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Determination of 2-naphthylamine in urine by a novel reversed-phase high-performance liquid chromatography method

Åse Marie Hansen, Otto Melchior Poulsen and Jytte Molin Christensen

Department of Chemistry and Biochemistry, Danish National Institute of Occupational Health, 105 Lersø Parkallé, DK-2100 Copenhagen (Denmark)

Steen Honoré Hansen

Department of Organic Chemistry, Royal Danish School of Pharmacy, 2 Universitetsparken, DK-2100 Copenhagen (Denmark)

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ABSTRACT

A high-performance liquid chromatographic method for the determination of 2-naphthylamine in urine using fluorescence detection was developed. The method validation analysis showed the method to be in analytical control, *i.e.* the distribution of the difference between the observed and true values of the method evaluation samples did not deviate significantly from the normal distribution. The recovery of the method was 85%. The entire run time of chromatography was 10 min using isocratic elution (acetonitrile–water, 35:65), and the retention time for 2-naphthylamine was 5.8 min. The relative short time of analysis in combination with the low limit of detection (0.272 nmol/l) makes the method potentially applicable for surveillance of occupational and environmental exposure to 2-nitronaphthalene. The developed method is presently used for measurement of 2-naphthylamine in urine samples from workers employed at factories, characterized by a low airborne exposure level of polycyclic aromatic hydrocarbons, *i.e.* in general less than 25 $\mu g/m^3$. The urine samples of exposed workers ($n = 95$) showed a 2-naphthylamine range of up to 9.4 nmol/l, whereas unexposed control individuals ($n = 114$) showed a range of up to 0.87 nmol/l.

INTRODUCTION

Polycylic aromatic hydrocarbons (PAHs) are present as components of the organic aerosols and are wide spread in the environment. Several of the PAHs have both mutagenic and carcinogenic properties. Studies in laboratory animal models have demonstrated that some of the volatile PAHs, *e.g.* pyrene, potentiate the effect of the carcinogenic PAH compound benzo[*a*]pyrene [1].

Naphthalene is often present in the work environment in relative high concentrations [2,3]. Naphthalene can react with nitrogen oxides, if present, to produce nitronaphthalene compounds, *e.g.* 1-nitronaphthalene and 2-nitronaphthalene [4]. Particularly in the liver, 1- and 2-nitronaphthalenes are metabolized to 1- and 2-naphthylamine, respectively [5]. The latter metabolite is a known human carcinogen, and when studied in laboratory animal models an increased incidence of bladder cancer was observed [6].

There is a growing interest of measuring the individual PAH exposure by determination of

Correspondence to: Dr. Åse Marie Hansen, Department of Chemistry and Biochemistry, Danish National Institute of Occupational Health, 105 Lersø Parkallé, DK-2100 Copenhagen, Denmark.

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the content of metabolites in the urine of exposed workers. Capillary gas chromatography (GC) with flame ionization detection (FID) [7] and mass spectrometry (MS) (8) for measuring 2-naphthylamine in urine of exposed workers have been described. In addition, two methods for the determination of 1- and 2-naphthylamine are based on normal-phase or amino-bonded-phase high-performance liquid chromatography (HPLC) and UV detection [9,10]. The detection limits for 2-naphthylamine in the two methods were 50 and 4.8 ng injected into the chromatographic column, respectively.

The objective of the present study was to develop a highly sensitive method for analyzing 2-naphthylamine in the urine of exposed workers using reversed-phase HPLC and fluorescence detection. The method should be sufficiently fast and sensitive to be applicable in large surveillance programs on occupational low-dose PAH exposure.

EXPERIMENTAL

Chemicals

The acetonitrile used was LiChrosolv grade (Merck, Darmstadt, Germany). Water was obtained from a Milli-Q water purification system (Millipore Waters, Taastrup, Denmark). A 7.000 μmol/ml 2-naphthylamine stock solution was prepared by dissolving 2-naphthylamine in acetonitrile. 2-Naphthylamine from Sigma (St. Louis, MO, USA) was used for the method evaluation samples (MEF samples) and β-naphthylamine from Aldrich (Milwaukee, WI, USA) was used for the five different standards.

Helix pomatia β-glucuronidase/sulfatase, 100 000/5000 U/ml (G-7017), was obtained from Sigma.

Apparatus

An HPLC system consisting of a Series 410 HPLC pump, a Model LS-4 variable-wavelength fluorescence detector and Omega-2 chromatography software (Perkin Elmer, Norwalk, CT, USA) was used. A WISP 710B autosampler (Waters Assoc., Milford, MA, USA) was used for automatic injection. The analytical column was a Perkin Elmer HC-ODS column (120 mm × 4.6 mm I.D.) packed with RP C_{18} (5-μm particles). The guard column, situated before the chromatographic column, was a Merck guard column (4 mm × 4 mm I.D.), packed with LiChrosorb RP-18 (5-μm particles).

Sample preparation

The five different standards were prepared by dilutions of the 2-naphthylamine stock solution in acetonitrile and mixing with pooled urine from unexposed persons (urine–acetonitrile, 8:2) to cover the range 0.34–7.00 nmol/l. In the same manner duplicate samples for method evaluation (MEF samples) were prepared covering the same range as the standards, but using a different batch of 2-naphthylamine.

Aliquots of 5.00 ml of the samples were buffered with 5.0 ml of 0.2 *M* sodium acetate buffer (pH 5.0) and hydrolyzed enzymatically with 100 μl of β-glucuronidase/sulfatase (13 200 U β-glucuronidase and 220 U sulfatase) for 20 h at 37.5°C in a water bath with shaker. After addition of 2.00 ml of acetonitrile, the samples were centrifuged for 10 min at 928 RCF at ambient temperature, and 25 μl of the supernatant were injected into the chromatographic column.

Chromatography

The analysis was carried out at ambient temperature. The optimal mobile phase for isocratic elution (acetonitrile–water, 35:65) was found after gradient elution and estimation of the acetonitrile concentration eluting 2-naphthylamine. The flow-rate was 1.0 ml/min. The optimal excitation and emission wavelengths used for quantification were 234 and 405 nm, respectively. These wavelengths were selected by scanning different excitation and emission wavelengths and seeking the highest specificity and sensitivity. The excitation and emission slits were 10 and 5 nm, respectively, and the response time was 8 s.

Statistics

Both the MEF samples and the standards were prepared by spiking pooled urine with 2-naph-

thylamine. In order to include the effect of batch variation in the method evaluation, two different batches of 2-naphthylamine were used for the preparation of standards and MEF samples, respectively.

Detailed information on the statistical models for the employed method evaluation design was described previously [11,12]. The general principles of the method evaluation design are as follows.

Any chemical method can be characterized by its method evaluation function (MEF) which is the estimated result of the chemical analysis, $E(Y|\mu)$, as a function of the true value of the analyte, μ. The equation of MEF is $E(Y|\mu) = \alpha + \beta\mu$. The method evaluation is based on a least-squares regression analysis of the MEF. The underlying theory is based on the assumption that the analytical method is in statistical control, *i.e.* the distribution of Y given μ is normal. The standard deviation (S.D.$_y$) is an expression of the combined uncertainty of the method.

The systematic error of the method is a combination of the so-called zero-point error (α) and the proportional error ($\beta - 1$). Ideally, an analytical method should be without any systematic error (*i.e.* $\alpha = 0$ and $\beta = 1$). However, it is in practice not possible to assure $\alpha = 0$ and $\beta = 1$ for all μ values. When a least-squares regression analysis of the MEF is performed and the standard deviation of α and β is calculated, the accuracy of the method is validated by testing if $\alpha = 0$ and $\beta = 1$ using a t-test [13]. If the uncertainty of the method increases profoundly with increasing true value (μ), *i.e.* the coefficient of variation increases more than 50% over a fifteen-fold concentration range [14], weight regression analysis should be used to estimate the value and standard deviation of α and β, respectively.

The limit of quantification is calculated using $RMSE^{1/2}$ (relative mean square error). In this context the limit of quantification is defined as the lowest concentration of μ which will produce an $RMSE^{1/2} < 33\%$ [15]. The limit of quantification can be obtained by plotting the central estimates of $RMSE^{1/2}$ *versus* μ [11,12].

The limit of detection (LD) was determined as the 3 S.D.$_y$ of twenty blank urine samples [16].

RESULTS

A representative chromatogram of a spiked urine sample (A) and a real sample (B) is presented in Fig. 1. The retention time for 2-naphthylamine was 5.8 min. Within 10 min the baseline was stable and the system was ready for a new injection.

Based on 36 calibration curves the MEF plot was produced for 2-naphthylamine. The linearity of the MEF plot was tested using a pure error lack of fit test, which was not significant at the 5% level, indicating that the plot was linear. The intercept and slope of the MEF plot are presented in Table I. Since the S.D.$_y$ was a function of μ, it was necessary to perform a weighted regression analysis using the reciprocal of the concentration as weights. The intercept, tested with a t-test, was not significantly different from zero (*i.e.* the confidence interval include zero). However, the slope was significantly different from 1 at the 5% level (t-test), indicating that the recovery was 85%.

The estimated limit of quantification based on $RMSE^{1/2}$ was 0.196 nmol/l. The LD of the method determined as 3 S.D. of twenty measurements of samples at the zero concentration level was 0.272 nmol/l. This level corresponds to 0.001 ng injected into the chromatographic column. The LD calculated by using the estimated S.D.$_y$ obtained in the MEF analysis was 0.162 nmol/l (3 $\times$ 0.054 nmol/l).

The developed method is presently used for the measurement of 2-naphthylamine in urine samples from workers employed in industrial plants with low airborne exposure of PAHs, *i.e.* in general less than 25 $\mu g/m^3$. The preliminary study of the urine samples of exposed workers ($n = 95$) showed a 2-naphthylamine range from the LD up to 9.4 nmol/l, whereas unexposed control individuals ($n = 114$) showed a range from LD up to 0.87 nmol/l.

DISCUSSION

The present paper is the first description of a reversed-phase HPLC–fluorescence method for the determination of 2-naphtylamine in human urine.

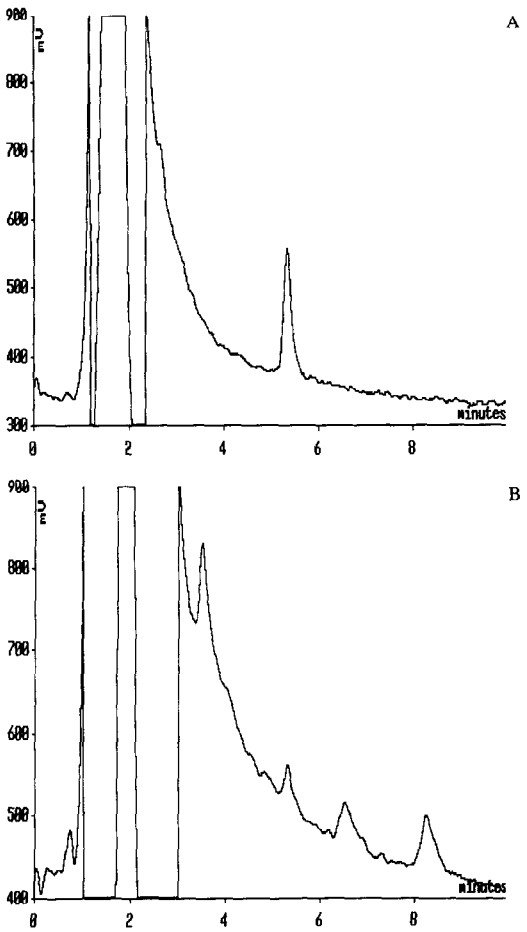

Fig. 1. (A) Chromatogram of a standard solution of 7.00 nmol/l 2-naphthylamine in urine. (B) Chromatogram of a real sample. Column: HC-ODS (120 mm × 4.6 mm I.D.) packed with RP C_{18} (5-μm particles); eluent: acetonitrile–water (35:65); fluorescence detection at 234 nm excitation and 405 nm emission; flow-rate: 1.0 ml/min; run time: 10 min.

TABLE I

METHOD EVALUATION PARAMETERS FOR β-NAPHTHYLAMINE

MEF parameter	Value
α (nmol/l)	−0.008
S.D.$_\alpha$ (nmol/l)	0.029
β	0.849
S.D.$_\beta$	0.032
S.D. (Y/μ)	0.054
DL (nmol/l)	0.272

The evaluation of the method was carried out on 36 MEF samples prepared in the same manner as the standards. The n-score test for normal distribution [19] did not reveal any significant deviation from the normal distribution (*i.e.* the method was in analytical control), and the pure error lack of fit test was significant demonstrating linearity of the MEF plot. The slope of the MEF plot (β = 0.85) was significantly different from 1, indicating that the recovery (85%) was significantly different from 100%. Hence, a correction factor of 0.85^{-1} is used when the method is used for routine analysis of 2-naphthylamine in urine. The stability of the method is controlled by injection of a reference material for each fifth sample analyzed. The recovery after correction was 100%.

The estimated S.D.$_y$ at the lowest concentration of μ was 0.054 (Table I) demonstrating, that the method had a good reproducibility, even at low concentrations. Furthermore, due to the low limit of quantification of the method it can measure low concentrations of 2-naphthylamine with a reasonable low analytical error.

The present method and several other methods for the determination of aromatic amines in different matrices are summarized in Table II. The present method has an LD ten-fold lower than that of the method of Von Blome *et al.* [7]. The method of Carlucci *et al.* [8], which could measure several aromatic amines simultaneously, was stated to have an LD of 0.4 pg 2-naphthylamine. However, Carlucci *et al.* [8] used a definition of LD (signal-to-noise ratio of 3:1) not in accordance with the IUPAC definition [16]. Hence it is not possible to compare the LDs of the two methods. In addition, the extraction procedure used prior to GC is much more time-consuming than the one used in the present HPLC method for biological samples. The recovery of the presented method is in the same magnitude as that of Carlucci *et al.* [8], who found a recovery of 86%.

According to Klaassen *et al.* [20] both primary and secondary amines wil undergo conjugation with glucuronic acid. In the present study it was, consequently, assumed that β-naphthylamine was present in urine as an N-glucuronide, which

TABLE II

METHODS FOR ANALYSIS OF 2-AMINONAPHTHALENE

Principle of analysis	Compound	Matrix	Limit of detection (ng)	Ref.
GC–FID	2-Naphthylamine	Human urine	0.01	7
GC–MS	Aromatic amines	Human urine	0.0004	8
Normal-phase HPLC–photometry	Benzidin, 1-naphthylamine, 2-naphthylamine	Water	50	9
Reversed-phase HPLC–electrochemical detection	Aromatic amines	Water and air	0.1	17
Normal-phase HPLC–UV	Six aromatic amines	Impurities in chemicals	4.8	10
Reversed-phase HPLC–fluorescence detection	1-Aminopyrene	Hemoglobin	Not specified	18
Reversed-phase HPLC–fluorescence detection	2-Naphthylamine	Human urine	0.001	Present study

was enzymatically hydrolyzed with *Helix pomatia* β-glucuronidase/sulfatase.

The short run time of the developed method makes it potentially applicable for large-scale surveillance of occupational PAH exposure. The method was recently used with the above-mentioned correction for recovery for measurement of 2-naphthylamine in urine samples of workers employed at factories, which are characterized by a low airborne exposure level of PAHs, *i.e.* in general less than 25 $\mu g/m^3$. Urine from 95 workers showed a 2-naphthylamine range from LD up to 9.4 nmol/l. Urine samples of control individuals ($n = 114$) were found to range between LD and 0.87 nmol/l. The preliminary results indicate that the method enables the detection of small differences in low-dose airborne naphthalene exposure.

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