Journal of Chromatography, 620 (1993) 254–259
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7097

Short Communication

Simple high-performance liquid chromatographic method to verify the direct barbituric acid assay for urinary cotinine

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(First received June 2nd, 1993; revised manuscript received August 24th, 1993)

ABSTRACT

An isocratic HPLC method is described to determine urinary concentrations of nicotine and cotinine after derivatization with cyanogen chloride and barbituric acid. This method has been used to assess the reliability of the direct barbituric acid assay to determine smoking status. It is concluded that the direct barbituric acid assay is a very reliable indicator of smoking status, provided that urine blank samples are prepared to correct for background absorbance. If the direct barbituric acid assay is in disagreement with self-reported smoking status, this HPLC procedure is a useful method to resolve the discrepancy.

INTRODUCTION

Since there is a worldwide tendency to put a penalty on the health insurance of smokers, it has become important to verify smoking status by objective laboratory tests. Self-reported assessment of tobacco comsumption is often unreliable [1], and the urinary level of cotinine has become a popular choice to indicate smoking status. Cotinine is an important metabolite of nicotine [2], is present in a high concentration in the urine of smokers [3] and has a long elimination half life [2,4].

A convenient method to measure urinary continine levels is the direct barbituric acid (DBA) assay [5–8], which is based on the König reaction [9,10]. This method requires the opening of the pyridine ring of cotinine by reaction with cyanogen chloride to form a glutaconaldehyde derivative, which reacts with barbituric acid to form an orange colored complex. The concentration of cotinine and other nicotine metabolites can then be determined by spectrophotometry at 490–510 nm [11]. However, the König reaction is not entirely specific for cotinine since other compounds (drugs) containing a pyridine ring structure may also react with barbituric acid. Not only are false positive results possible, but false negatives may also occur as a result of the instability of the formed chromogen. In fact, a recent study found that the quantiative DBA assay suffered from a false negative rate of 7% and a false positive rate of 3% [6].

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Since an error of up to 7% is not acceptable to the local insurance industry, alternative methods have to be implemented to validate data on smoking behavior. Urinary cotinine levels can be reliable assayed by HPLC [3,12–14] or GC [15], but these methods are expensive and require time consuming extraction procedures. The direct DBA assay is considerably more cost effective and may become the method of choice if false results can be eliminated.

In this study, we evaluated a simple HPLC procedure to validate the DBA assay. The HPLC procedure requires the same sample derivatization procedure as employed in the DBA assay, thus allowing for easy reanalysis of urine samples when the self-reported assessment of smoking behavior is at variance with the DBA test.

EXPERIMENTAL

Materials

All the reagents were obtained from Merck (Darmstadt, Germany), except barbituric acid, which was bought from BDH (Poole, England), while cotinine and nicotine (hydrogen tartrate salt) were supplied by Aldrich Chem. Co. (Milwaukee, WI, USA).

DBA assay

Urinary concentrations of nicotine metabolites were determined by the direct barbituric acid of Peach *et al.* [5] as modified by Barlow and coworkers [6]. Urine blanks were prepared [7] and used for background correction. Absorbance was measured at 490 nm using a double beam Lamda 2 spectrophotometer (Perkin Elmer, Ueberlingen, Germany). Cotinine standards in water (50–250 μmol/l) were used to calibrate the assay and results are expressed as apparent cotinine levels.

HPLC of nicotine metabolites after reaction with barbituric acid

A Waters (Milford, MA, USA) Model 510 HPLC pump, coupled to a Spark-Holland (Emmen, The Netherlands) Marathon autosampler, was fitted with a Spherisorb S 5ODS 2 (Phase Sep, Queensberry, UK) reversed-phase analytical column (25 cm × 4.6 mm I.D., particle size 5 μm). A Whatman (Clifton, NJ, USA) reversed-phase guard column (1 cm × 4.6 mm I.D., particle size 10 μm) was fitted between the analytical column and the autosampler. The mobile phase was 0.05 *M* sodium acetate buffer (pH 5.2)–acetonitrile (78:22, v/v), and the flow-rate was 1.5 ml/min. The column eluate was monitored at 490 nm by a Beckman (Berkeley, CA, USA) Model 165 variable wavelength detector coupled to a Spectra-Physics (San Jose, CA, USA) Model 4290 integrator.

Urine samples or a standard containing 12.5 μmol/l of cotinine and nicotine, were treated as described by Barlow *et al.* [6] for the DBA assay. Immediately after the final addition of sodium metabisulfite to stop the reaction, 50 μl was injected onto the column.

Chromogen stability as evaluated by HPLC

A standard solution containing 12.5 μmol/l of cotinine and nicotine, respectively, was reacted with barbituric acid as described above. Aliquots (50 μl) were analyzed by HPLC at 10 min intervals up to 260 min.

Assessment of smoking status in humans

Male police recruits (n = 133), aged between 18 and 22 years, volunteerd to donate a urine sample for the assessment of smoking status. The purpose of the study was explained and the volunteers were asked to complete a questionnaire on smoking behavior. Based on smoking habits, the volunteers were divided into 3 groups, *i.e.* non-smokers (n = 78), light smokers (< 10 cigarettes/day; n = 39) and moderate smokers (> 10 cigarettes/day; n = 16).

RESULTS

Fig. 1 depicts chromatograms obtained after (A) analysis of a standard containing nicotine and cotinine at 12.5 μmol/l respectively, and urine samples obtained from (B) a smoker and (C) a non-smoker. No peaks corresponding to nicotine and cotinine were observed in urine samples from non-smokers, while smokers' urine

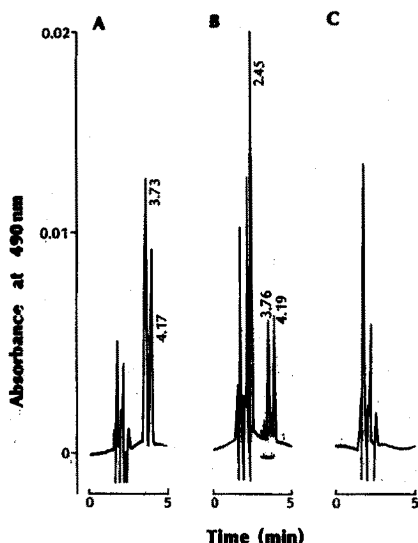

Fig. 1. HPLC analysis of the chromogens formed from cotinine (retention time: 3.7 min) and nicotine (retention time 4.1 min) after precolumn derivatization with cyanogen chloride and barbituric acid. (A) Standard solution containing 12.5 μmol/l of cotinine and nicotine, (B) analyses of a urine sample from a smoker, and (C) non-smoker. A large, unidentified peak at 2.45 min was present in urine samples from smokers.

contained, in addition to peaks corresponding with nicotine and cotinine, unidentified components that eluted early from the column.

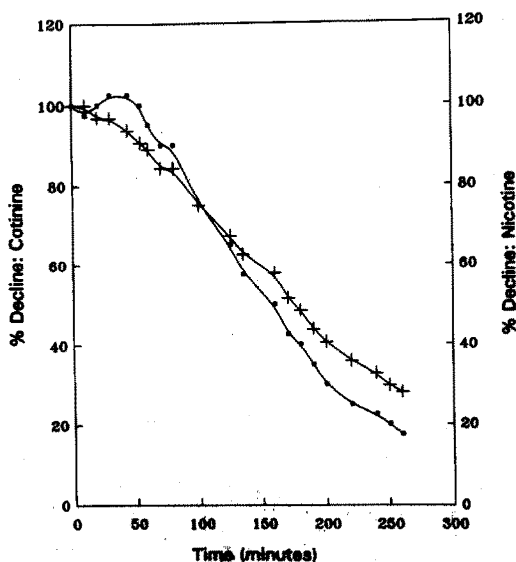

Fig. 2. Stability of nicotine and cotinine derived chromogens as assessed by HPLC. (+) Nicotine, (■) cotinine.

TABLE I

PERFORMANCE STATISTICS OF THE HPLC ASSAY FOR COTININE AND NICOTINE

Compound	Concentration range[a] (μmol/l)	Precision (coefficient of variation, %)	
		Within-day (n = 12)	Day-to-day (n = 12)
Cotinine	13.1–17.0	6.6	8.6
Nicotine	6.5– 7.8	6.9	7.1

[a] Concentration range refers to the minimum and maximum estimates of urinary cotinine and nicotine concentrations obtained during twelve consecutive days.

The chromogens derived from nicotine and cotinine are notably unstable, and 4 hours after addition of sodium metabisulfite to stop the reaction, only 32% and 22% of the original nicotine and cotinine derived chromogens could be detected by HPLC analysis (Fig. 2). The HPLC analysis should be done within 50 min after addition of sodium metabisulfite to ensure reliable estimation of the urinary cotinine and nicotine levels (Fig. 2). The rapid decay of the formed chromogens dictates that the number of samples per batch should be small in order to obtain acceptable analytical reproducibility. We limit the preparation of urine samples for HPLC to a maximum of 5 per batch; under these conditions both the within-day as well as the day-to-day reproducibility were found to be satisfactory (Table I).

Fifty-five volunteers indicated that they were smokers and the remaining 78 indicated that they were either non-smokers or ex-smokers who had ceased smoking more than a month ago. Apparent cotinine levels as determined by spectrophotometry were significantly higher in smokers compared to non-smokers, however, neither the spectrophotometric method nor the HPLC method could differentiate between light (<10 cigarettes/day) and moderate (>10 cigarettes/day) smokers (Table II). All the volunteers who indicated that they were non-smokers had urinary apparent cotinine levels <50 μmol/l, except for

TABLE II

ANALYSES OF COTININE AND NICOTINE IN URINE SAMPLES BY HPLC AND SPECTROPHOTOMETRY

Self-assessed smoking status	n	Daily cigarette consumption		Apparent cotinine[a] (μmol/l)	Cotinine HPLC (μmol/l)	Nicotine HPLC (μmol/l)
		Mean	Range			
Non-smoker[b]	78	0	0	11.4 (12.1)	0.06[c] (2.75)	0.01[c] (1.80)
Smoker (< 10 cigarettes/day)[d]	39	7.5 (2.6)	3–9	151.9 (85.4)	11.7 (7.9)	6.6 (8.7)
Smoker (> 10 cigarettes/day)[d]	16	16.2 (3.6)	10–20	150.1 (56.3)	9.9 (6.8)	4.9 (4.8)

Results are expressed as mean (S.D.).

[a] Apparent cotinine concentrations were measured by spectrophotometry.

[b] One individual who reported that he was a non-smoker, but had an apparent cotinine level of 110 μmol/l as well as relatively high urinary levels of both nicotine (15.8 μmol/l) and cotinine (23.7 μmol/l) in his urine, was omitted from this group.

[c] Only 5 non-smokers had measurable but low (< 10 μmol/l), cotinine and/or nicotine levels, which is ascribed to passive smoking.

[d] Urinary levels of cotinine and nicotine are significantly ($p < 0.001$) higher in smokers than non-smokers, irrespective of the method employed.

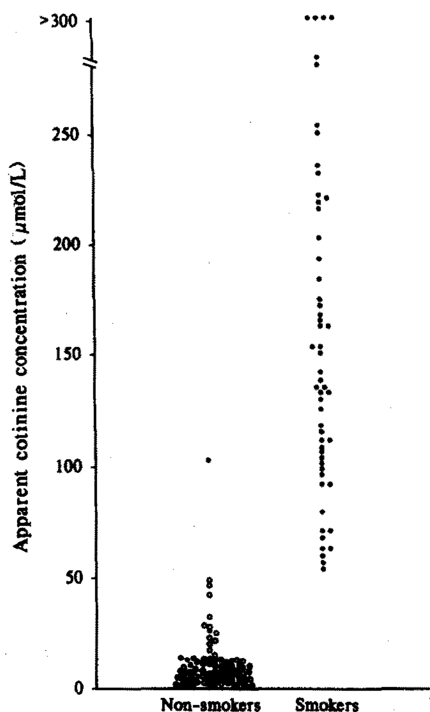

Fig. 3. Urinary levels of cotinine equivalents measured in smokers and non-smokers. Individuals were classified as smokers or non-smokers according to their self-reported assessment of smoking status. Urine samples where the presence of cotinine was confirmed by HPLC are indicated with (●); absence of cotinine is indicated by (○). Four smokers had apparent cotinine levels in excess of 300 μmol/l. Apparent cotinine levels ranged from 311 to 421 μmol/l in this subgroup.

one individual who had an apparent cotinine level of 110 μmol/l. HPLC analysis of this person's urine showed that both cotinine and nicotine were present, indicating that this individual's self-assessment of smoking status was wrong (Fig. 3). Smokers had urinary apparent cotinine levels > 50 μmol/l (Fig. 3). Peaks corresponding with cotinine were found by HPLC analyses of 5 self-reported non-smokers who also had appar-

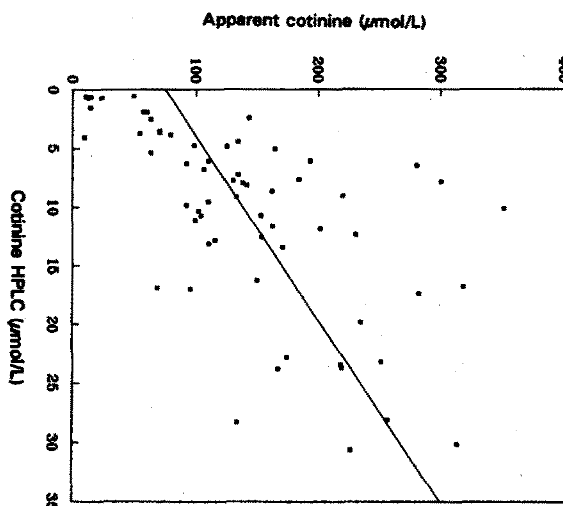

Fig. 4. Linear regression between urinary cotinine levels assessed by HPLC and the DBA assay. Regression equation: $y = 6.34x + 74.7$, correlation coefficient (r) = 0.61 ($p < 0.001$).

ent cotinine levels of < 50 μmol/l; the urine cotinine levels assessed by HPLC in these 5 cases were < 1.0 μmol/l.

Urinary cotinine levels measured by HPLC were significantly ($p < 0.001$) correlated ($r = 0.61$) to apparent cotinine levels as assayed by spectrophotometry (Fig. 4).

DISCUSSION

Our results indicate that the chromogens formed after reaction of nicotine and cotinine with cyanogen chloride and barbituric acid can be easily separated and quantified by using isocratic reversed-phase HPLC. Barlow *et al.* [16] analyzed the chromogens obtained from smokers' urine by using the same derivatization procedure, except that 1,3-diethylthiobarbituric acid was used as the final color reagent. Our results support the observations by Barlow and coworkers that cotinine is not the major urinary nicotine metabolite; from Fig. 1 it is clear that several components characteristic of smokers' urine elute early from the column. These components are included in the DBA assay and partially explains why this assay gives higher values for urinary cotinine when compared to HPLC. Since the DBA assay does not only measure cotinine, we refer to results obtained by this method as apparent cotinine levels. The contribution of these other urinary compounds explains why only 37% of the variation ($r^2 \times 100$) in apparent cotinine levels as measured by the DBA assay can be explained by cotinine levels measured by HPLC.

Non-smokers have urinary apparent cotinine concentrations or cotinine equivalents of up to 50 μmol/l. Since we used urine blank corrections, the apparent cotinine levels in non-smokers presumably reflect participation of endogenous urine components in the assay procedure. Nevertheless, no overlap was found between apparent cotinine levels measured in smokers and non-smokers, except for 1 individual who indicated that he was a non-smoker, but had a urinary apparent cotinine level of 100 μmol/l as measured by the DBA assay. HPLC analysis indicated the presence of cotinine and nicotine in this person's urine, and it was concluded that false information was furnished by this individual. A small number of non-smokers ($n = 5$) had low urinary levels of cotinine and nicotine, presumably the result of passive smoking.

In the population group studied, a cut-off point of 50 μmol/l for the urinary apparent cotinine level discriminates well between smokers and non-smokers. However, neither the DBA assay, nor the HPLC method was able to discriminate between light and moderate smokers. Similar observations were reported by Peach *et al.* [5], who also found that the urinary apparent cotinine level was a poor indicator of the daily cigarette consumption. These results may be explained by observations that the urinary cotinine level depends not only on the number of cigarettes smoked, but is also a function of puffing and inhaling patterns [5,15,18]. Moreover, the plasma half-life of cotinine has been shown to vary from 11 to 37 h [4], suggesting that individual differences in nicotine metabolism and the time elapsed since the last cigarette has been smoked, will also be important determinants of spot urinary cotinine levels. Therefore, although the urinary cotinine concentration is an excellent biochemical marker to distinguish between smokers and non-smokers, our data suggest that it cannot be used as quantative indicator of cigarette consumption.

Our results indicate that the DBA assay is a reliable indicator of smoking status, provided that urine blank samples are prepared to correct for background absorbance. Without blanks, results obtained in smokers and non-smokers showed considerable overlap (Results not shown). In epidemiological surveys or in routine medical evaluations for insurance purposes, we use the HPLC method in those cases were self-reported assessment of smoking status contradicts the DBA assay. The HPLC assay is therefore used to eliminate the possibility of false positive results when the DBA assay is at variance with self reported smoking status. We find this relatively simple HPLC procedure a useful adjunct to the DBA assay where accurate information on smoking status is required.

ACKNOWLEDGEMENTS

Mrs. Lia Goddard and Anne Schnell rendered excellent technical assistance.

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