FUROYLGLYCINE AND TOTAL PYROMUCIC ACID IN URINE OF PERSONS EXPOSED TO FURAL*

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Methods are proposed for the determination of furoylglycine and total pyromucic acid in the urine of persons exposed to fural vapours. Furoylglycine from urine is extracted once with ethyl acetate, converted with diazomethane to its methyl ester, and determined gas chromatographically. A similar procedure is followed in the determination of total pyromucic acid but the urine is submitted previously to alkaline hydrolysis. It is shown that for the purpose of exposure test the second of these methods is more advantageous.

Furoylglycine (pyromycuric acid) C_4H_3O—CO.NH.CH_2COOH is the main metabolite of fural, furyl alcohol and several other furan derivatives[1-3]. For its determination in the urine of experimental animals the method of spectral absorption in UV region has been proposed[3]. However, the accuracy of this method is unsufficient because numerous normal or accidental components of urine, for example hippuric acid and further free or conjugated acids[4,5], also absorb under the given conditions to a larger or smaller extent[6,7]. The disturbing effect could be partly suppressed by measuring at two different wave-lengths (255 and 280 nm), where the difference of the absorbances indicated the contents of furoylglycine[8].

Recently the method of gas chromatography is used successfully for the determination of toxicologically important urine components. As an example the determination of hippuric acid (a toluene metabolite) may be mentioned, which is extracted either directly[9] or after previous hydrolysis[10] from urine, then converted with diazomethane to methyl ester and chromatographed in this form. In view of the relatedness of the substances under consideration it could be expected that a similar procedure could be used even for the determination of furoylglycine or total pyromucic acid in the urine of persons exposed to fural vapours.

EXPERIMENTAL

Reagents and Apparatus

Pyromucic acid (m.p. 133°C, Kofler) was prepared from fural[11], furoylglycine (m.p. 168°C, Kofler) from pyromucic acid by its conversion to chloride[12] and reaction of the latter with aminoacetic acid in alkaline medium[13].

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The standard aqueous solution contained in 1 ml 754·5 μg of furoylglycine (corresponding to 500 μg of pyromucic acid); it was kept in a refrigerator and used for a maximum of 2 weeks. As internal standard a solution of phenacetine in ethyl acetate (1 000 μg/ml) was used for the determination of furoylglycine, or a solution of acetophenone (200 μg/ml) in ethyl acetate for the determination of pyromucic acid. The following chemicals were also used: dilute sulfuric acid (40 ml of 96% H_2SO_4/100 ml), solutions of sodium and potassium hydroxide (50 g/100 ml), ammonium sulfate, diethyl ether, N-nitrosomethylurea[14] and acetic acid.

The apparatus for the preparation of diazomethane and for the esterification of the acids present in urine extracts was described in a preceding paper[9]. The gas chromatograph of the firm Carlo Erba model GD, was provided with a FID, Honeywell recorder, inlet resistance of the amplifier was 10^{11} ohms, paper shift 12·7 mm/min. Working conditions: *a*) Furoylglycine: a stainless steel column 1 m × 2 mm, packed with 10% neopentylglycol succinate on acid washed Chromosorb W 60—80 mesh, temperature 190°C, injection space 250°C, detector block 200°C. The flow of nitrogen was 55 ml/min, of hydrogen 40 ml/min, and of air 300 ml/min. Injection 4 μl, record sensitivity 1/64 or 1/32. *b*) Total pyromucic acid: 2 m × 2 mm column packed with 10% polyethylene glycol 1 500 on acid washed Chromosorb W 60—80 mesh, temperature 130°C, injection space 200°C, detector block 200°C. Nitrogen flow 45 ml/min, hydrogen flow 28 ml/min, air 300 ml/min. Injection 4 μl, record sensitivity 1/128 or 1/64.

Working Procedure

a) *Determination of furoylglycine*: 2 ml of the urine were pipetted into a test tube with a ground glass stopper, 0·1 ml of dilute sulfuric acid was added and the mixture was saturated with ammonium sulfate (2 g) and shaken for one minute thoroughly with 2 ml of a phenacetine solution in ethyl acetate. After standing for 3—5 minutes both layers were well separated; if not, the mixture was centrifuged. The extract was worked up using the procedure described under *c*).

b) *Determination of total pyromucic acid*: The urine (2 ml) was pipetted into a test tube with a ground glass stopper, followed by 2 ml of sodium hydroxide solution, the mixture was mixed and heated on a boiling water bath for 20 minutes. After cooling 2 ml of dilute sulfuric acid were added under cooling and stirring, followed by 2 g of solid ammonium sulfate. The mixture was shaken with 2 ml of an acetophenone solution in ethyl acetate for one minute and then allowed to stand for 3—5 minutes, or it was centrifuged. The extract was worked up as under *c*).

c) *Esterification of acids*: In view of the high toxicity of diazomethane all operations had to be done in a well functioning fume cupboard. About one ml of the ethyl acetate extract was transferred into a test tube and diazomethane (prepared by decomposition of N-nitrosomethylurea with potassium hydroxide) was introduced with a mild stream of nitrogen. The introduction was interrupted when ether in the next control test tube assumed a yellow colour[9]. The liquid was then bubbled through shortly with nitrogen in order to eliminate excess diazomethane, and about 4 μl of the resulting solution were injected into the injection port of the gas chromatograph. On the record the heights of the peaks of the methyl ester and of the internal standard were measured and their ratio was calculated (standard = 1·0). Using the calibration curve the concentration of the analysed substances in urine may be read (in μg/ml).

d) *Calibration curves*: Into a set of test tubes gradually increasing volumes (0—2 ml, in 0·4 ml intervals) of the standard solution were measured and made up with water to 2 ml volume. Further procedure was the same as for the working up of samples. The calculated ratio of the peak heights of the methyl ester and the standard was plotted in a diagram against the concentration of furoylglycine or pyromucic acid in aqueous solution.

RESULTS AND DISCUSSION

Determination of Furoylglycine

The basis of the method is the extraction of furoylglycine from urine with ethyl acetate and its further conversion with diazomethane to methyl ester. Extraction is carried out once only and its effect is satisfactory if the analysed samples are previously saturated with ammonium sulfate. The amount of furoylglycine passing into the non-aqueous layer is about 84% and it is well reproducible. Since the yield of extraction is not quantitative the calibration curve should be constructed in the same manner, *i.e.* by extraction of the standard aqueous solutions. For chromatographic separation it is advantageous to use a shorter column (1 m) with a packing containing a larger amount of stationary phase (10% NPGS); more symmetrical peaks are obtained than when packings with a low concentration of the phase are employed (3% NPGS). Under the operating conditions the peaks of furoylglycine methylester (relative elution time 0·35, phenacetine = 1·0) and the internal standard are well separated from the peaks of other urine components so that the measurement of their parameters presents no difficulty. The dependence between the height of the chromatographic peaks (or the ratio of the peaks of methyl ester and internal standard) and concentration of furoylglycine is not strictly linear within the whole range of concentrations. Therefore it is recommended to determine 5 points at least.

Preceding authors[1] observed that in the urine of animals intoxicated with fural an addition compound of furoylglycine with urea may occur, and under certain conditions also a considerable amount of free pyromucic acid. We investigated how these compounds would appear on chromatographic records. The addition compound of furoylglycine with urea (the compound was obtained by crystallization of equimolar amounts of the components from a mixture of anhydrous ethanol and benzene; it melted at 120°C) was extracted from the aqueous solution with the same yield, and after esterification it gave a peak with the same elution time as furoylglycine. This fact indicates that the addition compound decomposes in aqueous solutions to its components. The determination of furoylglycine is in no way affected by the presence of urea. In contrast to this free pyromucic acid escaped determination under the given working conditions; its methyl ester has a relatively short elution time and the respective peak is overlapped by a large peak of the solvent used. This fact is rather disadvantageous for practice. The reason for this follows from the subsequent text.

Determination of Total Pyromucic Acid

The basis of the method is hydrolysis of furoylglycine to pyromucic acid which is then extracted, esterified and chromatographed. The time course of the hydrolysis of furoylglycine at various basicities of the reaction mixture is shown in Fig. 1. From the

curves it is evident that after a certain time of heating with sodium hydroxide the amount of pyromucic acid attains a maximum value which does not change on prolongation of the reaction time. At low basicity of the mixture the required heating time is relatively long, but with increasing sodium hydroxide concentration it is distinctly shortened. As operating condition a concentration of 25% w/v of sodium hydroxide in the reaction mixture was selected and the required heating time (5 minutes) was prolonged to the safe 20 minutes. The quantitative reaction course was checked by working up various weights of furoylglycine; in all instances furoylglycine disappeared from the solution and an equivalent amount of pyromucic acid was found. The hydrolysis of the addition compound of furoylglycine with urea takes place equally easily.

The extraction of the pyromucic acid from the acidified and with ammonium sulfate saturated reaction mixture is almost quantitative (97%) and the conversion of the acid to its methyl ester takes place equally smoothly as in the case of benzoic acid[10]. The chromatographic peak (rel. elution time 0·70, acetophenone = 1·0) is perfectly symmetric and well resolved from the peaks of other urine components. The advantage of the method consists in the relatively low operating temperature, strictly linear calibration curve and the fact that for standardization the better accessible pyromucic acid may be used instead of furoylglycine.

Extent of the Method and Reproducibility of Results

The extent of both methods is chosen so that it may cover the concentration range of both metabolites which may occur in practice in the analysis of urines of individuals exposed to fural vapours in industrial conditions. At higher concentrations (for example in the analysis of urines of experimentally intoxicated animals) when

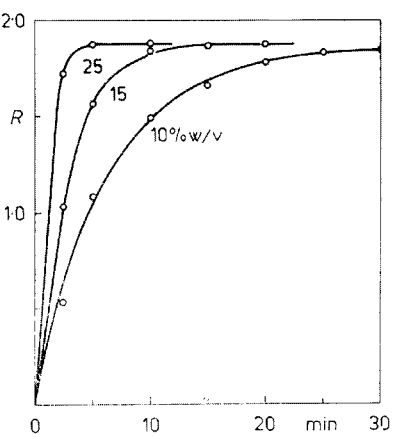

FIG. 1

Course of Hydrolysis of Furoylglycine during Heating (100°C) of the Reaction Mixtures of Various Basicity

R is the ratio of the heights of the chromatographic peaks of the methyl ester of pyromucic acid and of the internal standard. Concentration of sodium hydroxide in the reaction mixture 10, 15 and 25% w/v.

the mutual ratio of the peak heights of the methyl ester and the internal standard would be excessively high, the dilution of the urine with a known volume of water is recommended. In contrast to this, when normal levels of pyromucic acid in human urine are followed, the ratio of the heights of the respective peaks would be too small. It was found suitable to take a larger volume of the sample and of the reagents (5 ml each) and to use 2 ml of ethyl acetate solution of lower concentration of internal standard (50 μg of acetophenone/ml); in this case, of course, it is necessary to construct the calibration curve in the same manner.

The reproducibility of both methods was checked by a 20-times repeated urine analysis of a person exposed to a defined concentration of fural for 8 hours. Statistical evaluation of the furoylglycine determination gave a variation coefficient $V = 1{\cdot}34\%$, while for the total pyromucic acid determination the variation coefficient was $V = 0{\cdot}67\%$. Chromatograms obtained in the analysis of urines of exposed individuals are shown as examples in Figs 2 and 3.

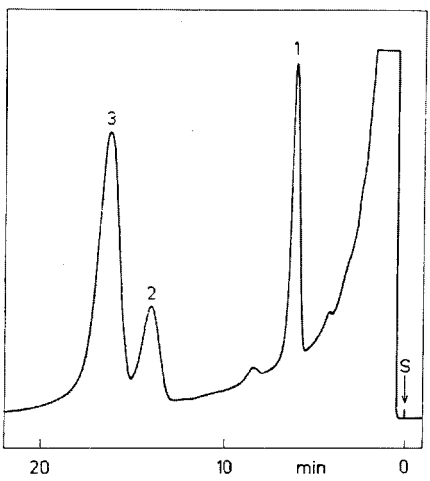

FIG. 2

Chromatogram of the Urine of a Person Exposed to Fural Vapours (Determination of Furoylglycine)

S Start of the analysis, 1 methyl ester of furoylglycine, 2 methyl ester of hippuric acid, 3 internal standard (phenacetine).

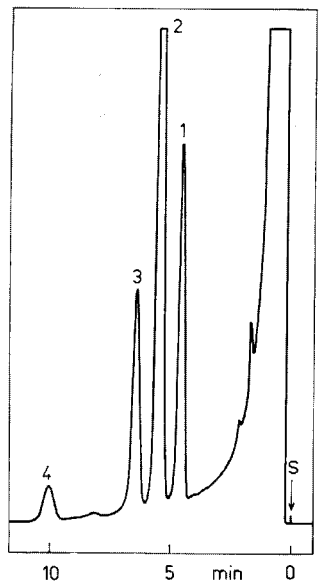

FIG. 3

Chromatogram of the Urine of a Person Exposed to Fural Vapours (Determination of Total Pyromucic Acid)

S Start of the analysis, 1 methyl ester of pyromucic acid, 2 methyl ester of benzoic acid, 3 internal standard (acetophenone), 4 methyl ester of phenylacetic acid.

Practical Applications

For the purposes of practice (*i.e.* for evaluation of the level of exposure according to the amount of the metabolites excreted in urine) the method of determination of total pyromucic acid was found more convenient. The reason is not only the already mentioned advantages of the operating procedure, but mainly the fact that the analysis comprizes both furoylglycine and the free pyromucic acid. Although it was shown[15] that in freshly excreted urine of persons exposed to the maximum allowable fural vapours concentration (Soviet norm 10 mg/m^3, American norm 20 mg/m^3) free pyromucic acid is present in trace amounts only, it is still unknown whether at higher exposures (for example in cases of accidents) the excreted amount would not increase considerably.

Free pyromucic acid appears further in the urine of exposed persons after the samples have been stored at room temperature for several days. An enzymatic or microbial splitting of furoylglycine takes place and its concentration decreases with time, while the concentration of free pyromucic acid increases. If the changes in the composition of the samples cannot be prevented by storage at low temperature (for example during the mailing of the samples), the determination of furoylglycine may lead to considerably distorted conclusions. In contrast to this the determination of total pyromucic acid affords even after a prolonged storage of samples (up to 14 days) results which agree with those carried out with fresh urines.

Both methods were used for the study of the kinetics of the excretion of the metabolites in persons exposed experimentally to fural vapours. The details and the results of this study will be published elsewhere[15].

REFERENCES

1. Jaffé M., Cohn R.: Ber. Deut. Chem. Ges. *20*, 2311 (1887).
2. Schempp E.: Hoppe-Seyler's Z. Physiol. Chem. *117*, 41 (1921).
3. Paul H. E., Austin F. L., Paul N. F., Ells V. R.: J. Biol. Chem. *180*, 345 (1949).
4. Armstrong M. D., Shaw K. N. F., Wall P. E.: J. Biol. Chem. *218*, 293 (1956).
5. Armstrong M. D., Shaw K. N. F., Gortatowski M. J., Singer H.: J. Biol. Chem. *232*, 17 (1958).
6. Castille M. A., Ruppol E.: Bull. Soc. Chim. Biol. *10*, 623 (1928).
7. Lang L.: *Absorption Spectra in the Ultraviolet and Visible Region*, Vol. 1—17. Akadémiai Kiadó, Budapest 1959—1972.
8. Kemka R., Klučík I.: Pracovní Lékařství *14*, 331 (1962).
9. Šedivec V., Flek J.: This Journal *35*, 3265 (1970).
10. Flek J., Šedivec V.: This Journal *36*, 3108 (1971).
11. Vogel A. I.: *Practical Organic Chemistry*, p. 792. Woolwich Polytechnik, London 1950.
12. Baum E.: Ber. Deut. Chem. Ges. *37*, 2949 (1904).
13. Sasaki T.: Biochem. Z. *25*, 272 (1910).
14. Arndt F., Noller C. R., Liebermann S.: *Organic Synthesis*, Coll. Vol. II, p. 461. Wiley, New York 1946.
15. Šedivec V., Flek J.: Pracovní Lékařství, in press.

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