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A simple high performance liquid chromatographic method for the quantification of total cotinine, total 3′-hydroxycotinine and caffeine in the plasma of smokers

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Abstract

A simple isocratic HPLC procedure has been developed for the quantification of caffeine and the nicotine metabolites cotinine, 3′-hydroxycotinine, cotinine glucuronide and 3′-hydroxycotinine glucuronide in the plasma of smokers. The glucuronide conjugates were determined indirectly via initial basic hydrolysis of the analyte sample followed by quantification of the resulting deconjugation product. Plasma was basified, extracted with dichloromethane, evaporated, the residue dissolved water and an aliquot part was analyzed by HPLC. The method utilized a Partisil-10 SCX cation-exchange column and an isocratic mobile phase of sodium phosphate buffer: methanol (92:8 v/v, 0.1 M, adjusted to pH 4.8 with triethylamine) at a flow rate of 1.5 ml/min. UV detection was at 254 nm. All solutes were separated with good resolution, and quantification was determined using an internal standard of *N*,*N*-diethylnicotinamide. The retention times were: caffeine 5.1 min, 3′-hydroxycotinine 7.2 min, *N*,*N*-diethylnicotinamide 9.5 min, and cotinine 15.5 min. Detection limits for caffeine, 3′-hydroxycotinine, cotinine, and total cotinine were 10 ng/ml; the detection limit for total 3′-hydroxycotinine was 20 ng/ml. The inter-day and intra-day variations for all analytes were between 1 and 8%. This analytical method is suitable for the determination of caffeine and nicotine metabolite levels in large numbers of clinical samples.

Keywords: Cotinine; 3′-Hydroxycotinine; Cotinine glucuronide; 3′-Hydroxycotinine glucuronide; Caffeine; Nicotine; Cationic exchange chromatography

1. Introduction

Human exposure to nicotine (NIC) during smoking or inhalation of environmental tobacco smoke has previously been estimated by measur-

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ing NIC and cotinine (COT) in body fluids [1–3]. In this respect, it is now generally accepted that plasma, saliva, and urine levels of NIC alone may be poor indicators for the actual intake of NIC during cigarette smoking because NIC has a relatively short plasma half-life (1–2 h) [4].

COT is the major metabolite of NIC [1,2] and since its plasma half-life (15–20 h) is considerably longer than that of NIC, plasma COT is now widely used as a more accurate indicator of NIC intake in humans [5,6]. However, COT undergoes further metabolism, particularly via C-hydroxylation and *N*-glucuronidation pathways. In this respect, *trans*-3′-hydroxycotinine (3HC) appears to be a major metabolite in the plasma of smokers, and the most abundant metabolite in smoker's urine [7–9]. Another important observation in these latter studies is that a significant proportion of COT and 3HC are excreted in the form of glucuronide conjugates in smokers′ urine. Several reports of the quantitation of conjugated NIC metabolites in smokers' urine using the enzyme β-glucuronidase, coupled with analysis of the liberated deconjugated metabolite have shown that the glucuronide metabolites of NIC include the quaternary ammonium conjugates NIC glucuronide and COT glucuronide as well as 3HC O-glucuronide. Together, these conjugates constitute almost 25–30% of total nicotine metabolites in smokers' urine [7–9]. Surprisingly, there are no reports in the literature on the analysis of glucuronides in the plasma of smokers. The advantage of analyzing the glucuronides in plasma rather than in urine is that plasma analysis offers a better understanding of the overall pharmacokinetics and pharmacodynamics of NIC and its metabolites.

Several epidemiological studies have been carried out to determine the relationship between smoking behavior and ethnic background [9–11]. Other studies have shown that certain neurological diseases may be less prevalent in smokers compared to non-smokers [12,13]. Because NIC metabolism may be one of the key variables influencing NIC pharmacology, elucidating NIC's metabolic profile in humans may provide a better understanding of the relationship between smoking and ethnic background, and may also provide insight into the possible neurological properties of NIC.

It has been concluded from epidemiological studies that smokers tend to drink more caffeinated beverages than non-smokers. These studies have shown a positive association between cigarette smoking and coffee drinking [14,15]. This may be related, at least in part, to the fact that tobacco products are inducers of caffeine (CAF) metabolism [16]. However, the interaction of CAF and NIC in human smokers is not well understood.

Several methods have been developed to quantify NIC metabolites in different biological fluids, including immunoassay and chromatography [17–20]. Immunoassays such as RIA and ELISA have been used for small volume samples but cross-reactivity with closely related molecules often leads to over estimation of metabolite concentrations. Currently, the most frequently used chromatographic method is gas chromatography with nitrogen phosphorous detection, or mass spectrometric detection, or flame ionization detection [21]. The main disadvantage of the GC method is the difficulty of indirectly detecting and quantifying the more polar metabolites of NIC, such as 3HC and the glucuronides conjugates. Sample preparation can be tedious, especially when using gas chromatography-mass spectrometry. An additional problem is that some polar components decompose at the high temperatures utilized in the analysis. HPLC systems, connected to ultraviolet (UV), mass spectrometric, or electrochemical detectors for quantifying major NIC metabolites, are frequently used to overcome the disadvantages of the above methods [21]. HPLC utilizing UV detection is considered more practical than using HPLC with other detectors such as mass spectrometers or electrochemical detectors due to simplicity and low cost.

This paper describes a simple isocratic HPLC method for quantifying COT, 3HC and their respective glucuronides conjugates (COT-GLU, and 3HC-GLU), as well as CAF, in smokers' plasma.

2. Method and materials

2.1. Chemicals

S-(−)-Cotinine, caffeine, *N*,*N*-diethylnicotinamide (Sigma, St. Louis, MO) and dichloromethane, triethylamine (both HPLC grade), monobasic phosphate (Fisher Scientific, Pittsburgh, PA) were purchased. *Trans*-3HC and COT-GLU were synthesized as previously described in the laboratory [22]. Millipore filtered water was obtained by passing distilled water through a Milli-Q system (Millipore, Milford, MA).

2.2. HPLC system

The analytical system comprised an HP 1090 HPLC (Hewlett Packard, Avondale, PA) unit connected to a HP Chemstation and data analysis unit. A Whatman Partisil SCX 10 column (25 × 0.46 cm, Whatman, Clifton, NJ) connected to a Phenomenex SCX security column (Phenomenex, Torrance, CA) was used. A mobile phase of sodium phosphate/methanol (92:8 v/v, 0.1 M, adjusted to pH 4.8 with triethylamine) was utilized. A flow rate of 1.5 ml/min was used and UV detection was carried out at 254 nm.

2.3. Standards and controls

Stock solutions of each analyte; COT, 3HC, CAF, and COT-GLU (1 mg/ml, 100, 10, and 1 μg/ml) and *N*,*N*-diethylnicotinamide (Fig. 1) as internal standard (20 μg/ml) were prepared in methanol. All solutions were stored at −20°C until utilized. Controls for the generation of calibration curves were freshly prepared prior to analysis using drug-free human plasma. Calibration curves for each analyte were generated from at least six points on each curve, with each point representing an average of three runs; concentrations ranged from 10 ng/ml to 1 μg/ml for COT and 3HC, and 10 ng/ml–10 μg/ml for CAF. Inter-day and intra-day variations were assessed using concentrations of 50, 200, 500 ng/ml for each of the three analytes: CAF, COT, and 3HC. The detection limit (signal-noise ratio is 2:1) and quantification limits (lowest concentration at which the coefficient of variation between repeated measurement > 20%) were assessed.

2.4. Biological sample preparation

Blood samples (10 ml) from smokers were collected between 2 and 4 pm in EDTA-containing tubes and centrifuged immediately at 1000 × *g* and 4°C for 15 min. The plasma supernatant was then collected and frozen at −70°C until analyzed.

2.5. Quantification of plasma cotinine, 3HC, and caffeine

Plasma (0.5 ml containing 500 ng of *N*,*N*-diethylnicotinamide as internal standard) was added to 0.5 ml of 0.5 M NaOH, and the mixture extracted once with dichloromethane (10 ml). The dichloromethane layer (8 ml) was separated by centrifugation, rapidly evaporated to dryness in a stream of air, and 300 μl of water was added to the resulting residue. A 200 μl aliquot part of this aqueous solution was injected onto the HPLC system. Each plasma sample was analyzed in duplicate. Analysis of CAF in plasma was also determined for comparative purposes using a previously reported gas chromatographic analysis procedure as an alternative method of quantification [23].

2.6. Quantification of total cotinine and total 3′-hydroxycotinine

A 0.5 ml volume of 5.0 M NaOH was added to 0.5 ml plasma. The mixture was heated at 70°C for 30 min. A 25 μl volume of methanol containing 500 ng of *N*,*N*-diethylnicotinamide as internal standard was added. The mixture was then extracted once with dichloromethane (10 ml). The dichloromethane layer (8 ml) was separated by centrifugation, rapidly evaporated to dryness in a stream of air, and 300 μl of water was added to the resulting residue. A 200 μl aliquot part of this aqueous solution was injected onto the HPLC system. Each plasma sample was analyzed twice. The amount of total COT and total 3HC (i.e.

resulting from both conjugated and non-conjugated forms) in each sample was calculated, and the conjugated metabolites determined indirectly by subtracting the values obtained from the initial analysis of the unconjugated metabolites.

Hydrolysis of COT-GLU was determined over time at 70°C under the basic hydrolysis conditions utilized in the assay, to test the efficiency of the deconjugation procedure. A 30 min incubation time was found to be optimal for conversion of COT-GLU to COT under these conditions (Fig. 2). The hydrolytic conversion value for 1000 ng/ml COT-GLU was determined to be $92 \pm 6\%$ at 30 min incubation time.

3. Results and discussion

CAF and the two major NIC metabolites, COT and 3HC were separated and quantified in plasma samples taken from a group of ten smokers (Table 1). The retention times were: CAF 5.1 min, 3HC 7.2 min, *N*,*N*-diethylnicotinamide (internal standard) 9.5 min, and COT 15.5 min (Fig. 3).

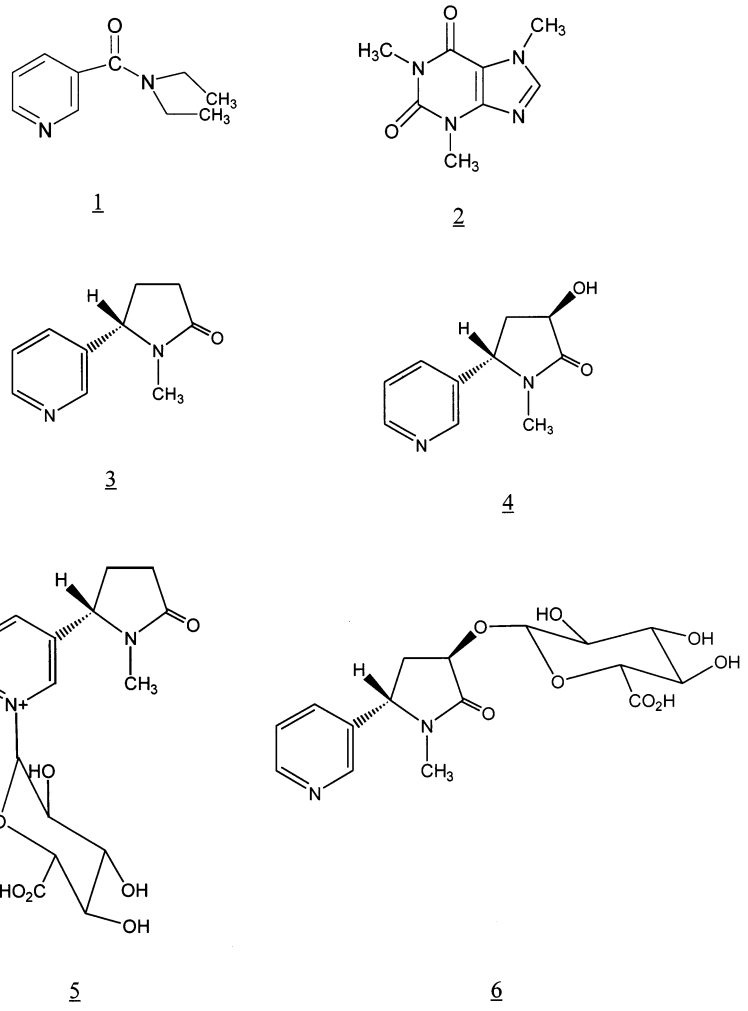

Fig. 1. The structures of the internal standard, *N*-*N*-diethylnicotinamide (1), caffeine (2), S-(−)-cotinine (3), *trans*-3′-hydroxycotinine (4), S-(−)-cotinine *N*-glucuronide (5), and *trans*-3′-hydroxycotinine O-glucuronide (6).

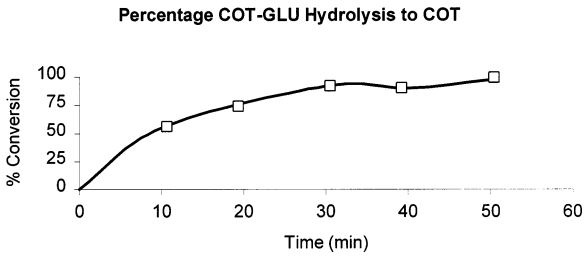

Fig. 2. Hydrolysis profile of cotinine *N*-glucuronide (1000 ng/ml) conversion to cotinine. Results are expressed as percentage of hydrolysis vs. time of incubation. Experiments were performed under the conditions described in Section 2.

The calibration curve for each analyte showed good linearity with detection limits for CAF of 10 ng/ml, for 3HC of 10 ng/ml, for COT of 10 ng/ml, for total COT of 10 ng/ml and for total 3HC of 20 ng/ml. The quantification limits were 30, 10, 10, 10, and 20 ng/ml, respectively. The following calibration curve equations were used: CAF ($y = 0.001x + 0.0329$), COT ($y = 0.0015x + 0.0202$), 3HC ($y = 0.0006x + 0.006$), total COT ($y = 0.0014x - 0.0017$), and total 3HC ($y = 0.0003x + 0.0019$). The correlation coefficient (r^2) was greater than 0.99 for all of the curves. Inter-day and intra-day variations for the components were determined, and were found to be between 1 and 8%. The recovery was 84–88% for CAF, 80–85% for COT, and 42–47% for 3HC.

COT-GLU hydrolysis to COT under the basic conditions described was assessed using authentic standards, and an average of $92 \pm 6\%$ of COT-GLU was converted to COT. Unfortunately the synthesis of 3HC-GLU has not been previously reported, thus, an authentic standard of this metabolite is not available. Consequently, quantitative base hydrolytic conversion of this compound to the deconjugated product could not be established.

It is important to note that in comparing 3HC and total 3HC curves, a loss of 3HC was observed under basic conditions during heating, which resulted in a decrease in the sensitivity for total 3HC determination. However, the results are not affected by loss, due to the establishment of a total 3HC calibration curve, which accounts for any loss in 3HC during heating. Thermal instability was not observed for COT and total COT determination, suggesting that the instability of 3HC could be due to the presence of the 3′-hydroxyl group.

HPLC data for nine smokers afforded caffeine levels in the range 0.4–2.6 μg/ml with a mean value of 1.42 ± 0.30 μg/ml. Undetectable levels of caffeine were found in one smoker. The above HPLC data correlated well [corr. coeff. = 0.98; $F(1,9) = 204.4$, $P < 0.001$] with data obtained from the same samples using the GC method of Greene et al. [23].

Table 1
The quantitation of caffeine (CAF), cotinine (COT), 3′-hydroxycotinine (3HC), cotinine glucuronide (COT-GLU) and 3′-hydroxycotinine glucuronide(3HC-GLU) in smokers' plasma

Sample no.	CAF[b]	CAF[c]	COT	COT-GLU	3HC	3HC-GLU
1	444[a]	501	150	283	56.3	114
2	2050	1860	187	19.1	96.7	0
3	807	611	102	74.2	16.7	47
4	2440	2240	35.9	281	83.3	144
5	547	590	139	16.5	94.1	79.6
6	2620	1940	92.2	28	45.8	0
7	0	0	83.9	5.7	79.2	101
8	1140	766	56.5	96.3	50.8	84.5
9	1640	1440	128	39.4	91.7	35.9
10	2490	1270	39.9	122	28.3	79.2
Mean ± S.E.M.	1420 ± 303	1120 ± 234	101 ± 15.7	96.5 ± 33.0	64.3 ± 9.10	68.5 ± 14.9

[a] Values in ng/ml.
[b] Caffeine levels measured by HPLC as described in Section 2.
[c] Caffeine levels measured by GC utilizing a published method [23].

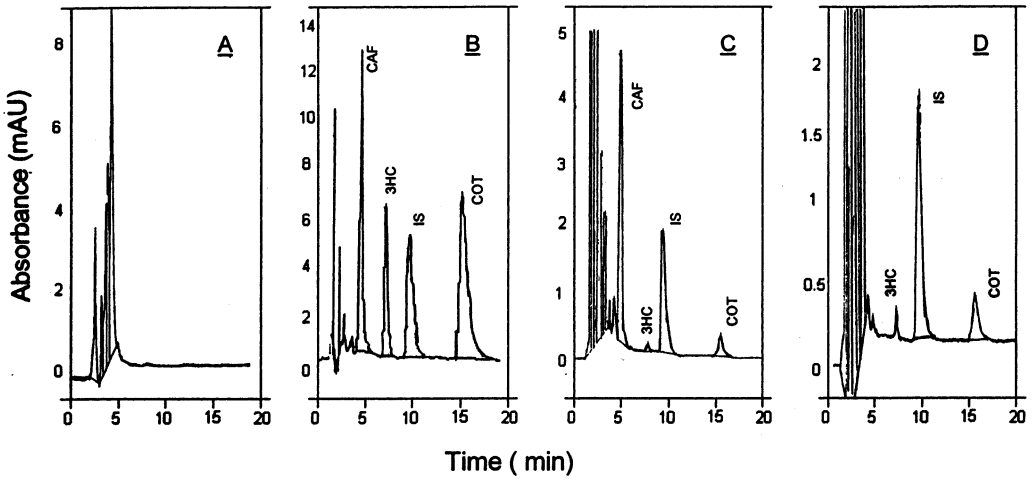

Fig. 3. Four chromatograms illustrating HPLC analysis of: (A) a drug-free human plasma sample; (B) an authentic metabolic standard mixture in water containing *N*,*N*-diethylnicotinamide as internal standard (IS), caffeine (CAF), cotinine (COT) and 3′-hydroxycotinine (3HC). (C) A smoker's plasma sample processed as described in Section 2 for the analysis of CAF, COT and 3HC. (D) A smoker's plasma sample processed as described in Section 2 for the analysis of total (deconjugated and unconjugated) COT and 3HC.

In the same analytical runs, from which caffeine levels were determined, it was also possible to quantify the four major NIC metabolites COT, 3HC, COT-GLU and 3HC-GLU. COT levels in the group of ten smokers examined ranged from 35.9 to 187 ng/ml with a mean of 101 ± 15.7, where 3HC levels were 16.7–96.7 ng/ml with a mean of 64.3 ± 9.1. The results are relatively close to the results obtained in an earlier study which measured COT and 3HC in smokers serum ($n = 20$). The average serum levels in that study were COT 134 ± 75.0 and 3HC 25.7 ± 19.1 [19]. Interestingly, plasma concentrations of the glucuronide conjugates were generally much more variable than the values obtained from the corresponding unconjugated metabolites. For COT-GLU, plasma levels ranged from 5.7 to 283 ng/ml (mean, 96.5 ± 33 ng/ml) and for 3HC-GLU from 0 to 144 ng/ml (mean, 68.5 ± 14.9 ng/ml). In two individuals, 3HC-GLU concentrations were below the level of detection for the HPLC assay, and COT-GLU concentrations were also low in these smokers. These results suggests that the glucuronide pathway for NIC metabolism may be deficient in some individuals.

The results generally show that the glucuronidation pathway for NIC metabolism is a major route in smokers, and that plasma concentrations of these conjugated biotransformation products may reach levels exceeding those of cotinine in certain individuals.

4. Conclusion

An HPLC analytical procedure has been developed for the separation and quantification of COT and 3HC, as well as CAF in human plasma. The glucuronide conjugates of COT and 3HC could also be indirectly quantified by this method via initial basic hydrolysis of the conjugates to their unconjugated forms prior to analysis.

This simple analytical HPLC method can be utilized for routine monitoring of levels of CAF and nicotine's two major metabolites in human plasma.

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