extract of 0.5-ml serum sample spiked with 200 ng/ml *trans*-3′-hydroxycotinine (1), 200 ng/ml cotinine (2) and 3 μg/ml N-ethylnorcotinine (3).

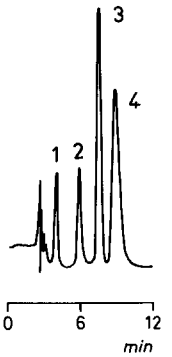

Fig. 3. Chromatogram from a smoker's serum. Peaks: 1 = *trans*-3′-hydroxycotinine (80 ng/ml); 2 = cotinine (200 ng/ml); 3 = caffeine (380 mg/ml); 4 = N-ethylnorcotinine (3 μg/ml)

To optimize the separation of the analytes on the reversed-phase column, we investigated the effect of pH in the range 4.1–5.0. We found that a pH value of 4.3 gave the best separation of the substances, and prevented the coelution of cotinine with caffeine, which was present in 80% of our serum samples (Fig. 3.).

The limit of quantitation observed with this method was 5 ng/ml for cotinine (coefficient of

TABLE I

NICOTINE DAILY INTAKE AND SERUM CONCENTRATION OF COTININE AND *trans*-3′-HYDROXYCOTININE

Subject	Nicotine daily intake (mg per day)	Concentration (ng/ml)	
		Cotinine	*trans*-3′-Hydroxycotinine
1	11.0	145.0	18.1
2	10.0	110.0	61.2
3	16.0	279.0	64.0
4	8.8	128.0	43.3
5	20.0	175.0	61.0
6	11.0	120.0	61.2
7	14.3	179.0	25.0
8	16.5	182.0	87.0
9	8.0	161.0	28.1
10	13.0	129.0	41.0
Mean ± S.D.	12.9 ± 3.8	160.8 ± 48.9	49.0 ± 21.5

variation = 8%) and 5 ng/ml for *trans*-3′-hydroxycotinine (coefficient of variation = 12%). At a detection wavelength of 254 nm and using the solid-phase extraction described, nicotine (retention time 2.7 min) was not detectable below the concentration of 200 ng/ml.

An example of the method's application was the determination of cotinine and *trans*-3′-hydroxycotinine in the serum from ten smokers. Table I shows serum levels of the compounds (ng/ml) compared with nicotine daily intake (mg per day), calculated from the number of cigarettes smoked per day and the mass of nicotine per cigarette.

Calibration curves, recovery and precision

The calibration curves of the peak-height ratio of analytes to the internal standard (y) *versus* the concentration of the analytes (x) were prepared and checked daily from spiked serum carried through the entire procedure; they were linear over the range 5–500 ng/ml (regression equations: $y = 0.59x - 0.04$, $r = 0.997$, for continine, and $y = 1.11x + 0.004$, $r = 0.999$, for *trans*-3′-hydroxycotinine. Over the concentration ranges tested in the analysis, recoveries were 88–90% for cotinine and 50–55% for *trans*-3′-hydroxycotinine.

The analytical imprecision was determined from analyses of five spiked serum samples performed for up to six days. The within-day and between-day coefficients of variation were 2.8 and 4.1% for 200 ng/ml cotinine, and 4.1 and 6.2% for 200 ng/ml *trans*-3′-cotinine in spiked serum.

CONCLUSION

This method is simple and easy to perform. The main advantages are that solid-phase extraction is a reliable and quick procedure, and there is the possibility of simultaneous determination of cotinine and *trans*-3′-hydroxycotinine. The serum levels of these metabolites should be of great help in observational studies of nicotine intake and in bioavailability studies of transdermal nicotine systems used in smoking cessation programs.

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