DETERMINATION OF TOXIC SUBSTANCES AND THEIR METABOLITES IN BIOLOGICAL FLUIDS BY GAS CHROMATOGRAPHY. VII.*

TOLURIC OR TOLUIC ACIDS IN URINE

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o-, *m*-, and *p*-Toluric acids (methylhippuric acids) present in the urine of people exposed to the vapours of isomeric xylenes are split by alkaline hydrolysis at 130°C to toluic acids (methylbenzoic acids). After acidification and saturation of the reaction mixture with ammonium sulfate the liberated toluic acids are extracted once with ethyl acetate containing acetophenone as internal standard. On reaction with diazomethane they are transformed to corresponding methyl esters which are then determined gas chromatographically on 10% polyethylene glycol 1 500 at 130°C, using FID. The method enables a rapid and specific determination of toluric (or toluic) acids when present together even in the presence of a large excess of hippuric (or benzoic) acid and other normal or accidental components.

For the determination of toluric (methylhippuric) acids in the urine of people exposed to xylene vapours the photometric method[1,2] has been proposed, based on a colour reaction with *p*-dimethylaminobenzaldehyde in acetic anhydride as medium. However, the method is not specific because hippuric acid, which is a normal urine component, also reacts with this reagent; therefore it must be separated beforehand by paper or thin-layer chromatography. Even this tedious and time-consuming sample preparation does not permit the determination of individual toluric acid isomers parallelly, but only the sum of *m*- and *p*-isomer (the *o*-isomer was not investigated[1]). In one of the preceding papers[3], we demonstrated that *m*- and *p*-toluric acids may be extracted from the investigated urines, transformed to corresponding methyl esters, and then determined even in the presence of a large excess of hippuric acid by gas chromatography; however, the determination of the *o*-isomer has not been possible because it had the same elution time as hippuric acid. Toluric acid may be hydrolysed with alkali quantitatively to corresponding toluic acids the methyl esters of which — including the *o*-isomer — have substantially longer elution times than the methyl ester of benzoic acid (as we have shown earlier[4]). This is the basis of their simple and specific determination.

The study of optimum conditions of toluric acids hydrolysis and the gas chromatographic determination of the formed toluic acids is the subject of this communication.

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EXPERIMENTAL

Chemicals and Apparatus

Toluric acids (methylhippuric acids) were prepared from toluic acids which were transformed to corresponding chlorides and then reacted with aminoacetic acid in alkaline medium. Their melting points (*ortho-* 164·5°C, *meta-* 138·0°C, *para-* 164·5°C) were determined on a microthermal block. The standard aqueous solutions were prepared by dissolving 0·1419 g of toluric acids in water alkalized with 0·25 ml of 50% (w/v) NaOH, and filling up to 100 ml; (1 ml corresponds to 1·00 mg of toluic acids). Further chemicals used were: acetophenone in ethyl acetate (200 μg/ml), H_2SO_4 (40 ml of 96% sulfuric acid/250 ml), aqueous sodium hydroxide solution (50 g/ /100 ml), ammonium sulfate of analytical grade, diethyl ether, N-nitrosomethylurea[5], and acetic acid.

The apparatus for esterification has been described in a previous communication[3]. Further a chromatograph of the firm Carlo Erba (model GD with FID) and a stainless-steel column (2 m/2 mm) filled with 10% polyethylene glycol 1 500 on Chromosorb W (60—80 mesh) were used; working temperature was 130°C, temperature of the injecting compartment was 180°C; nitrogen (25 ml/min), hydrogen (20 ml/min), air (300 ml/min), injection 5 μl, Honeywell recorder, paper shift 12·7 mm/min, input resistance 10^{10} Ohm, 1/8 of full sensitivity.

Procedure

a) *Hydrolysis*: 2 ml of the investigated urine were pipetted into a 25 ml flask followed by 2 ml of sodium hydroxide solution and the mixture was stirred and heated in a silicone oil bath (ultrathermostat Höppler) at 130°C for 60 minutes. Five ml of dilute H_2SO_4 were then slowly added to the mixture under stirring and cooling. The acidified mixture was then saturated with 3 g of ammonium sulfate under shaking. A solution of acetophenone (5 ml) was then added, the flask stoppered and the mixture again shaken for 1 minute. After five minutes standing the layers usually separated. If not, short centrifuging was necessary.

b) Esterification: Approximately 2 ml of the ethyl acetate layer were transferred into the esterification tube and diazomethane was introduced as described before[3]. When the reaction terminated excess diazomethane was eliminated by introducing shortly a nitrogen stream through the mixture which was then chromatographed. The relative height ratio of the peaks of toluic acid methyl esters and acetophenone (standard = 1·0) was calculated and the concentration of toluric or toluic acids in the analysed urine was read from the calibration curve.

Calibration curves: Into a set of flasks (25 ml) increasing amounts (0 to 2·0 ml) of standard solutions of *o-*, *m-*, *p*-toluric acids were pipetted and water was added up to the volume of 2·0 ml. 2·0 ml of NaOH solution were added and the mixture was worked up as described. The calculated ratio of the peak heights of the methyl esters and the standard were plotted in a graph against the concentration of toluic or toluric acids (Fig. 1).

RESULTS AND DISCUSSION

Hydrolysis of toluric acids. We investigated the course of hydrolysis of toluric acids in dependence on the basicity of the reaction mixture, temperature and time of heating. If some toluric acid remained unhydrolysed, it was determined by gas chromatography[3]. From the results it followed that at 100°C *m-* and *p*-toluric acids are already hydrolysed quantitatively after 20 minutes heating of the reaction

mixture in 25% (w/v) sodium hydroxide, *i.e.* at the same rate as hippuric acid[4]. *o*-Toluric acid hydrolyses under the same conditions only partially, the main part (85%) remains unchanged. By prolonging the reaction time the degree of hydrolysis increases slowly, but for a quantitative hydrolysis an excessively long time would be necessary. Therefore, the reaction temperature was increased to 130°C; under such conditions the hydrolysis was practically complete after 40 minutes heating (Fig. 2). When standard procedure was applied the reaction time was prolonged to 60 minutes. The quantitative course of the reaction was checked by processing various amounts of *o*-toluric acid; in all instances an equivalent amount of *o*-toluic acid was formed.

Extraction and esterification of toluic acids: The yields of the extraction of toluic acids from the aqueous phase saturated with ammonium sulfate were high (99%) and perfectly reproducible. Transformation to methyl esters takes place equally smoothly as with benzoic acid[4].

Choice of internal standard and conditions of chromatographic determination: In order to eliminate the errors caused by the change of the extract volume during the introduction of diazomethane, or inaccuracies during the injection of the samples,

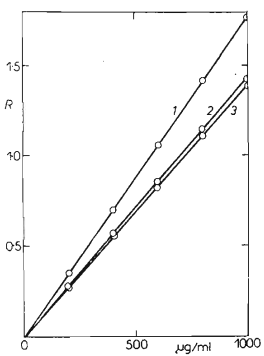

FIG. 1

Calibration Curves for the Determination of Toluic Acids

R ratio of the heights of peaks of methyl esters of *o*-, *m*-, and *p*-toluic acids and the internal standard; concentration of toluic acids is given in µg/ml of the analysed sample. *1 o*-Toluic acid, *2 m*-toluic acid, *3 p*-toluic acid.

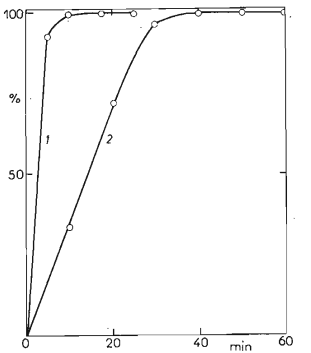

FIG. 2

Course of Hydrolysis of Toluric Acids

Hydrolysis of toluric acids is expressed in %; *t* reaction time (min), 25% NaOH (w/v), temperature 130°C. *1 m*- and *p*-toluric acid, *2 o*-toluric acid.

the method of internal standard was applied for the evaluation of the analyses. In order to keep the procedure identical with that for benzoic acid determination[4] acetophenone was chosen as internal standard.

Polyethylene glycol 1500 was chosen as the most suitable stationary phase because it enables a satisfactory separation of all envisaged isomers, especially methyl esters of *m*- and *p*-toluic acids. Relative elution times (acetophenone = 1·0) are the following: methyl benzoate 0·84; methyl ester of *o*-toluic acid 1·14; methyl ester of *m*-toluic acid 1·38; methyl ester of *p*-toluic acid 1·46. When higher molecular polyethylene glycols (20M) or polyester stationary phases were employed *m*- and *p*-isomers separated poorly. In addition to this, peaks of other substances (for example methyl salicylate) were shifted to the critical region of elution times.

Quantitative relationships were derived from the heights of the corresponding peaks[6]. From Fig. 3 it is evident that the peak of *o*-toluic acid methyl ester is perfectly separated not only from the methyl benzoate peak, but also from the methyl esters of both remaining isomers, so that a quantitative estimation does not present any difficulty. The amount of *m*-toluic acid may be equally easily determined; it is most abundant in the urine of persons exposed to xylene vapours (*m*-xylene is the main component of technical xylenes). Somewhat more complex is the evaluation of the chromatographic peaks of the *p*-isomer because it is not perfectly separated from the preceding peak, *i.e.* methyl ester of *m*-toluic acid. In consequence the peak is a little higher. The larger the ratio of the heights of the peaks of the *m*- and *p*-isomers, the larger the error in the determination of the *p*-isomer. Therefore, for analyses requiring special precision a correction is introduced[7].

Selectivity of the method and disturbing effects: The described method of determination of toluric acids in urine is very selective. Hippuric acid does not impair the determination even at high concentrations, such as can be met in the urine of people exposed to toluene vapours. Hydroxyhippuric acids which occur as incidental components of the urine also do not affect the precision of the determination; under the given conditions they are hydrolysed to corresponding hydroxybenzoic acids the methyl esters of which have longer elution times than methyl esters of toluic acid. Phenol and *p*-cresol which occur in normal urines in substantially lower concentrations than the considered acids also do not disturb the determination because their elution times are longer; if under the effect of diazomethane they are partly transformed to corresponding methyl ethers, then they are eluted at the beginning of the chromatographic curve.

The sole substances which could distort the determination of toluric acids could be toluic acids, either free or bound to substances other than aminoacetic acid (for example glucuronic acid).

Free toluic acids, however, do not occur in fresh urine of people exposed to xylene. This was shown by direct extraction of the urines with ethyl acetate (*i.e.* without

previous alkaline hydrolysis). After working up the extract no peaks of methyl esters of toluic acids could be found on the chromatographic curves. In contrast to this, if these urines were stored for several weeks, they gave on direct working up quite distinct chromatographic peaks of the considered substances. Evidently, bacterial cleavage of toluric acids to toluic acids took place during storing. The decomposition can be prevented by addition of sulfuric acid (0·4 ml of 40% sulfuric acid per 100 ml of urine).

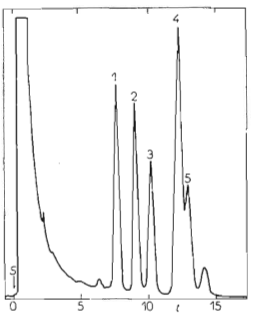

FIG. 3

Chromatogram of the Urine of a Person Exposed to a Mixture of Xylene Isomers

S Start, 1 methyl benzoate, 2 internal standard (acetophenone), methyl ester of 3 *o*-toluic acid, 4 *m*-toluic acid, 5 *p*-toluic acid.

The excretion of toluic acids bound to glucuronic acid[8] was observed only in the case of experimental rabbits exposed to xylene, but the concentration was low. However, it has not been observed in humans. According to the analogy with benzoic acid, which is usually excreted as hippuric acid but in the case of a heavy loading of the organism also, partly, as benzoylglucuronic acid[9,10], this alternative cannot be excluded. Should the analysed urines contain toluylglucuronic acids, they would be hydrolysed under the effect of sodium hydroxide analogously as benzoylglucuronic acid[11–13]. The results of the determination of toluric acids would then be higher than would correspond to reality, although the error would be only small with respect to the relative amount of the substances under consideration.

For practical purposes, *i.e.* for the determination of the degree of exposure of persons, it seems more advantageous to express the results of the urine analyses as the amounts of toluic acids, because this method includes not only toluric acids, but also the free toluic acids (in old and unsuitably stored urines) or other theoretically possible conjugates of toluic acids (toluylglucuronic acid).

Range of the method and reproducibility of results: The range of the method is chosen so that it would cover the concentration range of toluric or toluic acids, which may come into consideration during the analysis of urines of persons exposed to xylene vapours. Within the whole range (0–1 000 μg of toluic acids per one ml of urine) the calibration curves are linear. At very high concentrations, when the ratio of the heights of the peaks of toluic acids methyl esters and the internal standard would be excessive, it is recommended to dilute the urines with a known amount of water. The reproducibility of the method was checked by repeated analysis (20 times) of the same urine sample of an exposed person. Statistical evaluation of the results showed that the variation coefficient was as follows: for *o*-isomer V – 0·77, *m*-isomer V – 0·58, *p*-isomer V – 0·82%. Twenty to thirty urine samples can be analysed per day. A chromatogram obtained during the analysis of the urine of a person exposed to xylene vapours is shown in Fig. 3.

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