Journal of Chromatography, 132 (1977) 469–476

CHROM. 9626

ISOTACHOPHORETIC ANALYSIS OF MANDELIC ACID, PHENYLGLYOXYLIC ACID, HIPPURIC ACID AND METHYLHIPPURIC ACID IN URINE AFTER OCCUPATIONAL EXPOSURE TO STYRENE, TOLUENE AND/OR XYLENE

JAN SOLLENBERG
The National Board of Occupational Safety and Health, S-100 26 Stockholm 34 (Sweden)
and
ASTOR BALDESTEN
LKB-Produkter AB, S-161 25 Bromma 1 (Sweden)
(Received August 9th, 1976)

SUMMARY

A simple, rapid and sensitive analytical method has been developed for the determination of phenylglyoxylic acid, mandelic acid, hippuric acid and methylhippuric acid; *o*-, *m*- and *p*-methylhippuric acids are partly separated. These compounds are found as metabolites after occupational exposure to styrene, toluene and xylene. The method has been applied successfully to samples extracted from human urine by diethyl ether. The method can be used to accurately and simultaneously determine as little as 0.5 nmole of all of these acids in less than 20 min.

INTRODUCTION

The biotransformation products phenylglyoxylic acid (PGA), mandelic acid (MA), hippuric acid (HA) and methylhippuric acid (MHA) can be found in human urine. HA is a common constituent of urine, whereas the other acids are found less frequently and in considerably smaller amounts. Drastically increased levels of PGA and MA, HA and MHA are found after occupational exposure to the organic solvents styrene, toluene and xylene, respectively. The use of these biotransformation products as biological indicators of the degree of exposure to such solvents is of great importance. Hitherto, routine determination of these compounds has been hampered by the low sensitivity and specificity of the known methods[1,2]. Recently, some improvements have been achieved by the use of gas chromatography after derivatization[3,4]. However, these methods are time-consuming and elaborate. In a search for a better and simpler technique, the isotachophoretic method[5–7] has been tried in the expectation that it would offer advantages over previously described techniques.

EXPERIMENTAL

The isotachophoretic separations and determinations were made in an LKB 2127 Tachophor. The design of the instrument has previously been described by Arlinger[8]. The instrument was equipped with a 430-mm capillary of 0.5-mm I.D. The starting voltage was 5 kV with a final voltage of 20 kV at 125 μA and 23°. When *o*-, *m*- and *p*-MHA had to be determined the detection was made in a 630-mm capillary at 70 μA in order to sharpen the zone boundaries and to improve the separation. UV absorption at 254 nm was used for identifications and quantifications. The analysis time was *ca.* 20 min for the 430-mm capillary.

The chemicals used were analytical grade, generally commercially available if not specifically stated. *o*-, *m*- and *p*-MHA were synthesized from the corresponding methylbenzoyl chlorides and glycine[9]. The water used was twice distilled from glass.

Electrolytes

Leading: 5 m*M* HCl Suprapur (E. Merck, Darmstadt, G.F.R.); 20 m*M* β-alanine (Sigma, St. Louis, Mo., U.S.A.) and 0.4% hydroxypropylmethylcellulose (Methocel 90 HG 15000 cps; Dow Chem., Midland, Mich. U.S.A.) (pH 3.75). Terminating: 5 m*M* caproic acid (Fluka, Buchs, Switzerland).

Urine samples

The normal urine samples were from laboratory personnel. The samples from cases exposed to xylene were from laboratory assistants working with xylene in microscopy preparations, and the urine samples from cases exposed to styrene were obtained from a medical centre. The origin was unknown. The samples were kept frozen if not analyzed immediately.

Extraction

0.5 ml of urine were saturated with NaCl, acidified with 0.02 ml of 3 *M* HCl and extracted with 5 ml of diethyl ether by intensive shaking for 10 min. The ether layer was separated and evaporated to dryness. The residue was dissolved in 0.5 ml or 1.0 ml of 0.01 *M* HCl. 1–10 μl of the samples were injected directly into the Tachophor. Extractions were made with diethyl ether, diethyl ether–ethyl acetate and ethyl acetate after saturation of the urine with $(NH_4)_2SO_4$ or NaCl. The recoveries with all of these methods were approximately the same, but the extraction into the organic phase of interfering impurities was more pronounced when ethyl acetate was used.

Evaluation

The quantities of the compounds were determined by measuring the zone lengths with a graticule after a seven-fold magnification. The chart speed was 5 cm/min. The zone lengths of the unknown samples were compared with the zone lengths from standard runs of known amounts of the corresponding acids.

RESULTS

The Tachophor was checked daily by running a blank containing only the electrolytes and by running a standard containing PGA, MA, HA and *m*-MHA (Fig. 1).

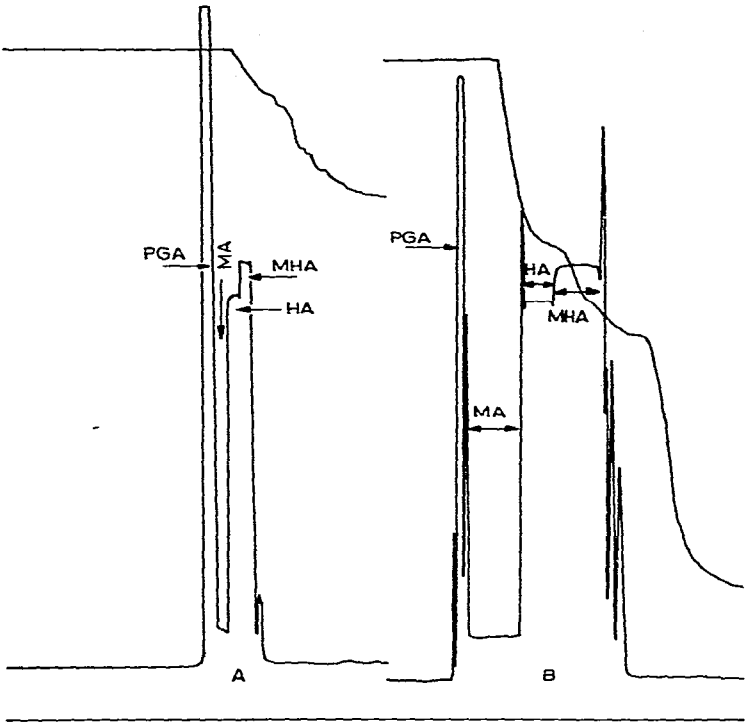

Fig. 1. (A) Standard run of 2 nmoles of PGA, MA, HA and *m*-MHA. (B) Isotachophoretic run of urine to which had been added 0.6 nmole of PGA, 6 nmoles of MA and 5 nmoles of *m*-MHA. The zone due to HA shows the natural amount in this urine sample. The detection in the LKB Tachophor was by measuring the light transmission at 254 nm. A zone starts at the point where the first change in the light transmission is detected, and continues until another change shows that a new zone is passing through the detector, as is shown by the arrows. The thermosignal, which indicates the joule heat dissipated, runs from the top left to the bottom right corner of the figures.

The values for the standard were then used for the evaluation of these compounds in the authentic samples. It was possible to determine 0.5–35 nmoles of the acids in the urine samples, where the length of the UV-absorbing zone was directly proportional to the amount of acid. With larger amounts of injected acids, equilibrium was not obtained with the length of capillary used. If the amount of any of the four acids was outside this range the injection volume was adjusted so that the amount fell within this range. Some of the values given in this paper thus originate from different runs with the same sample. The maximum sample volume injected was 10 μl, and therefore the minimum measurable concentration was 0.05 μmole/ml.

Two types of standard runs were performed. For the recovery studies, urine from both healthy females (8) and males (6) were used. An aliquot portion of each sample was withdrawn, then the same amount of PGA (0.6 μmole), MA (6 μmoles) and MHA (5 μmoles) was added per millilitre of all of the samples. No HA was added. Fig. 1B shows the result of an analysis of a sample of urine containing the added acids. None of the untreated urine samples contained MA. PGA was found in two untreated urine samples: one sample contained 144 nmoles of PGA per ml of urine

TABLE I

PERCENTAGE RECOVERIES OF THE DIFFERENT ACIDS AFTER ADDITION TO URINE

In experiment A the same amount of the acids was added to 14 different urine samples. In experiment B varying amounts of the acids were added to the same urine sample.

Compound	*Type of experiment*	*No. of samples*	*Mean recovery (%)*	$\pm S_{\bar{x}}$
PGA	A	14	96	3.2
PGA	B	8	94	5.2
MA	A	14	91	5.2
MA	B	8	87	3.9
HA	B	8	93	2.2
MHA	A	14	76	3.5
MHA	B	8	86	3.0

and the other contained 196 nmoles/ml. One of the untreated samples contained 57 nmoles of MHA/ml. All of the untreated urine samples contained HA in amounts ranging from 1.2 to 8.6 μmoles per ml of urine. No difference was noticed between the samples obtained from women and men.

In another experiment, the acids were added in increasing amounts to the same urine sample as follows (μmoles/ml): PGA, 0.06–1.3; MA, 0.6–13; HA, 0.5–11; and

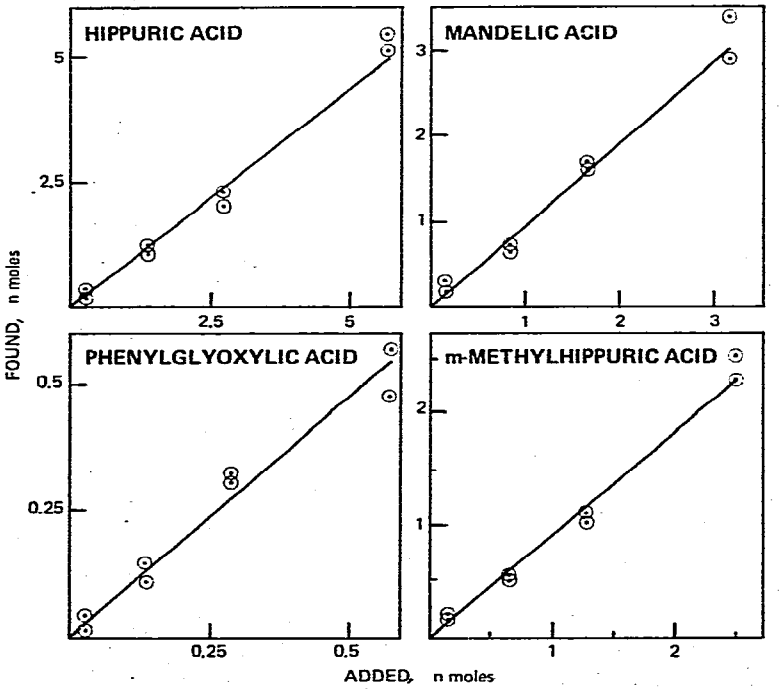

Fig. 2. Recovery curves for PGA, MA, HA and *m*-MHA after extraction from human urine and isotachophoretic analysis, showing the amounts found for the four acids as a function of amounts added in nmoles/μl of sample.

m-MHA, 0.5–10. The quantities used were the values expected after occupational exposure to the corresponding solvents. In the untreated sample, 120 nmoles of PGA, 220 nmoles of MA and 4.4 μmoles of HA were found, all per ml of urine, but no MHA was present. The values found in the untreated urine sample have been subtracted from the results shown in Table I and Fig. 2.

In order to test the method, extracts of urine samples from cases exposed to styrene or xylene were run on the Tachophor. Typical curves showing the increased levels of MA, PGA and MHA are given in Figs. 3A and 4A. To prevent uncertainty, it may occasionally be necessary to identify the acids in an authentic sample by addition of a known compound, as is shown in Figs. 3B and 4B. The broadened zones were easily distinguishable after the addition of all of the four acids to the sample. Each zone has a characteristic light absorption.

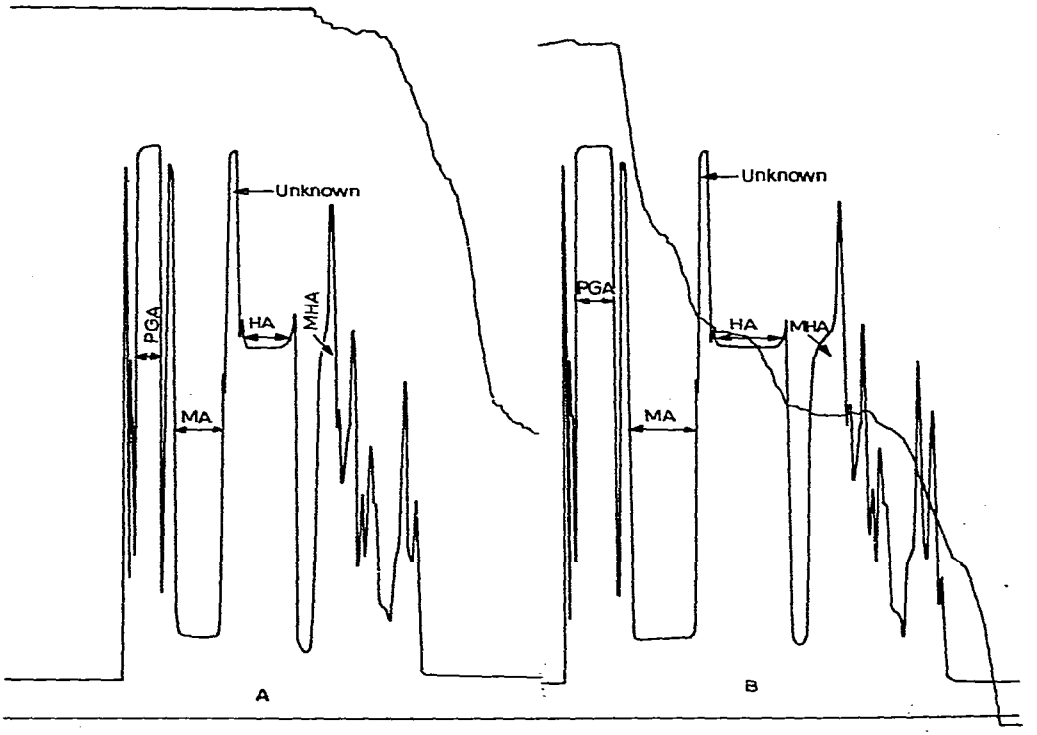

Fig. 3. (A) Isotachophoretic run of urine from a person exposed to styrene. The occurrence of MHA indicates that the person may also have been exposed to xylene. (B) The same sample as in A after addition of PGA, MA, HA and *m*-MHA for identification. For details see Fig. 1.

The isotachophoretic determination of MA in urine samples from persons exposed to styrene was compared with gas chromatographic measurements where, however, the MA first was converted into the trimethylsilyl derivative. The results are summarized in Table II, and coincide within the limits of the experimental errors.

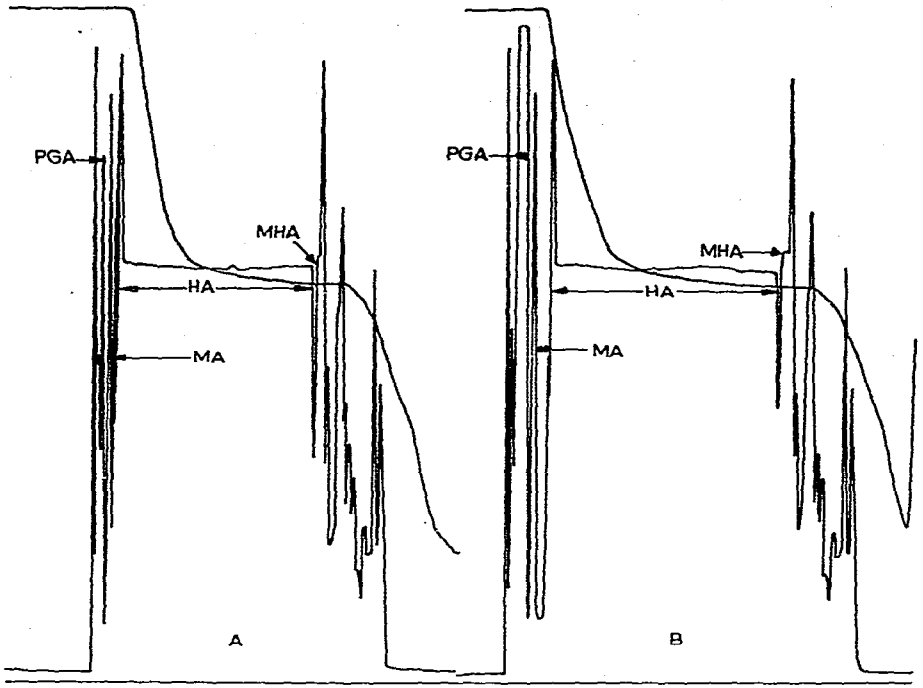

Fig. 4. (A) Isotachophoretic run of urine from a person exposed to xylene. This sample contains small amounts of PGA and MA, and the level of HA is unusually high. The amount of MHA is minute, indicating only a slight exposure to xylene. (B) The same sample as in A after addition of PGA, MA, HA and *m*-MHA for identification. For details see Fig. 1.

TABLE II

COMPARISON OF THE RECOVERIES OF MANDELIC ACID DETERMINED FROM THE SAME SAMPLES WITH AN LKB 2127 TACHOPHOR AND BY GAS CHROMATOGRAPHY OF THE TRIMETHYLSILYL DERIVATIVES

The values are in mmole/l and show the increased levels found after occupational exposure to styrene.

Isotachophoresis	*Gas chromatography*
2.1	2.0
1.6	1.9
1.0	0.9

DISCUSSION

The primary objective of this investigation was to develop a simple and rapid method for the simultaneous determination of PGA, MA, HA and MHA in urine. There is increasing interest in the occupational and environmental control of chemicals, and such a method should be of great advantage since other available methods are time-consuming and have some methodological difficulties. Isotachophoresis is a method of high resolution for the separation of compounds according to their net mobility in a chosen electrolyte system. This method enables the separation of closely related compounds, and it is possible to simultaneously quantify the separated mole-

cules. In a given electrolyte system, the separation pattern is very reproducible and the identification generally does not cause problems. When there is any ambiguity, it is sufficient to add a known standard to an actual sample and to see which peak becomes broader (see Figs. 1, 3 and 4). A fraction collector, which allows collection of the different zones from an isotachophoretic run for further studies, has been described by Arlinger[10].

The reproducibility of the isotachophoretic analysis is noteworthy. The mean recoveries in the experiments shown in Table I include all of the manipulation errors from the sample preparation to the evaluation of the zones. The simple extraction procedure of the urine samples by diethyl ether was chosen to facilitate the rapid routine handling of a large number of samples. The method is still sufficiently sensitive, as the quantity of the acids excreted in urine after exposure to the corresponding solvent is often very large. After exposure to 50 ppm of styrene in air (present Swedish threshold-limit value, TLV), the excretion of MA can reach levels of 15 μmoles per ml of urine[11]. It is thus possible to measure less than one hundredth of that concentration. The excretion of PGA is found to be about one quarter of that of MA[11]. The TLVs of xylene and toluene are double that of styrene. The concentrations in urine of MHA and HA found after an exposure at this level are in the mM range[12] and are thus easy to measure.

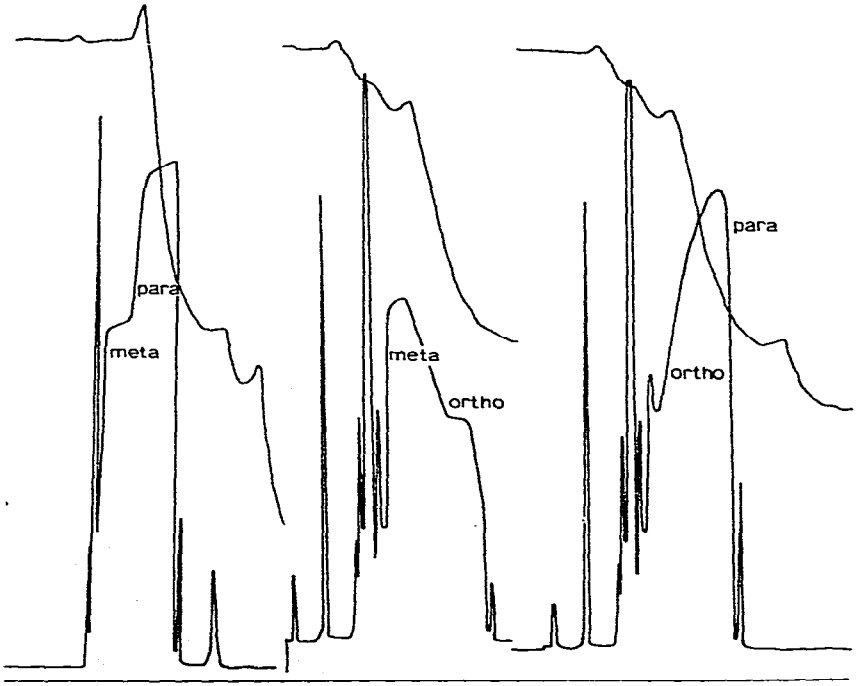

Fig. 5. Isotachophoretic runs of pairs of *o*-, *m*- and *p*-MHA. The *meta* and *para* isomers are well separated and can be quantified. Since the mobility of the *ortho* form is intermediate between those of the other two isomers, a mixture of the three isomers gives a mixed zone which, however, can be used for quantification. The separations were performed in a 630-mm capillary. For details see Fig. 1.

In the 630-mm capillary, *m*- and *p*-MHA were well separated, whereas the addition of the *ortho* isomer caused mixed zones (Fig. 5). However, the MHA originating from technical xylene consists mainly of the *meta* and *para* isomers since this grade contains very little of the *ortho* isomer. Accordingly, the *ortho* isomer is normally found in urine in minute amounts which do not effect the results. The separation and quantification of MHA from an authentic urine sample is also possible in a 430-mm capillary, as is evident from Fig. 4, since the entire zone must have the same concentration of the isomers whether they are separated or present as a mixture. As HA can be present in urine in considerable amounts, its use as an indicator of toluene inhalation is meaningful only in cases of heavy exposure.

The present method can be used for occupational health control, not only in cases of exposure to one solvent but also in cases of exposure to a mixture of solvents.

REFERENCES

1 H. Ohtsuji and M. Ikeda, *Brit. J. Ind. Med.*, 27 (1970) 150.
2 M. Ogata, K. Tomokuni and Y. Takatsuka, *Brit. J. Ind. Med.*, 26 (1969) 330.
3 K. Engström and J. Rantanen, *Int. Arch. Arbeitsmed.*, 33 (1974) 163.
4 J. Flek and V. Sedivec, *Collect. Czech. Chem. Commun.*, 38 (1974) 1754.
5 H. Haglund, *Sci. Tools*, 17 (1970) 2.
6 R. J. Routs, *Thesis*, University of Eindhoven, 1971.
7 A. J. P. Martin and F. M. Everaerts, *Proc. Roy. Soc. (London)*, *A*, 316 (1970) 493.
8 L. Arlinger, in H. Peeters (Editor), *Protides of the Biological Fluids*, Vol. 22, Pergamon, Oxford, New York, 1975, p. 661.
9 A. Gleditsch and H. Moeller, *Justus Leibigs Ann. Chem.*, 250 (1889) 376.
10 L. Arlinger, *J. Chromatogr.*, 119 (1976) 9.
11 H. Härkönen, P. Kalliokoski, S. Hietala and S. Hernberg, *Work Environ. Health*, 11 (1974) 162.
12 M. Ogata, Y. Takatsuka and K. Tomokuni, *Brit. J. Ind. Med.*, 28 (1971) 382.