ELSEVIER

Journal of Chromatography B, 734 (1999) 211–217

JOURNAL OF
CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Assay of 2-naphthol in human urine by high-performance liquid chromatography

Heon Kim[a,*], Yong-Dae Kim[a], Holik Lee[a], Toshihiro Kawamoto[b], Mihi Yang[b], Takahiko Katoh[c]

[a]*Department of Preventive Medicine, College of Medicine, Chungbuk National University, 48 San Kaeshin-dong, Hungdok-gu, Cheongju, Chungbuk 361-763, South Korea*

[b]*Department of Environmental Health, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807, Japan*

[c]*School of Health Science, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807, Japan*

Received 11 March 1999; received in revised form 19 July 1999; accepted 22 July 1999

Abstract

This paper describes a novel liquid chromatographic method for the quantitation of 2-naphthol in human urine. Urine samples were extracted after enzymatic hydrolysis of glucuronides and sulfates; 2-naphthol was then separated using reversed-phase high-performance liquid chromatography. The corresponding detection limits were 0.04 ng/ml for the standard sample in acetonitrile and 0.13 ng/ml for urine samples. The level of urinary 2-naphthol in 100 Korean shipyard workers was analyzed using this new method. The level ranged from 0.21 ng/ml (0.26 μmol/mol creatinine) to 34.19 ng/ml (59.11 μmol/mol creatinine), and the mean±standard deviation was 5.08 ng/ml (6.60 μmol/mol creatinine)±5.75 ng/ml (9.22 μmol/mol creatinine). The mean±standard deviation of urinary 2-naphthol level of smokers, 7.03 ng/ml (8.49 μmol/mol creatinine)±6.16 ng/ml (10.23 μmol/mol creatinine), was significantly higher than that of non-smokers, 2.49 ng/ml (4.10 μmol/mol creatinine)±3.92 ng/ml (7.03 μmol/mol creatinine). © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Naphthalene; 2-Naphthol

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are important carcinogens which are found in cigarette smoke, coke oven emissions, and diesel exhaust; they are absorbed through the respiratory and gastrointestinal tracts, as well as through the skin [1]. Among PAHs, naphthalene is rapidly absorbed when inhaled, but its absorption is slower following ingestion or dermal exposure [2]. It has been reported that naphthalene can form toxic and carcinogenic metabolites [3]. Absorbed naphthalene undergoes rapid hydroxylation to 1- and 2-naphthol, and conjugation to form more water-soluble derivatives, which are excreted in urine [4,5]. Urinary 2-naphthol concentration is a better biomarker for inhalation exposure to PAHs than urinary 1-naphthol concentration [6].

Most methods for determining 2-naphthol levels

*Corresponding author. Tel.: +82-431-2612-864; fax: +82-431-2742-965.

E-mail address: kimheon@med.chungbuk.ac.kr (H. Kim)

0378-4347/99/$ – see front matter © 1999 Published by Elsevier Science B.V. All rights reserved.
PII: S0378-4347(99)00350-3

are not sensitive enough, or require expensive equipment or a complicated sample preparation technique. For example, the method using gas chromatography–mass spectrometry (GC–MS) is the most sensitive [6], but the procedure for preparing samples is quite complicated. In this report, the authors describe a sensitive and simple method for measuring 2-naphthol in urine using high-performance liquid chromatography (HPLC) with a fluorescence detector. The method was tested by analyzing the distribution pattern of urinary 2-naphthol levels in 100 Korean shipyard workers.

2. Experimental

2.1. Study subjects

The study subjects were 100 male shipyard workers. Demographic data are presented in Table 1. A questionnaire was used to determine smoking habits, and dietary and occupational exposure to PAHs. Spot urine was collected from every subject and kept at −20°C before analysis.

2.2. Chemicals

Acetonitrile and 2-naphthol were obtained from Wako (Osaka, Japan). A standard stock solution of 2-naphthol was prepared by dissolving 2-naphthol in acetonitrile. β-Glucuronidase with sulfatase activity (G-0876) was obtained from Sigma (St. Louis, MO, USA).

2.3. Apparatus

A HPLC system consisting of a pump (Hitachi L-6210, Tokyo, Japan), a variable-fluorescence detector (Hitachi LaChrom L-7480, Tokyo, Japan), an automatic injector (Hitachi L-7200), and an integrator (Hitachi D-2500 Chromato-Integrator), was used. The column was 250 mm×4.6 mm I.D. (J'sphere ODS-H80 reverse phase; YMC, Wilmington, NC, USA).

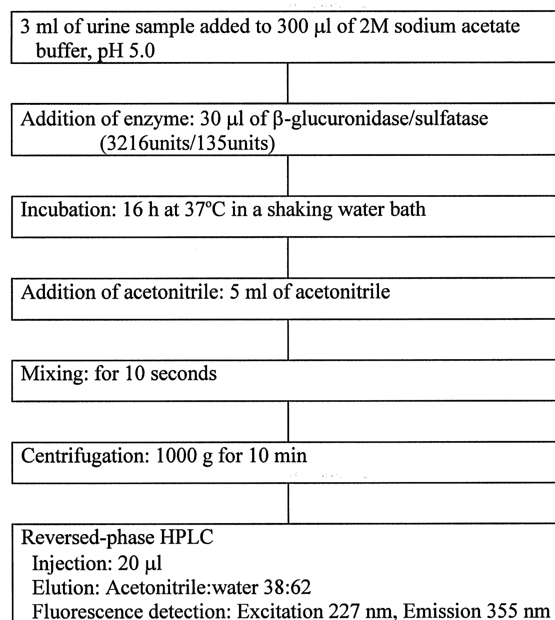

Fig. 1. Flow diagram of sample treatment and chromatography.

2.4. Sample preparation

Fig. 1 shows a flow chart of the steps involved in sample pretreatment and chromatography for quantitative determination of 2-naphthol in urine.

Aliquots of the samples were spiked with 2-naphthol stock solution, in order to check whether the addition of 2-naphthol increased the height of the peak that was thought to represent 2-naphthol in the native solution. In a dark room, 3-ml aliquots of

Table 1
Demographic data and extent of daily smoking of the study subjects

Variable	Total subjects (n=100)		Smokers (n=57)		Non-smokers (n=43)	
	Mean	SD	Mean	SD	Mean	SD
Age (years)	38.2	7.8	36.0	8.1	41.0	6.5
Height (cm)	169.8	5.4	169.6	6.0	170.1	4.6
Weight (kg)	64.6	7.4	63.3	7.1	66.3	7.6
Daily smoking amount (cigarettes/day)	16.1	6.4	17.2	5.0	–	–

spiked or unspiked samples were buffered with 300 μl of 0.2 *M* sodium acetate buffer (pH 5.0), and hydrolyzed enzymatically with 30 μl of β-glucuronidase and sulfatase, for 16 h at 37°C in a shaking water bath. After hydrolysis, 5 ml of acetonitrile was added and the samples were centrifuged at 1000 *g* for 10 min [7]. A 20-μl volume of the supernatant was injected into the HPLC system.

2.5. Chromatography

The mobile phase used was acetonitrile–water (38:62), at a flow-rate of 1 ml/min. The excitation and emission wavelengths (selected after scanning at different wavelengths) were 227 nm and 355 nm, respectively.

2.6. Wavelength scans with fluorescence detector

To determine the peak of standard samples and of urine samples retained for the same time, emission wavelength scanning at 227 nm excitation wavelength and excitation wavelength scanning at 355 nm emission wavelength were carried out, using a variable-wavelength detector for comparison.

2.7. Calibration and calculations

The urine was variably spiked with 2-naphthol (0.5–40 ng to 1 ml of urine), and hydrolyzed under the aforementioned conditions. The 2-naphthol concentration of each urine sample was calculated as the product of fluorescence signal and its gradient, and was adjusted by urinary creatinine concentration.

2.8. Recovery, reproducibility and repeatability

Ten urine samples were spiked with 2-naphthol at two levels (10 and 40 ng in 1 ml of urine, respectively), hydrolyzed at the same time, and analyzed within a day. These procedures were repeated three times. The recovery rates were measured and the mean values and standard deviations, both within- and between-days, respectively, were calculated.

2.9. Statistical methods

Mean urinary 2-naphthol concentrations were compared statistically between the smokers and the non-smokers, using Student's *t*-test.

3. Results

Fig. 2 shows the intensity of the fluorescence signal according to the amount of enzyme added into the urine sample in the preparation step. From this figure, 30 μl of the enzyme (representing 3216 units of β-glucuronidase and 135 units of sulfatase) is sufficient to completely hydrolyze the conjugates in 3 ml of urine [8].

Representative chromatograms of the unspiked

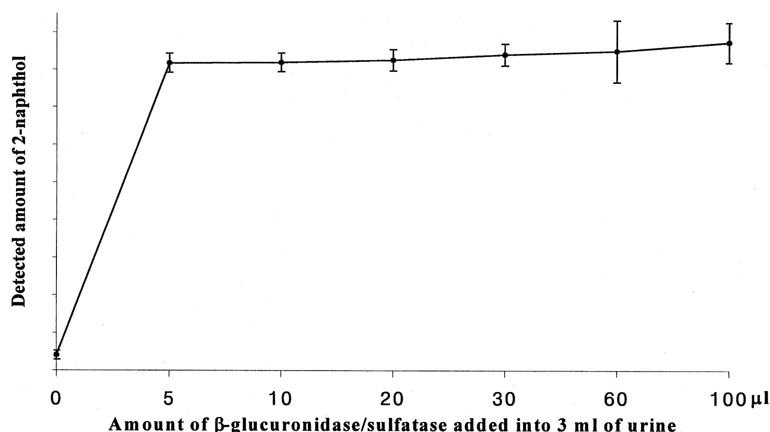

Fig. 2. Amount of 2-naphthol detected according to the amount of β-glucuronidase/sulfatase added to a urine sample. The 2-naphthol concentration of the urine sample was 34.19 ng/ml.

and spiked urine sample of a non-smoker are shown in Fig. 3. The chromatogram of the spiked urine sample shows that the peak with the same retention time as that of the 2-naphthol standard increased after the addition of 2-naphthol to the sample. Emission and excitation wavelength scans of the peaks of the standards and of the urine samples with the same retention time could be regarded as identi-

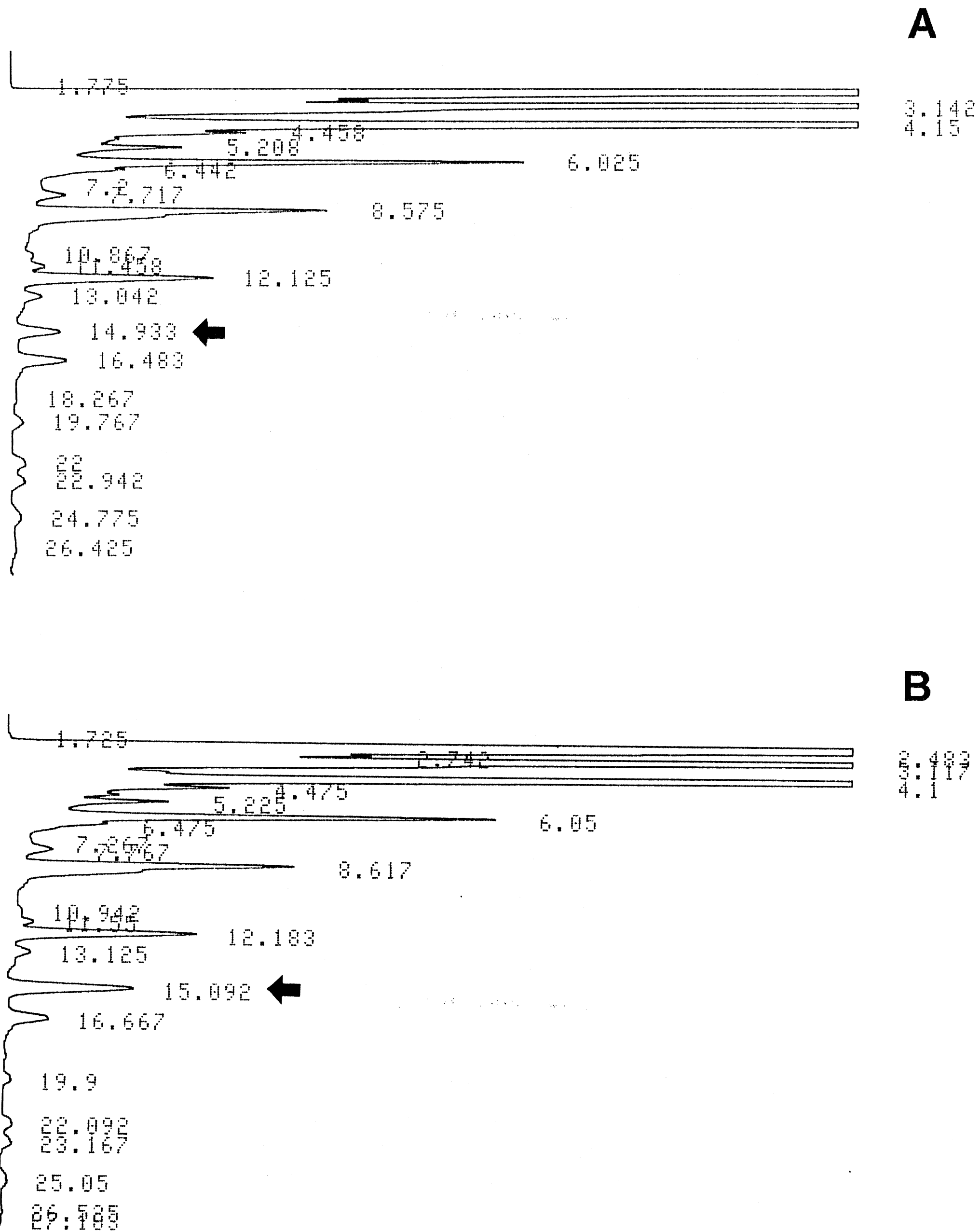

Fig. 3. Chromatograms of the unspiked (A) and the spiked (B) urine sample. In the case of the spiked sample, 22.5 ng of 2-naphthol was added to 3 ml of urine before sample treatment. The 2-naphthol concentration of the urine sample A was 8.0 ng/ml.

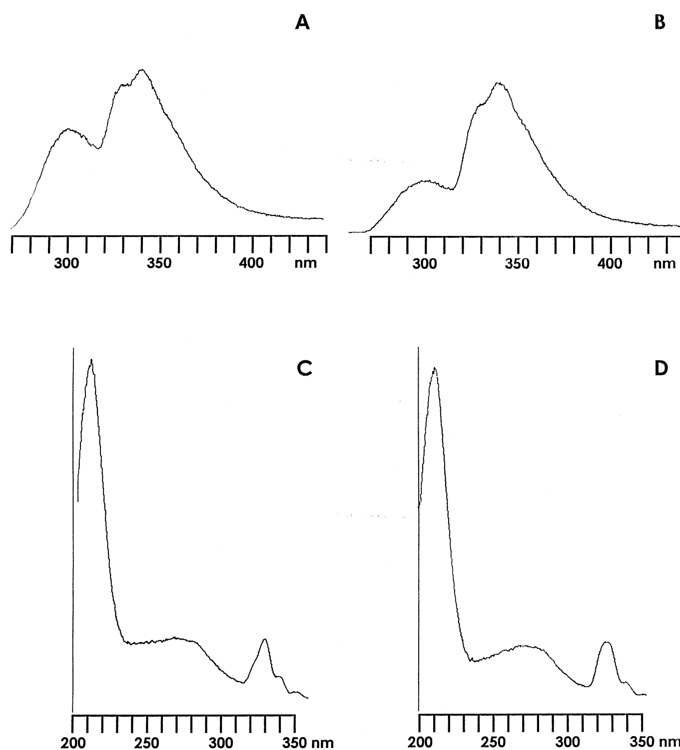

Fig. 4. Emission and excitation wavelength scans for a standard and a urine sample. (A) (standard) and (B) (urine sample) represent the emission scans at 227 nm excitation wavelength. (C) (standard) and (D) (urine sample) represent the excitation scans at 355 nm emission wavelength.

cal (Fig. 4). The fluorescence level of urine samples spiked with 2-naphthol is plotted in Fig. 5. There is a linear regression between the fluorescence signal amplitude and the amount of spiked 2-naphthol.

The detection limits, which were determined as three-times the standard deviation of a blank or an unspiked urine sample divided by the slope [9], were 0.04 ng/ml for 2-naphthol in acetonitrile, and 0.13 ng/ml for 2-naphthol in urine, respectively.

The recovery rates of 10 ng/ml and 40 ng/ml

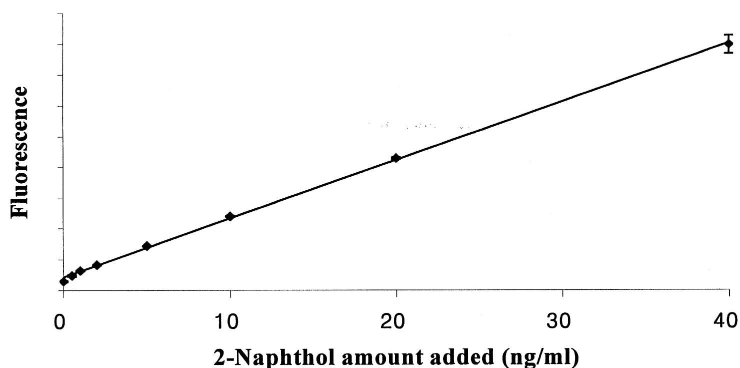

Fig. 5. A calibration curve constructed on the basis of standard urine samples. The correlation coefficient is 0.9996.

Table 2
Recovery and reproducibility of the 2-naphthol assay method

Amount spiked (ng/ml)	Amount detected (ng/ml)	Recovery (%)	Within-day RSD[a] (%)	Between-day RSD (%)
0	2.25	–	5.05	6.30
10	11.67	94.2	2.81	1.44
40	38.33	90.2	2.98	3.17

[a] RSD=Relative standard deviation.

Table 3
Urinary 2-naphthol levels of the study subjects[a]

2-Naphthol level	Total subjects (n=100)	Smokers (n=57)	Non-smokers (n=43)
Range			
(ng/ml)	0.21–34.19	0.42–34.19	0.21–16.82
(μmol/mol creatinine)	0.26–59.11	0.86–59.11	0.26–37.19
Arithmetic mean±SD[b]			
(ng/ml)	5.08±5.75	7.03±6.16	2.49±3.92
(μmol/mol creatinine)	6.60±9.22	8.49±10.23	4.10±7.03
Geometric mean±SD			
(ng/ml)	2.83±3.12	5.03±2.39	1.33±2.71
(μmol/mol creatinine)	3.08±3.21	6.00±2.11	1.69±3.42

[a] Arithmetic and geometric means were significantly different between smokers and non-smokers ($P<0.01$).
[b] SD=Standard deviation.

spiked urine samples were 94.2% and 90.2%, respectively. The within- and between-day relative standard deviations (RSDs) were 5.05% and 6.30%, respectively for the unspiked urine sample, 2.81% and 1.44%, respectively for the 10 ng/ml spiked urine sample, and 2.98% and 3.17%, respectively for the 40 ng/ml spiked urine sample (Table 2).

The urinary 2-naphthol levels of 100 Korean shipyard workers ranged from 0.21 ng/ml (0.26 μmol/mol creatinine) to 34.19 ng/ml (59.11 μmol/mol creatinine), and the mean±standard deviation was 5.08 ng/ml (6.60 μmol/mol creatinine)±5.75 ng/ml (9.22 μmol/mol creatinine). The mean±standard deviation of the urinary 2-naphthol level in smokers was 7.03 ng/ml (8.49 μmol/mol creatinine)±6.16 ng/ml (10.23 μmol/mol creatinine). This was significantly higher than the mean±standard deviation of the urinary 2-naphthol levels found in non-smokers, 2.49 ng/ml (4.10 μmol/mol creatinine)±3.92 ng/ml (7.03 μmol/mol creatinine) (Table 3).

4. Discussion

The HPLC method developed in this study for the determination of 2-naphthol in urine was found to be more sensitive than previous methods [2,10–12]. The limits of detection of these methods were around 10 ng/ml [2,11]. In contrast, the GC–MS method, reported to be the most sensitive, requires expensive measurement instruments, and the procedure for preparing samples is complicated [6]. According to our previous study, the detection limit of the GC–MS method was 0.27 ng/ml [6]. The detection limit of our new measurement method for urine samples, 0.13 ng/ml, is sufficiently low for the compound 2-naphthol to be measured in human urine. Furthermore, the urine sample that has been enzymatically hydrolyzed and extracted with acetonitrile can be used for the measurement of other PAHs, such as 1-hydroxypyrene [7].

To determine the optimal wavelength for HPLC analysis, we carried out excitation and emission

wavelength scanning of 1- and 2-naphthol. For 2-naphthol, the maximal peak was detected at 227 nm excitation and 355 nm emission. For 1-naphthol, however, no useful fluorescence peak was apparent. Since, from our earlier study, the 2-naphthol concentration in urine had shown a better correlation with the level of urinary cotinine, a biomarker for smoking, than the 1-naphthol concentration [6], 2-naphthol in urine is considered to be a better exposure marker for inhaled PAHs. The mean value of the urinary 2-naphthol concentration was much higher in the urine of smokers than in the urine of non-smokers.

Twenty-four hour urine would be more appropriate than spot urine for the estimation of total urinary excretion of 2-naphthol. However, we used spot urine samples because much more time and labor were required for the collection of 24-h urine samples. The 2-naphthol concentration in spot urine was corrected for urinary creatinine concentration.

When compared to the geometric mean of the urinary 2-naphthol level in Korean workers found in this study (2.83 ng/ml), the equivalent value for Japanese workers (geometric mean: 3.09 ng/ml) was similar [6]. On the other hand, the mean values of the urinary 1-naphthol level found in other studies are remarkably high [9,11,13]. This may be due to the fact that the limit of detection for 1-naphthol in the methods used in these previous studies was so high, that a considerable number of low values were not included in the estimation of the mean.

In conclusion, it can be stated that our proposed method for determining 2-naphthol levels in urine, using HPLC and fluorescence detection, is sensitive, simple and useful for monitoring the inhalation exposure to naphthalene.

References

[1] J.G.M. Van Rooij, M.M.S. Veeger, M.M. Bodelier-Bade, P.T.J. Scheepers, F.J. Jongeneelen, Int. Arch. Occup. Environ. Health 66 (1994) 55.
[2] S.D. Keimig, D.P. Morgan, Appl. Ind. Hyg. 2 (1986) 61.
[3] A.S. Wilson, C.D. Davis, D.P. Williams, A.R. Buckpitt, M. Pirmohamed, B.K. Park, Toxicology 114 (1996) 233.
[4] M.D. Tingle, M. Pirmohamed, E. Templeton, A.S. Wilson, S. Madden, N.R. Kitteringham, B.K. Park, Biochem. Pharmacol. 46 (1993) 1529.
[5] G.M. Pacifici, M. Franchi, L. Giuliani, A. Rane, Dev. Pharmacol. Ther. 14 (1989) 108.
[6] M. Yang, M. Koga, T. Katoh, T. Kawamoto, Arch. Environ. Contam. Toxicol. 36 (1999) 99.
[7] Å.M. Hansen, O.M. Poulsen, J.M. Christensen, S.H. Hansen, J. Anal. Toxicol. 17 (1993) 38.
[8] F.J. Jongeneelen, R.B.M. Anzion, P.T. Henderson, J. Chromatogr. 413 (1987) 227.
[9] G.L. Long, J.D. Winefordner, Anal. Chem. 55 (1983) 712A.
[10] Å.M. Hansen, J.M. Christensen, D. Sherson, Sci. Total Environ. 163 (1995) 211.
[11] P. Heilkkilä, M. Luotamo, L. Pyy, V. Riihimäki, Int. Arch. Occup. Environ. Health 67 (1995) 211.
[12] Á. Vinze, M.L. Huszár, J. Liq. Chromatogr. 12 (1989) 2887.
[13] G. Bienniek, Occup. Environ. Health 51 (1994) 357.