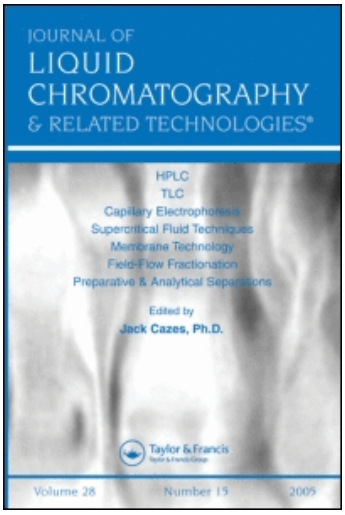

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Determination of α-Naphthol in Human Urine by High Performance Liquid Chromatography

Åse Marie Hansen[a]; Otto Melchior Poulsen[a]; Jytte Molin Christensen[a]; Steen Honoré Hansen[b]
[a] Department of Chemistry and Biochemistry, Danish National Institute of Occupational Health, Copenhagen, Denmark [b] Department of Organic Chemistry, Royal Danish School of Pharmacy, Copenhagen, Denmark

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DETERMINATION OF α-NAPHTHOL IN HUMAN URINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

ÅSE MARIE HANSEN[1], OTTO MELCHIOR POULSEN[1], JYTTE MOLIN CHRISTENSEN[1], AND STEEN HONORÉ HANSEN[2]
[1]*Danish National Institute of Occupational Health*
Department of Chemistry and Biochemistry
105 Lersø Parkallé
DK-2100 Copenhagen, Denmark
[2]*Royal Danish School of Pharmacy*
Department of Organic Chemistry
2 Universitetsparken
DK-2100 Copenhagen, Denmark

ABSTRACT.

A high performance liquid chromatography/-fluorescence (HPLC) method for quantitative analysis of α-naphthol in urine was developed. The method validation analysis showed the method to be in analytical control meaning that no significant systematical errors could be demonstrated. The entire run time of chromatography was 10 min using isocratic elution (acetonitrile:water (35:65)), and the

Correspondence to be addressed to Åse Marie Hansen.

retention time for α-naphthol was 5.9 min. The short run time in combination with the low limit of detection (0.038 µmol/l) make the method potentially applicable for surveillance of naphthalene exposure in the working environment. The developed method is presently used for measurement of α-naphthol in urine samples from workers employed who are characterized by a low airborne exposure level of polycyclic aromatic hydrocarbons in general is low, i.e. less than 25 µg/m^3. The preliminary results of 123 workers showed a range of α-naphthol in urine from the limit of detection (LD) up to 1.7 µmol/l, whereas non-exposed control individuals (n = 113) showed a range from LD up to 0.7 µmol/l.

INTRODUCTION.

Polycyclic aromatic hydrocarbons (PAH) are present as components of the organic aerosols and several of the PAH compounds have both mutagenic and carcinogenic properties. Studies in laboratory animal models have demonstrated that some of the volatile polycyclic aromatic hydrocarbons, e.g. pyrene potentiate the effect of the carcinogenic PAH compound benzo(a)pyrene (1). Furthermore, an increased incidence of lung cancer has been demonstrated in workers exposed to high levels of airborne PAH and exposure to airborne PAH is now considered a major environmental and occupational health problem (2).

It has been observed that the relative content of the different PAH compounds in air samples containing one major PAH compound was relative constant for different air samples (3). It was suggested that measurement of one of the major PAH compounds might be used as marker to predict both the exposure of total PAH and the exposure of total carcinogenic PAH, but choosing benzo(a)pyrene (b(a)p) was not considered optimal, since b(a)p most frequently is present in trace amounts only (3). Recently, we analyzed to what extend six different volatile PAH compounds could function as marker for the total concentration of six different carcinogenic particulate PAH compounds present in the air of smoke houses (4). Even though a significant positive correlation was observed between the concentration of each of six volatile compounds and the total concentration of carcinogenic PAH compounds, only pyrene and naphthalene seemed to function as markers for total carcinogenic PAH-exposure in both smokehouses and iron foundries, and these two compounds may be generally applicable as markers for PAH-exposure.

There are a growing interest of measuring the

individual PAH exposure, by determine the content of metabolites in urine of exposed workers. Methods for measuring 1-hydroxypyrene in urine of exposed workers has been described previously (5,6). These were HPLC methods with fluorescence detection. Only one method to determine ß-naphthol using HPLC and UV-detection has been described (7). Hitherto, to the authors knowledge no method has been published for quantitative measurement of α-naphthol in urine.

The purpose of the present study was to develop a highly sensitive method for analyzing α-naphthol in urine of exposed workers using HPLC and fluorescence detection. The method should be sufficiently fast to be applicable in large surveillance programs on occupational PAH exposure.

MATERIALS AND METHODS.

The acetonitrile used was LiChrosolv (Merck, Darmstadt, FRG). Water was obtained from a Milli-Q water

purification system (Millipore Waters, Taastrup, Denmark). A 7.000 mmol/l α-naphthol stock solution was prepared by dissolving α-naphthol in acetonitrile. α-Naphthol was pro analysi (Merck, Darmstadt, Federal Republic of Germany) from two different batches of chemical, i.e. one batch for the method evaluation samples and one batch for the five different standards.

Helix pomatia ß-glucuronidase with sulfatase activity, 100,000/5,000 units/ml (G-7017) and metabolites of α-naphthol, i.e. α-naphthyl-β-D-glucuronide, sodium salt and α-naphthyl sulfate, potassium salt were obtained from Sigma, St. Louis, USA. The metabolites were dissolved in pooled urine from nonexposed persons and used to test the efficacy of the enzyme treatment.

APPARATUS.

A HPLC system consisting of one HPLC-pump series 410, a variable fluorescence detector model LS-4 and Omega-2 chromatography software (Perkin Elmer Corp., Norwalk, United States) was used. A WISP

710B autosampler, Waters Associates Inc. Milford, United States was used for automatic injection. The analytical column was a Perkin Elmer HC-ODS column 120 X 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 X 4 mm, packed with liChrosorb RP-18, 5 µm.

SAMPLE PREPARATION.

The five different standards were prepared by serial dilution of the α-naphthol stock solution in pooled urine from unexposed persons to produce standards in the range 0.174 - 1.000 µmol/l. In the same manner duplicate samples for method evaluation (MEF-samples) were prepared covering the range of the standards, using a different batch of α-naphthol. Aliquot of 10.0 ml of the samples were buffered with 10.0 ml 0.2 N sodium acetate buffer pH=5.0 and hydrolyzed enzymatically with 200 µl ß-glucuronidase/-sulfatase (26400 units ß-glucuronidase and 440 units sulfatase) for 20 hours at 37.5°C in a water bath/shaker. Hereafter 10.00 ml of the buffered

samples were extracted with 10.00 ml cyclohexane:diisopropanol (9:1) by shaking for 20 minutes. The cyclohexane:isopropanol phase were finally extracted with 300 µl 0.1 N sodium hydroxide, followed by addition of 100 µl 0.4 N hydrochloric acid. All phases were separated by centrifugation. Samples of 25 µl of the final neutralized water extracts were injected to the 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, which eluted α-naphthol. The flow rate was 1.0 ml/min. The optimal excitation and emission wavelengths used for quantification were 282 nm and 455 nm, respectively, found by scanning for the excitation and emission wavelengths which produced the highest specificity and sensitivity.

PREPARATION OF CALIBRATION PLOTS AND METHOD EVALUATION POINTS.

Both the MEF-samples and the standards were prepared by spiking pooled urine with α-naphthol, and in order to include the effect of batch variation in the method evaluation two different batches of α-naphthol were used for the preparation of standards and MEF-samples, respectively.

STATISTICS.

Detailed information on the statistical models for the employed method evaluation design was described previously (8,9). 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, $E(Y \mid \mu)$, as a function of the true value of the analyt, μ. The equation of MEF is:

$MEF(\mu) = E(Y \mid \mu) = \alpha + \beta\mu$

The method evaluation is performed by statistical 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 (SD_y) is an expression of the combined uncertainty of the method.

The systematic error of the method is the combination of the socalled zero point error (α) and the proportional error (β-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 values of μ. When a least square regression analysis of the MEF is performed and the standard deviation of α and β is calculated, the accuracy of the measurements is validated by testing if $\alpha = 0$ and $\beta = 1$ using a T-test (10). 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 15-fold range of concentration (11) weight regression analysis should be used to estimate the value and standard deviation of α and β, respectively.

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 a $RMSE^{1/2} < 33\%$ (12). The limit of quantification can be obtained by plotting the central estimates of $RMSE^{1/2}$ versus μ (8,9).

The limit of detection was determined as the mean + 3SD of 20 urine blank samples (13).

RESULTS.

Figure 1 shows the time dependant efficacy of the enzyme treatment measured as the amount of α-naphthol released. Nearly complete hydrolysis was obtained after 20 hours of hydrolysis using a enzyme/substrate ratio of 760/38 units/μg. A period of hydrolysis of 20 hours and an at least a 5 fold increase in the enzyme/substrate ratio was selected for real samples to build in a high degree of certainty, that hydrolysis was complete.

A representative chromatogram of a sample of

Enzymatic hydrolysis of α-naphthyl-β-D-glucuronide and α-naphthylsulphate.

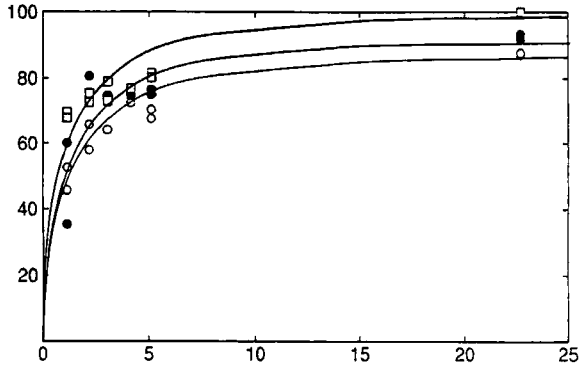

Figure 1.

Samples of 7.0 ml of pooled urine from non-exposed individuals was added 7.0 ml sodium acetate buffer, pH=5.0 and spiked with 18.44 µg α-naphthyl-β-glucuronide and 18.24 µg α-naphthylsulphate. After heating to 37.5°C 14,000/700 units (A), 21,000/1,050 units (B) and 28,000/1,400 (C) Helix pomatia β-glucuronidase were added. Enzymatic hydrolysis was allowed to proceed for increasing period (i.e. 1, 2, 3, 4, 5 and 20 hours) before termination of reaction by acetonitrile dilution 1:1. The degree of hydrolysis was expressed relative to the total possible release of α-naphthol, i.e. 1.34 µg/ml.

A chromatogram of a human urine sample spiked with 0.521 µmol/l α-naphthol.

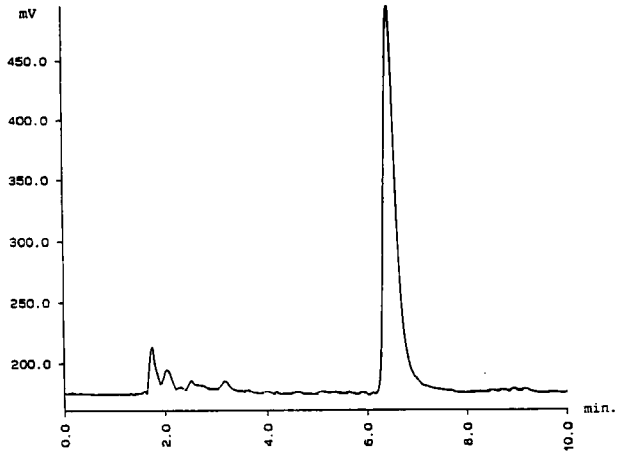

Figure 2.

0.521 µmol/l α-naphthol in urine (1). Eluent acetonitrile:water 35:65, fluorescence detection ex/em 282 nm/455 nm, flow 1.0 ml/min., run time 10 min.

naphthol spiked to urine is presented in figure 2. The retention time for α-naphthol is 6.1 min and within 10 min the baseline was stable and the system ready for a new injection.

MEF-plot of α-naphthol in urine.

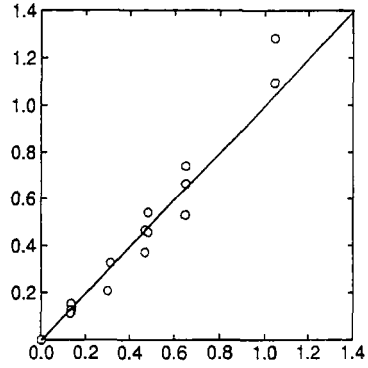

Figure 3.

The measured concentrations E(Y | μ) of the 21 MEF-samples plotted against the corresponding true concentration (μ).

Based on 11 independent calibration curves the concentration of α-naphthol was measured in 21 MEF-samples to produce the MEF-plot (Figure 3). 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 1. It must be emphasized that, since the SD

TABLE 1. Method evaluation parameters for α-naphthol.

Element	Number of MEF points	Intercept (α) µmol/l	SD_α	Slope (β)	SD_β	Lim. quant.[a)] µmol/l	SD for Y given µ µmol/l
α-naphthol	21	-0.006	0.013	1.010	0.062	0.058	0.019

of Y is a function of μ, it was necessary to perform a weight regression analysis using the reciprocal of the concentration as weights. The intercept was tested with a T-test to be not significantly different from zero (i.e. the 0.95 confidence interval included zero). Similarly, the slope was not significant different from 1, indicating that the recovery of the method in not significantly deviating from 100 %.

Figure 4 shows the central estimate of $RMSE^{1/4}$ of the method plotted versus μ. Furthermore the upper 95% limit of the tolerance interval around the $RMSE^{1/4}$ of an ideal method (i.e. $\alpha = 0$, $\beta = 1$) is indicated. The limit of quantification, i.e. the true value μ at which $RMSE^{1/4}$ = 33 %, was 0.058 μmol/l. The limit of detection (LD), 0.038 μmol/l, of the method was determined as 3*SD of 20 measurements of samples at the zero concentration level. The limit of detection obtained by estimating SD of the MEF using weight regression analysis was 3*0.019 μmol/l = 0.058 μmol/l, see Table 1.

Central estimate of $RMSE^{1/4}$ plotted versus the true value of concentration.

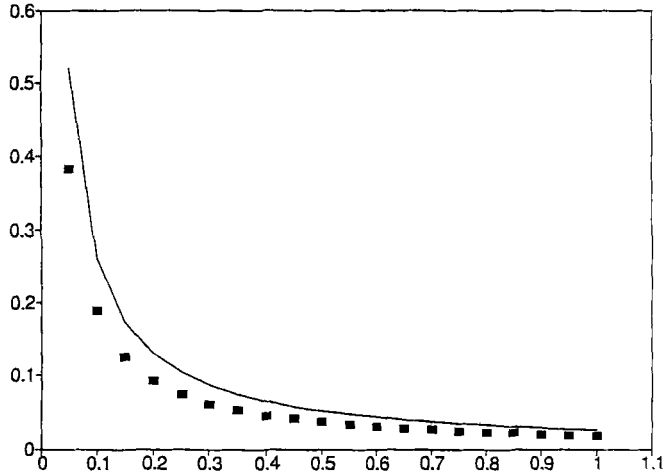

Figure 4.

The full line is the upper limit of the tolerance interval for the ideal $RMSE^{1/4}$ (i.e. $\alpha = 0$, $\beta = 1$, $SD_y = 0.02$). The squares are the central estimators of $RMSE^{1/4}$ of the method (i.e. $\alpha = -0.006$, $\beta = 1.010$ and $SD_y = 0.019$).

A preliminary study on low dose PAH exposure workers, i.e. in general less than 25 $\mu g/m^3$, showed the range of α-naphthol in urine (N=123) to be from LD up

to 1.7 µmol/l. Urine samples of controls (n=113) was found to be between LD and 0.7 µmol/l.

DISCUSSION.

The evaluation of the method was carried out on 21 MEF-samples prepared in the same manner as the standards. The n-score test for normal distribution (14) did not reveal any significant systematical errors (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 was not significantly different from 1, indicating a recovery not significantly different from 100 %.

The estimated SD_y at the lowest concentration of µ was 0.019 (Table 1) demonstrating, that the method had a good reproducibility even at low concentrations.

Furthermore, the low limit of quantification of the method indicated, that the method can measure low concentrations of α-naphthol with a reasonable low analytical error.

The short run time of the developed method makes it potentially applicable for large scale surveillance of occupational PAH exposure, and the method has presently been used for measurement of α-naphthol in urine samples of workers employed who are characterized by a low airborne exposure level of polycyclic aromatic hydrocarbons, i.e. in general less than 25 μg/m^3. Urine from 123 workers showed a range of α-naphthol from LD up to 1.7 μmol/l. Urine samples of control individuals (N=113) was found to be between LD and 0.7 μmol/l. The preliminary results may indicate that the method enable the detection of small differences in low dose airborne naphthalene exposure.

Naphthalene was previously demonstrated to function as a marker for total airborne carcinogenic PAH exposure (5), and consequently, α-naphthol in urine may be a marker for biological monitoring of carcinogenic PAH-exposure.

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