urnal of Chromatography, 229 (1982) 141—148
omedical Applications
lsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

HROMBIO. 1184

# )ETERMINATION OF URINARY HIPPURIC ACID AND *o*-CRESOL, AS NDICES OF TOLUENE EXPOSURE, BY LIQUID CHROMATOGRAPHY )N DYNAMICALLY MODIFIED SILICA

TEEN HONORÉ HANSEN*

*oyal Danish School of Pharmacy, Department of Organic Chemistry, 2 Universitetsparken, )K-2100 Copenhagen (Denmark)*

nd

IARTIN DØSSING

*ledical Department A, Division of Hepatology, Rigshospitalet, 9 Blegdamsvej, DK-2100 openhagen (Denmark)*

First received October 20th, 1981; revised manuscript received November 17th, 1981)

## SUMMARY

Two simple and sensitive high-performance liquid chromatographic methods for the letermination of hippuric acid and *o*-cresol in urine have been developed. The chromato-raphic system is the same for the two methods and is based on dynamically modified silica.

The detection limits were found to be 0.05 mg/ml and 0.05 μg/ml of urine for hippuric cid and *o*-cresol, respectively, when using UV detection at 254 nm. The recovery for ippuric acid was about 100% and for *o*-cresol 33—36%. The detection limit for *o*-cresol ould be lowered by a factor of ten by using fluorescence detection.

The methods were used for investigations of the urine from persons exposed to 100 ppm oluene for 6.5 h. The method for *o*-cresol may also be used for determination of other henols in urine.

## NTRODUCTION

Toluene is a solvent widely used in industry as a replacement for the carcinogenic benzene, but toluene also exhibits considerable toxicity [1]. Its main metabolite in man is benzoic acid, which is excreted as its glycine conjugate, hippuric acid. Hippuric acid is an endogenous metabolite common in human urine, but on exposure to toluene the level is enhanced [2—6]. Toluene

0378-4347/82/0000—0000/$02.75 

is, amongst several other substances, metabolised to *o*-cresol [1, 7, 8], which until now has not been reported as a usual component in human urine. It may therefore be regarded as a more specific metabolite and thus a better index of toluene exposure.

In this paper we present a chromatographic system based on dynamically modified silica [9, 10], in which the content of both hippuric acid and *o*-cresol in human urine may be determined.

## EXPERIMENTAL

### *Apparatus*

A Waters liquid chromatograph consisting of a Model 6000A pump, a Model 710A WISP autoinjector, a Model 440 ultraviolet (UV) absorbance detector (254 nm), a Model 730 data module and a Model 720 system controller was used. The columns were thermostated in a LC 250/3 Kratos oven. A Kontron SFM 23 LC spectrophotofluorimeter was used as the fluorescence detector (excitation at 273 nm, and emission at 298 nm).

For centrifugation a Sigma 3K-1 was used at 3000 *g* in all cases.

### *Chemicals*

Acetonitrile HPLC S grade was obtained from Rathburn Chemicals (Walkerburn, Great Britain). All other chemicals were of analytical-reagent grade and obtained from E. Merck (Darmstadt, G.F.R.).

### *Sample preparation*

*Hippuric acid.* A 1-ml volume of urine was mixed with 1 ml of acetonitrile and centrifuged for 5 min; 10 µl of the supernatant were injected onto the column.

*o-Cresol.* To 10 ml of urine, 1 ml of internal standard (0.005% 2,5-dimethylphenol or 0.015% 2,3,5-trimethylphenol) and 1 ml of concentrated sulphuric acid were added. The mixture was placed in a boiling water bath for 1 h. After cooling, 3 ml were extracted with 7.5 ml of cyclohexane and the phases were separated by centrifugation. The cyclohexane phase was then first extracted with 2 ml of 0.2 *M* potassium phosphate buffer and finally extracted with 300 µl of 0.1 *N* sodium hydroxide; 25 µl of the sodium hydroxide phase were injected onto the column. Careful centrifugation was necessary before separating the phases.

### *Chromatography*

The column set-up has been described previously [9, 10]. The analytical column was a Knauer column, 120 × 4.6 mm I.D., packed with LiChrosorb Si 60 (5-µm particles). The guard column (100 × 4.6 mm I.D.), situated between the pump and the autoinjector, was dry-packed with LiChroprep Si 60. Both columns were operated at 40°C. The mobile phase was acetonitrile—0.2 *M* potassium phosphate (pH 7.5)—water (30:5:65) containing 1.25 m*M* N,N,N-trimethylhexadecylammonium bromide; the flow-rate was 1.5 ml $min^{-1}$.

*Toluene exposure*

Forty-two printing workers (occupationally exposed to toluene during their daily work for ten years or more) were matched with control persons according, among others, to age and smoking habits. All persons were men. Each matched pair was randomly selected for either exposure to 100 ppm or 0 ppm of toluene in air for 6.5 h. The exposure was performed in an exposure chamber [11]. Three portions of urine were collected from each person at the following intervals: 2.5 h before the start of the experiment to time zero; from the start of the exposure to 3 h later; and from 3 to 6.5 h after the start of the exposure. The samples were stored at −20°C until analysed.

## RESULTS AND DISCUSSION

*Recovery, reproducibility and detection limits*

Hippuric acid can be determined by injecting diluted urine onto the column. Plotting the detector response in arbitrary units of area for hippuric aicd in the concentration range 0.1—10 mg/ml gave a rectilinear standard curve with the regression equation $Y = 23237X + 3.7$ ($r = 0.999$). The reproducibility and accuracy of this simple method were determined for urine using the standard addition method. The results in Table I show a recovery close to 100% with a good precision. Typical chromatograms are shown in Fig. 1.

TABLE I

DETERMINATION OF HIPPURIC ACID IN URINE

| Added (mg/ml) | Found (mg/ml) | Recovery ± R.S.D. (%) ($n = 4$) |
|---|---|---|
| 0 | 0.23 | — |
| 0.20 | 0.44 | 102.7 ± 2.1 |
| 0.50 | 0.73 | 100.3 ± 1.5 |
| 1.00 | 1.20 | 96.9 ± 1.8 |
| 2.00 | 2.19 | 98.1 ± 2.0 |

The phenolic metabolites (*o*-cresol and *p*-cresol) of toluene are present in urine as their sulphate and glucuronide conjugates. As *p*-cresol is also an endogenous metabolite only the specific metabolite *o*-cresol is quantitated, and the first step in the procedure is cleavage of the conjugates. As the concentration range of total *o*-cresol in urine is between zero and a few ppm, the development of a sufficiently reliable method involved investigation of the pre-chromatographic extraction and concentration procedures. The lower phenols including *o*-cresol are known to be volatile, and consequently it was decided to include the concentration step in the extraction procedure.

Several organic solvents were investigated with respect to efficiency and selectivity of extraction for *o*-cresol. In Table II the recoveries are shown for three of the solvents. Mixtures of hexane and diethyl ether were also investigated but a great tendency to emulsify was observed. Furthermore, the final extracts from these extractions caused severe baseline disturbance when injected onto the column, probably due to diethyl ether dissolved in the

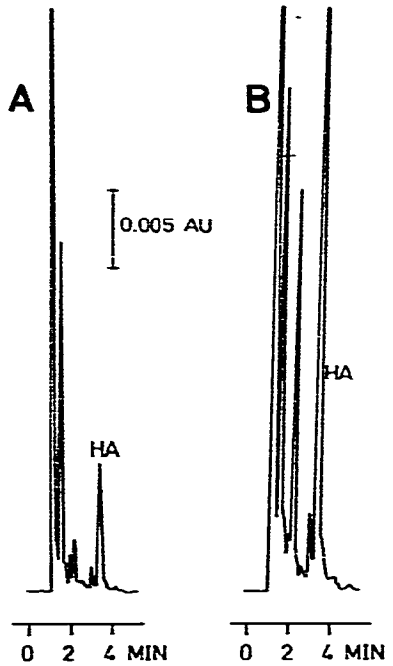


Fig. 1. Determination of urinary hippuric acid (HA). (A) Normal urine (HA = 0.29 mg/ml). (B) Urine from a person exposed to toluene (HA = 3.35 mg/ml).

TABLE II

RECOVERY OF *o*-CRESOL (1 ppm) FROM 3 ml OF 2 *M* SULPHURIC ACID EXTRACTED ONCE WITH 7.5 ml OF THE STATED SOLVENTS AND RE-EXTRACTED AS DESCRIBED IN THE PROCEDURE

Values are given in per cent ($n$ = 4).

| | Recovery | $S_{rel}$ | $S_{rel}$ after correction by internal standardization |
|---|---|---|---|
| Dichloromethane | 72 | 9.6 | 2.6 |
| Cyclohexane | 33 | 12.5 | 2.4 |
| Hexane | 27 | 15.0 | 3.1 |

aqueous phase. Dichloromethane gave the highest recovery, but when using urine too many co-extractants compromised the determinations. Extraction with hexane and cyclohexane gave low recoveries with a relative standard deviation of 12—15%, but the latter problem was solved by using an internal standard. Due to the purification step with phosphate buffer pH 7.0, only a few co-extractants from urine were found in the final extract.

The recovery of phenols from urine was found to be less than from 2 *M* sulphuric acid (Table III). The relative change in partition ratio was different for the three compounds and the relative standard deviations were larger when using urine. It is therefore necessary to use urine when preparing the standard solutions. These data were reproduced several times during a three-month

TABLE III

PERCENTAGE RECOVERY OF PHENOLS FROM 2 *M* SULPHURIC ACID AND URINE USING THE METHOD DEVELOPED

Number in parentheses are the relative standard deviations in per cent ($n$ = 4).

| | 2 *M* $H_2SO_4$ | Urine |
|---|---|---|
| *o*-Cresol | 35 (± 10.1) | 33 (± 13.7) |
| Dimethylphenol | 46 (± 4.1) | 38 (± 9.2) |
| Trimethylphenol | 33 (± 9.8) | 24 (± 20.7) |

TABLE IV

RECOVERY OF *o*-CRESOL FROM URINE USING THE METHOD DEVELOPED

All values are given in per cent ($n$ = 4).

| Amount added (ppm) | Recovery | $S_{rel}$ | $S_{rel}$ using internal standardization |
|---|---|---|---|
| 0.1 | 29 | 20 | 5.5 |
| 1.0 | 36 | 11.5 | 4.1 |
| 5.0 | 33 | 7.2 | 4.1 |

period with different samples of urine. The recovery of *o*-cresol at different concentration levels is given in Table IV.

The detection limits for practical work with the methods developed were found to be 0.05 mg of hippuric acid per ml of urine (UV, 254 nm), 0.05 μg of *o*-cresol per ml of urine (UV, 254 nm) and 0.005 μg of *o*-cresol per ml of urine (fluorescence, excitation 273 nm, emission 298 nm). The determination of *o*-cresol was first developed for use with a UV detector, but in Fig. 2 it is seen that fluorescence detection is more selective, and it is most likely that the extraction procedure could be simplified when using fluorescence detection alone.

*Occupational studies*

The described methods have been used in a large investigation of workers employed in the printing industry compared with workers employed elsewhere [11].

No difference in the metabolic pattern of toluene to hippuric acid and *o*-cresol in the two groups was observed (Table V), and the excretion of urinary hippuric acid and *o*-cresol showed a linear rise during the exposure. Urinary *o*-cresol discriminated better between exposed and non-exposed persons, especially during the first 3 h of exposure. This is due to the very low concentration of *o*-cresol found in urine before exposure. The low levels of *o*-cresol found in all persons may be due to the toluene pollution in the atmosphere.

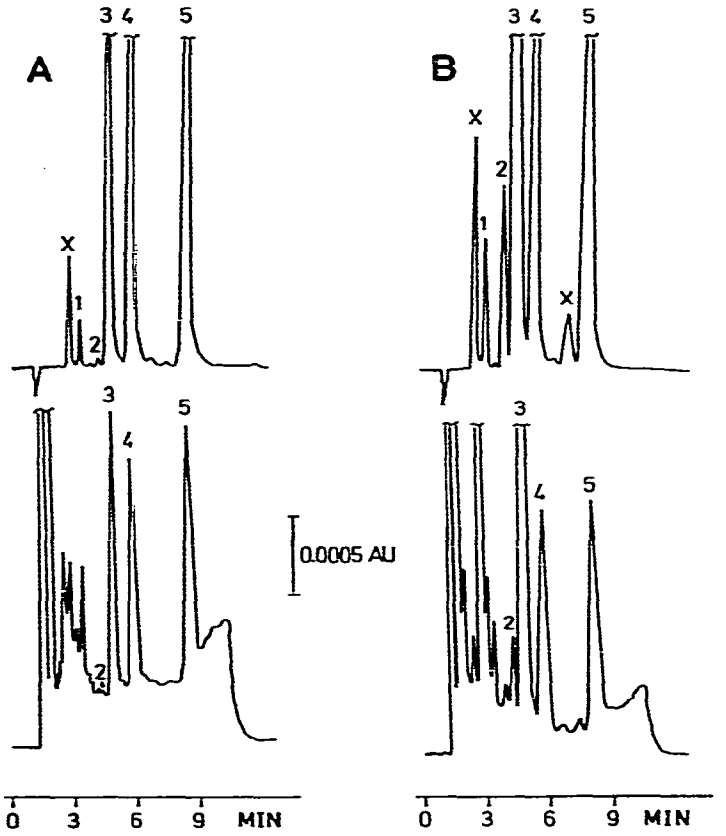


Fig. 2. Determination of urinary *o*-cresol. (A) Normal urine (0.03 μg *o*-cresol per ml). (B) Urine from a person exposed to toluene (0.9 μg *o*-cresol per ml). Upper traces: fluorescence detection (excitation 273 nm, emission 298 nm). Lower traces: UV detection at 254 nm. Peak identification: 1 = phenol, 2 = *o*-cresol, 3 = *m*- and *p*-cresol, 4 = 2,5-dimethylphenol, 5 = 2,3,5-trimethylphenol, and X = unknowns.

TABLE V

URINARY EXCRETION OF HIPPURIC ACID AND *o*-CRESOL IN 42 PRINTING WORKERS AND 43 CONTROL PERSONS DURING 6.5 h EXPOSURE TO 100 ppm OR 0 ppm TOLUENE IN AN EXPOSURE CHAMBER

| | Dose of toluene (ppm) | No. of persons | Urinary hippuric acid excretion (mmol) | | Urinary *o*-cresol excretion (μmol) | |
|---|---|---|---|---|---|---|
| | | | Median | (quartiles) | Median | (quartiles) |
| Printing workers | 100 | 19 | 6.7 | (6.3—7.4) | 4.6 | (4.0—4.9) |
| | 0 | 23 | 1.1 | (0.9—1.5) | 0.2 | (0.1—0.4) |
| Control persons | 100 | 21 | 6.7 | (5.5—7.7) | 4.2 | (3.4—5.4) |
| | 0 | 22 | 1.5 | (0.7—2.2) | 0.1 | (0.1—0.3) |

TABLE VI

URINARY EXCRETION OF HIPPURIC ACID AND *o*-CRESOL IN SMOKERS AND NON-SMOKERS EXPOSED TO 0 OR 100 ppm TOLUENE FOR 6.5 h IN AN EXPOSURE CHAMBER

| | Smokers | | | | | Non-smokers | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | No. of persons | *o*-Cresol (μmol) | | Hippuric acid (mmol) | | No. of persons | *o*-Cresol (μmol) | | Hippuric acid (mmol) | |
| | | Median | (quartiles) | Median | (quartiles) | | Median | (quartiles) | Median | (quartiles) |
| Before exposure −2.5–0 h | 53 | 0.14 | (0.08–0.25) | 0.87 | (0.62–1.25) | 31 | 0.04 | (0.02–0.05) | 0.54 | (0.40–1.29) |
| Exposed to 100 ppm 0–6.5 h | 33 | 4.54 | (3.68–4.92) | 6.68 | (5.98–7.52) | 7 | 4.08 | (3.35–6.05) | 6.69 | (5.68–7.18) |
| Exposed to 0 ppm 0–6.5 h | 20 | 0.38 | (0.15–0.62) | 1.20 | (0.84–1.91) | 24 | 0.13 | (0.07–0.20) | 1.13 | (0.86–1.89) |

Smoking was also found to have an influence on the *o*-cresol levels in the urine. Table VI shows that the levels are higher for smokers than for non-smokers during the whole experiment. Cigarette smoke is known to contain *o*-cresol [12], but smoking was not allowed during the experiment and at present no simple explanation for this observation can be given.

ACKNOWLEDGEMENTS

This work was supported by the Danish Medical Research Council Grant No. 12-0649.

The occupational part of the study was supported with grants from the Working Environment Fond.

The Central Laboratory, Rigshospitalet, is thanked for performing the creatinine analyses.

REFERENCES

1 K.-H. Cohr and J. Stokholm, Scand. J. Work. Environ. Health, 5 (1979) 71.
2 M. Ogata, R. Sugihara and S. Kira, Int. Arch. Occup. Environ. Health, 39 (1977) 199.
3 H. Veulemans and R. Masschelein, Int. Arch. Occup. Environ. Health, 43 (1979) 53.
4 J. Angerer, Int. Arch. Occup. Environ. Health, 43 (1979) 63.
5 H. Veulemans, E. van Vlem, H. Janssens and R. Masschelein, Int. Arch. Occup. Environ. Health, 44 (1979) 99.
6 H. Matsui, M. Kasao and S. Imamura, J. Chromatogr., 145 (1978) 231.
7 W. Woiwode, R. Wodarz, K. Drysch and H. Weichardt, Arch. Toxicol., 43 (1979) 93.
8 P. Pfäffli, H. Savolainen, P.-L. Kalliomäki and P. Kalliokoski, Scand. J. Work. Environ. Health, 5 (1979) 286.
9 S.H. Hansen, J. Chromatogr., 209 (1981) 203.
10 S.H. Hansen, P. Helboe, M. Thomsen and U. Lund, J. Chromatogr., 210 (1981) 453.
11 M. Døssing, S.H. Hansen, J. Bælum and G.R. Lundqvist, in preparation.
12 M.S. Baggett and G.P. Morie, Tob. Sci., 17 (1973) 30.