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Note

Thin-layer chromatographic detection of some N-acylglycine conjugates in urine

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In a number of organic acidurias, an abnormal excretion of N-acylglycine conjugates of one of the volatile fatty acids is found. Tanaka and Isselbacher[1] were the first to detect an abnormal compound in the urine from a patient suffering from isovaleric acidemia, which was identified as N-isovalerylglycine. Other abnormal N-acylglycines were detected in propionic acidemia[2,3], β-methylcrotonylglycinuria[4,5] and in a recently described disorder of isoleucine catabolism[6].

Ando and Nyhan[7] described a simple thin-layer chromatographic (TLC) method for the detection of N-isovalerylglycine in urine extracts. This system, however, gives only a poor separation of the N-acylglycine conjugates that may be found in pathological aciduria. In this paper, we describe a chromatographic system for the separation of the glycine derivatives which are of clinical interest and which can be used for screening those conditions characterized by a pathological excretion of N-isovaleryl-, N-β-methylcrotonyl- and N-tiglylglycine.

MATERIALS

Pre-coated Polyamid-6-UV_{254} (Polygram) sheets (0.1 mm thickness) were purchased from Machery, Nagel & Co. (Düren, G.F.R.).

Cellulose MN 300 plates were prepared by suspending 15 g of cellulose MN300 (Machery, Nagel & Co.) and 100 ml of distilled water in a Waring blender. The plates (0.25 mm thickness) were dried at room temperature.

All solvents used were obtained from Merck (Darmstadt, G.F.R.) and were of analytical-reagent grade. They were used without further purification.

METHODS

N-Isovaleryl-, N-β-methylcrotonyl-, N-tiglyl-, N-butyryl- and N-isobutyrylglycine were synthesized according to Bondi and Eisler[8] and purified by recrystallization and silicic acid column chromatography.

Propionylglycine was synthesized from propionic anhydride and glycine according to De Forrest Abbott[9].

The purity and identity of the glycine derivatives were confirmed by mass spectrometry, NMR and IR studies.

Urine samples were extracted as described by Ando and Nyhan[7]. The creatinine content of the samples was measured according to the colorimetric method of Jaffe[10].

Extraction procedure

Samples of 1–2 ml were acidified to pH 1–2 with 0.2 volume of 5 N hydrochloric acid, followed by extraction with 5 volumes of chloroform–*n*-butanol (5:1). After centrifugation to separate the phases, 2 ml of the organic phase were evaporated under a stream of nitrogen with slight heating. The residue was dissolved in 0.2 ml of the chloroform–*n*-butanol mixture and an amount equivalent to 15–20 μg of creatinine was spotted on to the plate (usually 10 μl).

Thin-layer chromatography

With Polyamid plates, a mixture of diethyl ether, light petroleum (b.p. 40–60°) carbon tetrachloride, water and formic acid (50:20:20:8:1) was used as the mobile phase. The plates were developed until the front had ascended about 15 cm, and after development they were dried at 105° for 1 h. The spots were detected by spraying the plates with 0.1 % bromocresol purple in ethanol.

Cellulose MN 300 plates were developed with ethanol —33 % ammonia— water (100:16:12) as the mobile phase. After drying, the acids were detected by spraying the plates with aniline–xylose reagent (1 % of each in methanol), which were then dried at room temperature followed by heating for 30 min at 105° for optimal color development (brown spots on a white background).

RESULTS AND DISCUSSION

When a mixture containing the four derivatives of clinical interest and hippuric acid (benzoylglycine), which is normally excreted in variable amounts, were chromatographed on Polyamid thin-layer sheets three distinct spots were obtained. The R_F values obtained are given in Table I.

As the N-acylglycine conjugates are not commercially available, hippurate can be used as a reference compound. As can be seen from Table I, the glycine conjugates are not completely separated. β-Methylcrotonylglycine and tiglylglycine, as well as hippuric acid and propionylglycine, were found to coincide. With a number of other solvent systems used, it was not possible to obtain a satisfactory separation between these two isomers. However, as discussed below, the separation obtained between the glycine conjugates permits a differentiation between the various conditions associated with an abnormal N-acylglycinuria.

The values reported in the literature on the daily excretion of glycine conjugates range from 500–1500 mg in isovaleric acidaemia[1] to 200–300 mg in β-methylcrotonylglycinuria[4]. Gompertz and Draffan[5] reported excretion of tiglylglycine in β-methylcrotonylglycinuria ranging from 100 to 200 mg per 24 h. In propionic acidaemia, Rasmussen *et al.*[2] found in the urine of all of the patients studied small amounts of propionylglycine (1–10 mg per 24 h). He also found a tiglylglycine excretion in two of these patients, ranging from about 10 to 60 mg per 24 h. The minimal amount that can be detected with bromocresol purple as the detection reagent is about 10 μg, and

TABLE I

R_F VALUES OF SOME ORGANIC ACIDS AND GLYCINE CONJUGATES ON POLYAMID-6 AND CELLULOSE MN 300 THIN-LAYER PLATES

Solvent systems: Cellulose MN 300, ethanol–33% ammonia–water (100:16:12); Polyamid-6, diethyl ether–light petroleum (b.p. 40–60°)–carbon tetrachloride–formic acid–water (50:20:20:8:1).

Compound	*Polyamid-6*	*Cellulose MN 300*
Isovalerylglycine	0.52	0.77
β-Methylcrotonylglycine	0.43	0.72
Tiglylglycine	0.43	0.71
Propionylglycine	0.32	0.63
Hippuric acid	0.32	0.69
β-Hydroxybutyric acid	0.52	0.66
Adipic acid	0.55	0.37
Glutaric acid	0.47	0.32
Succinic acid	0.38	0.27
Fumaric acid	0.49	0.30
Methylmalonic acid	0.43	0.22
Pyruvic acid	0.50	0.31

the minimal concentration, with respect to one of the glycine conjugates, that can be detected using the extraction procedure described is about 0.5 mg/ml. Although the concentration of glycine conjugates in urine in one of the conditions mentioned above depends on the diet, urine volume, etc., the sensitivity of the method should be sufficient for the detection of abnormal glycine conjugates in isovaleric acidaemia and β-methylcrotonylglycinuria. The excretion of glycine conjugates in propionic acidaemia is too low to be detected with this procedure. However, in those cases where excretion of tiglylglycine does take place, a tiglylglycine spot may be found, depending on the amount excreted.

A number of urine samples from normal children and newborns were extracted and chromatographed following the procedure described above. In the chromatograms obtained from the samples of newborns no spots could be seen, while one spot was observed in most chromatograms obtained when about 10 μl of the urine extract of samples from older children were spotted. The latter spot had the same R_F value as authentic hippurate. Other pathologic conditions may, however, be associated with an abnormal excretion of some organic acids that may be present in detectable amounts in the urine extract. A number of organic acids were found to interfere in the detection of glycine conjugates on Polyamid thin-layer sheets.

The use of a second TLC system, Cellulose MN 300 thin-layer plates with ethanol–33%ammonia–water (100:16:12) as the mobile phase, permits the separation of the glycine conjugates from the interfering acids. This system was described by Higgins and Von Brand[11] for the separation of lactic acid and some acids of the tricarboxylic acid cycle. The R_F values of these interfering acids and the glycine conjugates on Polyamid and Cellulose MN 300 thin-layer plates are given in Table I. Definitive identification can, however, only be made after using other techniques, *e.g.*, GLC and mass and NMR spectrometry.

A combination of the two TLC systems will provide a powerful tool for detecting abnormal glycine conjugates in organic acidurias.

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