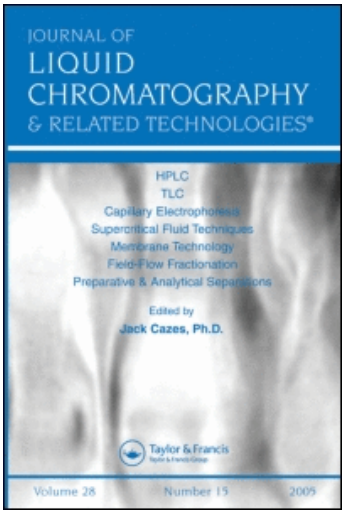

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ANALYTICAL IMPROVEMENTS IN BARLOW REACTION COUPLED TO HPLC DETECTION OF NICOTINE AND ITS METABOLITES

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ABSTRACT

The aim of this work was to optimize the conditions of a Barlow reaction coupled with HPLC to assay nicotine and its metabolites cotinine, trans-hydroxycotinine, and pyridylcarbinol. Thiobarbituric acid was chosen as chromogen and the incubation time for the reaction was set at 15 minutes. The mobile phase was a mixture of acetate buffer 0.05 mol/L pH 5.2, acetonitrile, tetrahydrofuran, triethylamine (86.0/11.4/2.5/0.1 v/v), and acetic acid to pH 5.4. Applied to a population of 30 smokers, the method was shown to be simple, reliable, and rapid (under 20 minutes).

INTRODUCTION

The specific markers of tobacco impregnation are nicotine, responsible for dependence,[1,2] and its metabolites. The urine assay of these components has allowed us to measure how severely a smoker is intoxicated,[3] and how to monitor tobacco withdrawal by substitution treatment.[4] The analytical methods currently most often used are gas chromatography[5] and high performance liquid chromatography (HPLC),[6-9] but both require a time-consuming extraction step. Multidimensional techniques including liquid chromatography-mass spectrom-

etry/mass spectrometry[10] are superior to conventional ones but likewise time-consuming and very expensive. A simpler method preferred by some workers[11,12] is to apply the colorimetric Barlow reaction directly on the urine of smokers. Unfortunately, this reaction is not specific: it detects all compounds containing an unsubstituted pyridine ring, as well as aldehydes and ketones.[12] Some workers have already proposed the Barlow reaction followed by HPLC,[13-15] but their procedures are often useless for routine use. Rustemeier et al.[14] assay nicotine and 12 metabolites on a ternary gradient taking more than 35 minutes; Moore et al.[13] use solid-phase extraction before injection on a binary gradient taking more than 55 minutes. Others[15] only detect nicotine and cotinine but not trans-hydroxycotinine, which can account for up to 90% of the markers of tobacco impregnation in urine.[16] The aim of this work was to develop a simple, reliable and interference-free method of separation and assay using the Barlow reaction followed by isocratic HPLC (termed «Barlow/HPLC»), which would separate nicotine and three of its metabolites (cotinine, trans-hydroxycotinine, and pyridylcarbinol) in the urine of smokers in less than 20 minutes.

EXPERIMENTAL

Chemicals

Nicotine (NIC), cotinine (COT), pyridylcarbinol (PC), and all other chemicals including thiobarbituric acid (TBA), diethylthiobarbituric acid (DETBA), and HPLC solvents were purchased from Merck (F-94736 Nogent sur Marne). Trans-hydroxycotinine (THOC) was a gift from SEITA (F-45401 Fleury-les-Aubrays).

Standards and Controls

NIC, COT, PC, and TOHC were dissolved in methanol to give 10 working solutions with final concentrations from 0.05 to 25 μg/mL. Dilutions were made fresh daily for each analysis.

Studied Population

Thirty smokers (12 males, 18 females; age = 40 ± 11 (median = 37)), recruited in the "Tobacco withdrawal Consulting Center", Laennec Hospital, Paris, France, were listed after screening, using the following exclusion criteria: hepatic and renal failure (blood ASAT, ALAT and γGT > 40 UI/L, urea > 7.5 mmol/L and creatinine > 110 μmol/L), respiratory failure and smoke non-inhaled. They smoked 25.0 ± 9.7 (median = 20.0) cigarettes per day. Exhaled

CO was 16.0 ± 9.7 (median = 13.0) ppm. Blood HbCO was 4.3 ± 1.6 (median = 4.4)% and Fagerström index was 7.8 ± 2.3 (median = 8.0). Their morning preprandial urine samples were collected. The micturition volume was 347 ± 174 (median = 310) mL and urinary creatinine 12.1 ± 6.1 (median = 9.9) mmol/L.

Instrumentation

The chromatographic analysis has been performed on an isocratic HPLC system (WATERS, F-78056 St-Quentin-en-Yvelines), consisting of a model 501 solvent pump, a U6K injector, a model 481 multiwavelength detector, a model 745 data recorder and electronic integrator. The Barlow assay absorbances were measured on a DU 7400 spectrophotometer (BECKMAN, F-93220 Gagny). Use of the U6K injector, with no extraction phase for the sample to be injected, made an internal standard unnecessary.

Chromatographic Conditions

We used a Nucleosil (AIT, F-78600 Le Mesnil Le Roi) C18 column (22 cm x 4.6 mm I.D.; particle size 5 µm). The flow rate was 1mL/mn. The column was equilibrated with the mobile phase for 30 minutes before analysis of samples.

Barlow Assay

Standards or urine (200 µL) were pipetted into a 5 mL glass centrifuge tube. A 100 µL volume of 4 mol/L acetate buffer pH 4.7, 40 µL of 1.5 mol/L potassium cyanide in water, 40 µL of 0.4 mol/L chloramine T in water and 200 µL of 78 mmol/L chromogen (TBA or DETBA) in 0.05 mol/L NaOH were added. The mixture was shaken and centrifuged at 2500 g for 10 min. The supernatant was filtered on 0.45 µm filters (MILLIPORE, F-78056 St-Quentin-en-Yvelines) and 25 µL was injected onto the HPLC column.

Optimizing Procedure

The most frequently proposed mechanism for the Barlow reaction is complex.[12-16] Cyanogen chloride is formed in situ by addition of chloramine T and potassium cyanide (KCN). It cleaves nitrogen-unsubstituted pyridine rings to form a pyridinium ion, which is converted in aqueous medium to glutaconic aldehyde, which condenses with TBA or DETBA to give a red-orange coloration. This reaction and the HPLC operating conditions were optimized by acting on four main variables:

Reaction Time

Due to the instability of the Barlow coloration, the reading time was optimized by following the time course of the coloration of a "standard mixture solution" containing 6.25 μg/mL of NIC, COT, PC, and TOHC, and varying the time, from 10 to 25 minutes, between adding the chromogen and reading. The measurements were carried out either directly on the spectrophotometer or after injection into the HPLC system. The efficiency of sodium metabisulfite added to the reaction medium in stabilizing the reaction was tested.

Choice of Chromogen

The two chromogens, TBA and DETBA, were studied by comparing the absorption spectra and chromatography profiles of the reagent blank and "standard mixture solution" after Barlow reaction with each chromogen.

Chloramine T Interference

From the procedure proposed by Barlow,[12] the concentration of chloramine T was reduced by 25, 50, and 75% and the different chromatography profiles obtained were compared. KCN and chloramine T were replaced by cyanogen bromide (final concentration 1 mol/L) with or without addition of sodium metabisulfite.

Mobile Phase

The mobile phase was optimized using a mixture of 0.05 mol/L acetate buffer pH 5.2, acetonitrile, tetrahydrofurane, and triethylamine (86.0/11.4/2.5/0.1 v/v), and the pH was adjusted with acetic acid in the range of 5.0 to 5.6.

The method was validated by repeatability tests on three standard solutions containing 0.625 μg/mL, 9.375 μg/mL, and 18.750 μg/mL (17), and then applied to a population of 30 smokers.

Expression of Results

The results were expressed as mean ± standard deviations and median [M ± SD (m)]. Given the small sample size and not-gaussian distribution of the results, the correlation coefficients (r) were calculated using Spearman's non-parametric test. Each metabolite appears on the chromatogram as two peaks. The surface areas of the two peaks were summed and expressed compared to the concentration of the standard. Each of the two peaks was also quantified separately.

RESULTS AND DISCUSSION

Reaction Time and Instability of the Coloration

The coloration of the "standard mixture solution" submitted to a Barlow reaction using TBA appeared in about 5 minutes and then fades by over 40% in 30 minutes (Figure 1). The addition of sodium metabisulfite causes an almost immediate and total decoloration of the reaction product. This observation conflicts with the suggestion of some workers that sodium metabisulfite can be used to quench the reaction.[12] Others report stabilization, even though partial: after four hours, only 32% of the colored derivative of NIC remains, and 22% of that of COT.[15]

The exact times for addition of the reagents and incubation have to be strictly observed. After these conditions, the extraction of the colored derivatives by organic solvents (shown in previous works[18-20] to be incomplete) and the solid-phase extraction,[13] expensive and time consuming are unnecessary. Furthermore, the recovery is often poor.

The KCN, chloramine T and TBA were added successively with a time lag of 15 seconds as recommended by Smith et al.[18] Figure 2 has shown the

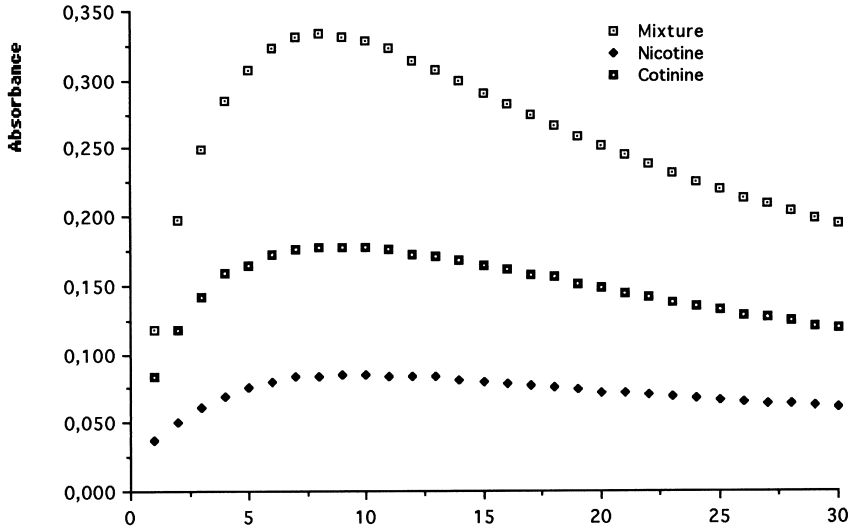

Figure 1. Time course at 490 nm of the Barlow reaction on a mixture of NIC, COT, TOHC and PC (6.25 μg/mL) and on NIC and COT solutions.

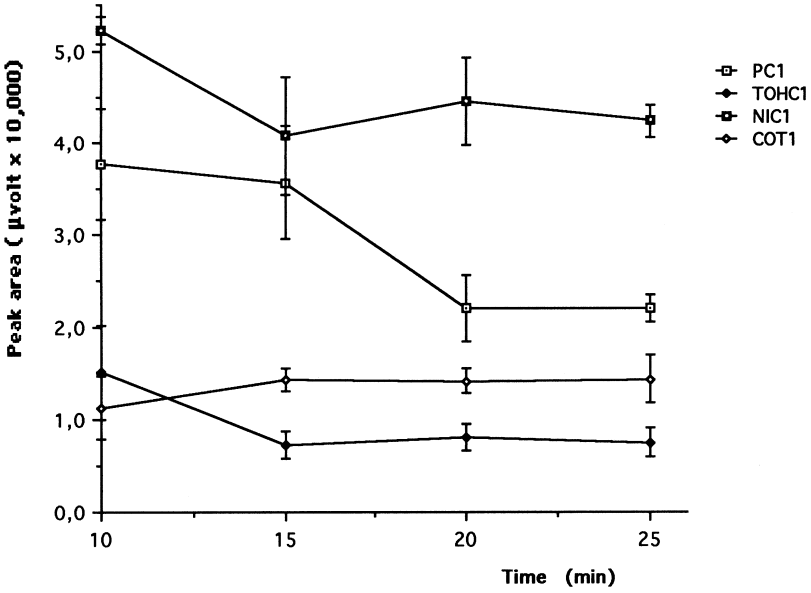

Figure 2. Time course of peak surface area for 6.25 µg/mL NIC, COT, TOHC and PC against Barlow reaction incubation time.

time course of the peak areas after HPLC injection against incubation time. For NIC, COT, and TOHC, it is relatively stable between 10 and 25 minutes. In contrast, the PC peaks decreased after 15 minutes. As COT is the marker currently used in the evaluation of nicotine substitution treatment, we preferred the COT assay with an incubation time of 15 minutes to retain a maximum peak and obtain a high sensitivity. These conditions are those used by most workers, who plead for incubation times between 15 and 20 minutes.[11,12]

Choice of Chromogen

Assay of the "standard mixture solution" by the Barlow reaction using either TBA or DETBA, after an incubation time of 15 minutes, showed a vivid pink-mauve coloration with DETBA (λmax = 536 nm, absorbance = 0.38), and a less intense pale orange coloration with TBA (λmax = 490 nm, absorbance = 0.27). HPLC injection of a reagent blank solution containing acetate buffer 4 M, pH 4.7, KCN, chloramine T, and TBA (Figure 3), showed that TBA generates a group of peaks with very short retention time (2 to 3 minutes), which do not impede reading of the chromatogram. Under the same conditions,

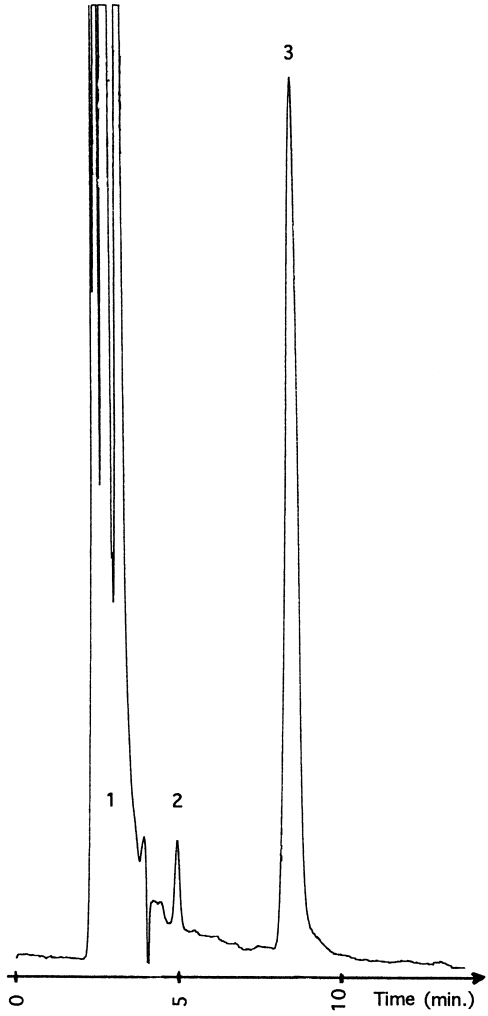

Figure 3. HPLC chromatogram of blank, showing TBA "group" (1) and chloramine T (2 & 3). (Chromatographic conditions are given in text).

DETBA gave a peak that come out between 6 and 10 minutes, masking the standard peaks. Though DETBA was more sensitive than TBA, it cannot be used as a chromogen under the conditions selected. TBA was, accordingly, chosen and the detector wavelength was set at 490 nm.

Chloramine T Interference and Specificity

The reagent blank chromatogram (Figure 3) has shown, in addition to the group due to TBA, two peaks at 5 and 9 minutes due to chloramine T. These peaks were not reported in previous works. The peak at 9 minutes can be an impediment as its occurs in the area of the NIC and NIC-metabolite peaks. Reducing the concentration of chloramine T in the reaction mixture reduces the size of its peaks, but also reduces the coloration. Replacing the KCN-chloramine T mixture with cyanogen bromide caused an immediate development of the coloration, followed by total decoloration (Figure 4). Adding 1 mol/L sodium metabisulfite did not stabilize the reaction: the solution underwent the same decoloration and then gradually turned yellow (formation of bromine water).

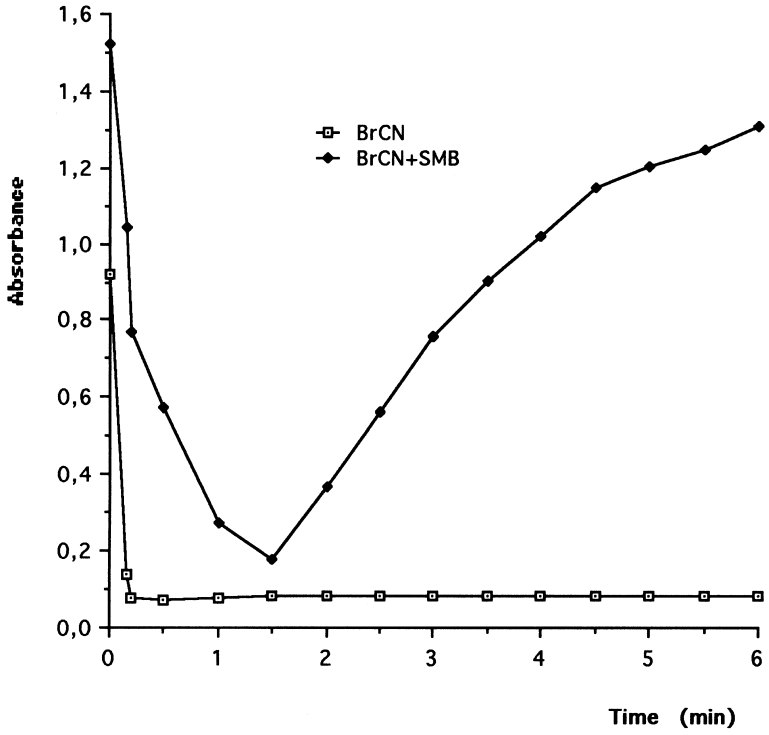

Figure 4. Time course of Barlow reaction by use of cyanogen bromide, on a solution of 100 μg/mL NIC, in presence or absence of sodium metabisulfite (SMB).

Mobile Phase

Varying the pH of the mobile phase from 5.6 to 5.0 caused a shift of the metabolite peaks with optimal separation at pH 5.4. The following mobile phase was, therefore, chosen: acetate buffer 0.05 mol/L pH 5.2, acetonitrile (CH3CN), tetrahydrofurane (THF), triethylamine (86.0/11.4/2.5/0.1 v/v), and acetic acid to pH 5.4. Figure 5 shows the chromatographic profile obtained under these conditions with a standard solution (A) and a smoker's urine (B).

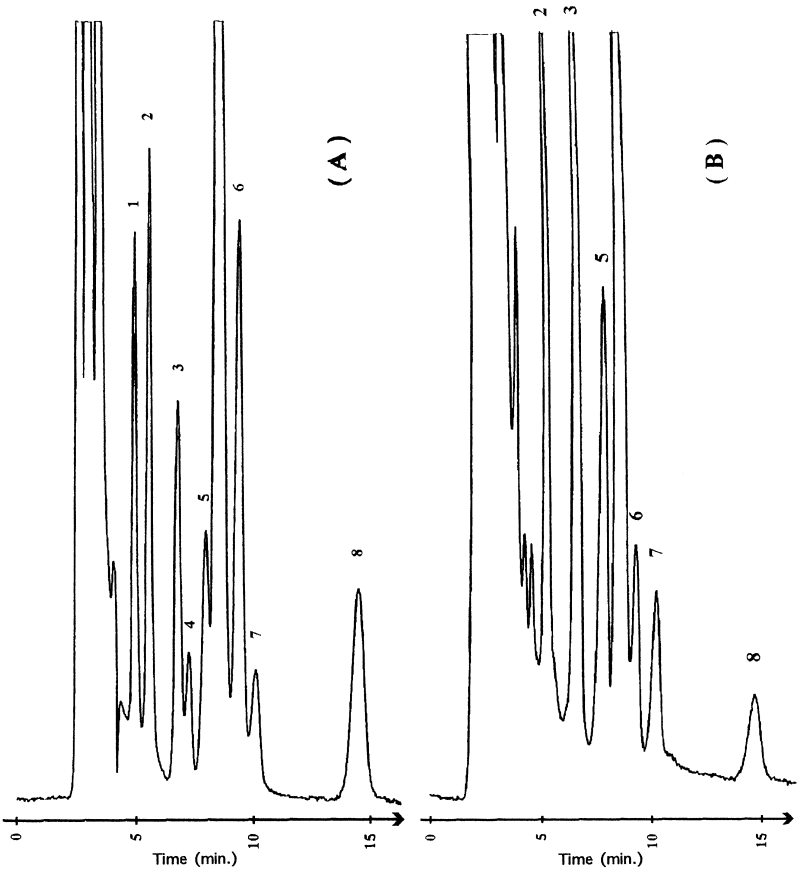

Figure 5. HPLC chromatogram of (A) 6.25 µg/mL "standard mixture solution" and (B) smoker's urine. Peaks: 1=PC1 ; 2=TOHC1 ; 3=NIC1 ; 4=PC2 ; 5=TOHC2 ; 6=COT1 ; 7=NIC2 ; 8=COT2.

Appearance of the Chromatogram

With the "Barlow/HPLC" method, as shown in Figure 5, the chromatogram profile of the "standard mixture solution" has shown two peaks for each compound. Most of the workers using the Barlow reaction followed by HPLC have not reported this effect. Only Rustemeier et al.[14] have drawn attention to the occurrence of two peaks: 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. Several assumptions may be put forward to explain this peak doubling. The first suggests that impurities or breakdown of the reference solutions, even under optimal storage conditions, might explain the presence of two peaks for a single injected solution. However, neither extemporaneous preparation of the standard solutions, nor the use of NIC bitartrate, which is known for its very high stability, prevent the peak doubling. The most likely explanation is found in the mechanism of the Barlow reaction. Rustemeier et al.[14] propose that after cleavage of the pyridine ring by the KCN-chloramine T mixture, the glutaconic aldehyde formed binds with one TBA or DETBA molecule, whereas O'Doherty et al.[21] suggest a formula with condensation of two TBA or DETBA molecules. These two molecular structures could coexist together (Figure 6), which would produce two peaks for NIC and its derivatives.

Validation

Figure 7 depicts the COT calibration curves. The correlation coefficients between peak surface area and theoretical concentrations were 0.993 for PC1 and 0.999 for NIC 1&2, COT 1&2, TOHC 1&2, and PC2.

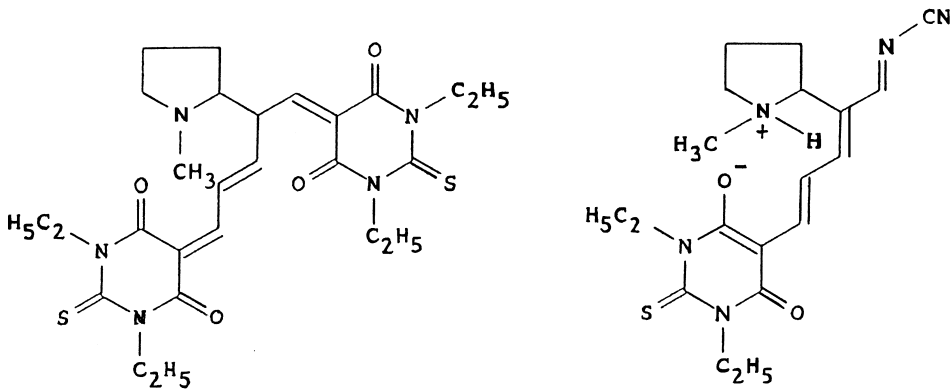

Figure 6. Molecular structures of the colored derivatives of NIC after Barlow reaction proposed by (1) O'Doherty and (2) Rustemeier.

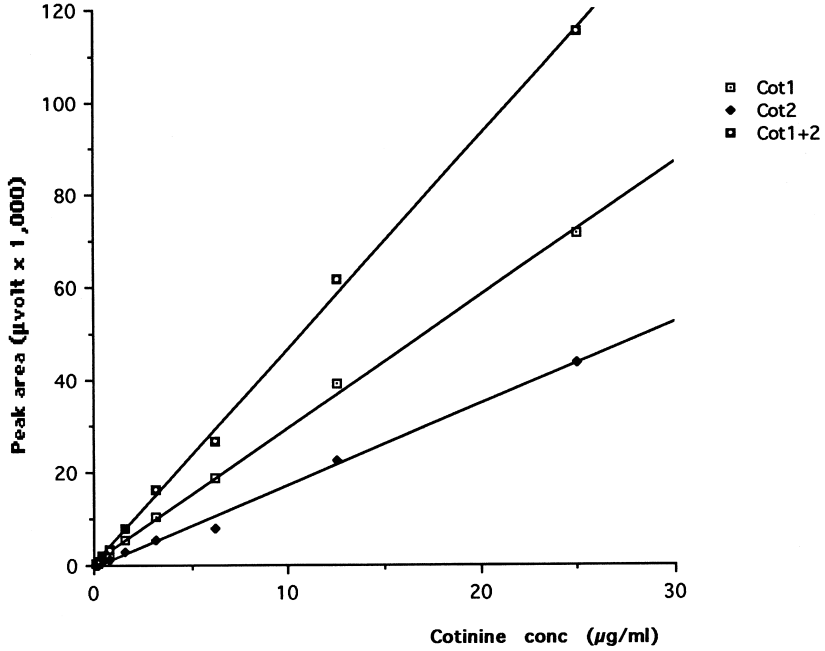

Figure 7. Calibration range of COT for summed and separate double peaks

Both the intra- and the inter-assay reproducibility were found to be satisfactory (Table 1) except for TOHC for the low level. However, this poor result was not useful, because this marker is found in very large amounts in smokers.[16] For peak 1, the detection threshold was 0.05 µg/mL and the quantification threshold was 0.10 µg/mL. For peak 2, which were smaller in area, the detection threshold was 0.10 µg/mL and the quantification threshold was 0.50 µg/mL.

Application to a Sample of Smokers

After validation, the "Barlow/HPLC" method was applied to a sample of 30 smokers. The value of NIC and its metabolites are given in Table 2. The results are expressed classically against urinary creatinine concentration. Although this expression, which is affected by inter- and intra-individual variations in creatinine levels,[22] may not be the best one,[23] we chose it in order to be able to compare our results with those of the literature.[14,24,25] The NIC concentrations were appreciably higher. Among the metabolites of NIC, PC was detected in none of the 30 smokers, but its occurrence in human urine has been challenged.[26] The results of TOHC, which is considered to be the main metabo-

Table 1

Performance Statistics of the HPLC Assay for Nicotine, Cotinine and Trans-Hydroxy-Cotinine

Compound	Theoretical Concentration (µg/mL)		Precision (CV%) Intra-Assay (n = 15)	InterAssay (n = 15)
Nicotine	0.625	Peak 1	4.9	5.5
		Peak 2	5.3	5.9
	9.375	Peak 1	4.0	4.6
		Peak 2	4.7	5.2
	18.75	Peak 1	3.8	4.2
		Peak 2	4.1	4.5
Cotinine	0.625	Peak 1	5.3	5.6
		Peak 2	4.5	4.7
	9.375	Peak 1	4.3	4.7
		Peak 2	4.3	4.6
	18.75	Peak 1	2.7	3.5
		Peak 2	2.7	3.3
Trans-OH-Cotinine	0.625	Peak 1	15.3	17.9
		Peak 2	19.5	20.4
	9.375	Peak 1	6.9	7.4
		Peak 2	8.3	9.0
	18.75	Peak 1	6.2	6.7
		Peak 2	7.7	7.9

Table 2

Urine Concentration of NIC and its Metabolites in a Population of 30 Smokers Measured by the "BARLOW"/HPLC Method Relative to Urinary Creatinine Level (µmol/mmol Creatinine)*

	Peak 1	Peak 2	Peak 1 + 2
NIC	4.77 ± 1.88 (4.53)	1.50 ± 1.60 (0.80)	3.82 ± 1.55 (3.55)
COT	0.57 ± 0.60 (0.45)	0.50 ± 0.57 (0.36)	0.55 ± 0.58 (0.44)
TOHC	4.19 ± 2.43 (3.48)	5.55 ± 2.65 (5.45)	4.74 ± 2.43 (4.36)

* Calibration of NIC, COT, and TOHC versus peak 1, peak 2 or peak 1 + 2.

lite of nicotine,[26,27] were similar[25] or slightly higher. The concentrations of COT, which is the more useful marker in all routine analytical laboratories, were of the same order.

In the case of NIC, the ratio of peak 1 to peak 2 concerning the standards and the urine samples is not the same. This difference could be caused by the fact that method validation was studied on standard solutions prepared in methanol and by the matrix (methanol) of these standard solutions, but we noted no significant difference when preparing the standards in water, buffer pH 4.0, buffer pH 10.0, or urine of a non-smoker unexposed to cigarette smoke. Another more credible hypothesis is that a urinary tobacco constituent could influence the derivatization reaction. Neither Moore et al.[13] nor Ubbink et al.[15] investigated the by-products that eluted early from the column. An unknown "peak C" with short retention-time was investigated by Rustemeier et al.;[14] after incubation with β-glucuronidase, "peak C" disappeared, indicating a glucuronidation. In our chromatogram, such a peak does not appear, because, unlike Rustemeier et al.,[14] our derivatization was performed using the 4 mol/L, pH 4.7 aceto-acetate buffer proposed by Barlow et al.[12,20] According to Schepers et al.,[28] our results suggest that this acid buffer hydrolyses the NIC-glucuronide and induces the NIC1 increase. To test this assumption, we observed that a 24 hours incubation of five urine samples obtained from smokers, with β-glucuronidase (1000 UI/l; pH 5.0; T = 37°C) did not induce any transformation of the chromatogram.

Due to the fact that NIC was deglucuronidated, we chose the calibration versus peak 1 + 2 including NIC and NIC-glucuronide.

CONCLUSION

The "Barlow/HPLC" method separates NIC and three of its metabolites. Full consideration of the complex mechanism of the Barlow reaction helped us to set optimal conditions for its application. Fortunately, the chromatography step does not require a mobile phase gradient. Overall, the "Barlow/HPLC" method has proved reliable, simple, and rapid. It takes less than 20 minutes to perform, making it suitable for many routine analytical applications. This approach is well suited to applications in screening for tobacco abuse, and monitoring therapeutic withdrawal.

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