Determination of Ornithine Conjugates of Some Carboxylic Acids in Birds by High-Performance Liquid Chromatography

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A high-performance liquid chromatographic (HPLC) method for the determination of ornithine conjugation of some carboxylic acids *in vitro* has been developed. The ornithine conjugates of benzoic acid, *p*-nitrobenzoic acid, furancarboxylic acid and phenylacetic acid in an incubation mixture with kidney mitochondria were well separated on a reversed-phase C18 column using a mixture of 10 mM ammonium acetate buffer and methanol as the mobile phase. In addition, by varying the pH of the mobile phase and utilizing the absorption wavelengths (nm) of the conjugates it was possible to resolve and specifically detect each conjugate. The calibration curves were linear in the range of 0.2—16 μg/ml for all compounds and the detection limits were about 50 ng/ml except for the ornithine conjugate of phenyl acetic acid ($S/N=2$). The ornithine conjugation of some carboxylic acids with chicken kidney mitochondria were determined by this assay method. The activity of ornithine conjugation of benzoic acid, furancarboxylic acid, *p*-nitrobenzoic acid and phenylacetic acid were 14.5, 5.5, 0.5 and 6.9 nmol/mg of protein, respectively. Moreover, the ornithine conjugation and the glycine conjugation of benzoic acid were examined in birds and rodents. The ornithine conjugation was observed only in chicken (14.5 nmol/mg of protein) and mallard (0.99 nmol/mg of protein).

Keywords carboxylic acid; ornithine conjugation; glycine conjugation; HPLC; kidney mitochondria; birds; rodents; metabolism

Introduction

A wide range of carboxylic acids in drugs, herbicides and pesticides can undergo an amino acid conjugation. The nature of the amino acid utilized for the conjugation reaction varies markedly according to the animal species and the chemical structure of the carboxylic acid. In general, the amino acids encountered in the conjugation reaction are glycine, glutamine, ornithine and taurine. Glycine is most commonly encountered and is utilized by a wide range of species for the conjugation of carboxylic acid. Taurine conjugation is also relatively widespread in its species occurrence, but is restricted in terms of acids undergoing this conjugation to arylacetic and cholic acid derivatives.[1-4] There have been only a few reports on ornithine conjugation and they indicate that ornithine conjugation is restricted in the species occurrence.[5-7] To date it has been found only in some birds and reptilian species but has not been reported in mammalian species. Thus the ornithine conjugation has not been investigated in detail, as it is a specific pathway for carboxylic acids.

In the present paper, we describe the quantitative determination of ornithine conjugates of some carboxylic acids using the high-performance liquid chromatographic (HPLC) method. Moreover, we examined the ornithine conjugation of carboxylic acid in birds and rodents *in vitro*.

Experimental

Chemicals and Reagents Benzoic acid, *p*-nitrobenzoic acid, 2-furancarboxylic acid and phenylacetic acid were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). *m*-Chlorobenzoic acid, *p*-nitrophenol and *p*-hydroxybenzoic acid, used as an internal standard (I.S.), were also obtained from Nacalai Tesque, Inc. Methanol of HPLC grade, ethylenediaminetetraacetic acid (EDTA), 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES), hippuric acid, L-(+)-ornithine hydrochloride and glycine were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Adenosine triphosphate (ATP) was obtained from Kojin Reagent Co., Ltd. (Tokyo, Japan). All other chemicals were of reagent grade.

Synthesis of Ornithine Conjugates Di(benzoyl)-, di(*p*-nitrobenzoyl)-, di(furancarboxyl)- and di(phenylacetyl)-L-(+)-ornithine were prepared by a modification of the method of Neuberger and Sanger.[8] The acyl chloride (6 mmol) in benzene was added dropwise to a stirred solution of ornithine (6 mmol) in 2 M NaOH. The mixture was stirred for 30 min at room temperature. The aqueous layer was separated and acidified with 2 M HCl (pH 2—3) followed by washing with diethyl ether in order to remove the unreacted material. The aqueous layer was concentrated *in vacuo* at 35 °C. The precipitated solid was filtered and recrystallized from aqueous ethanol. The resulting white crystals of the conjugates described above gave the following sharp melting points: di(benzoyl)-ornithine, 180—181 °C; di(*p*-nitrobenzoyl)-, 100—101 °C; di(furancarboxyl)-, 130—132 °C; and di(phenylacetyl)-, 139—140 °C. In addition, a molecular ion of each conjugate was confirmed by gas chromatography–electron impact-mass spectrometry (GC-EIMS)(Hitachi M-60 system, GC column: 2% OV-101) as its methyl ester: di(benzoyl)-ornithine methyl ester at *m/z* 354 (0.4%), di(*p*-nitrobenzoyl)- at *m/z* 444 (0.3%), di(furancarboxyl)- at *m/z* 334 (20%), di(phenylacetyl)- at *m/z* 382 (40%).

Instruments and Analytical Conditions The HPLC system consisted of a Shimadzu model LC-3A constant flow pump and a Shimadzu model SPD-2A ultraviolet (UV) detector. A Cosmosil 5C18 column (particle size 5 μm, 4.6 mm × 150 mm, Nacalai Tesque, Inc.) was used. The mobile phase consisted of 10 mM ammonium acetate buffer and methanol (25—50%). The pH of the mobile phase and the wavelength (nm) of the detector were modified for the determination of each compound. The mobile phase was passed through the column at a flow-rate of 0.8 ml/min.

Analytical Procedure for Assay To 1 ml of incubation mixture were added 0.05 ml of 6 M HCl, 0.1 ml of the appropriate I.S. solution (30—200 μg/ml) (*m*-chlorobenzoic acid for benzoic acid, *p*-nitrophenol for *p*-nitrobenzoic acid and phenylacetic acid, *p*-hydroxybenzoic acid for furancarboxylic acid) and 2 ml of ethyl acetate. The mixture was shaken for 1 min and centrifuged for 5 min at 600 × *g*. After the centrifugation, 1 ml of the organic supernatant was transferred to another tube and evaporated to dryness in the presence of conc. NH_4OH (20 μl). The residues were redissolved in 0.1 ml of methanol, and a 10 μl of sample was injected onto the HPLC column.

***In Vitro* Ornithine Conjugation** The birds used were chicken (male, 2.4—3.4 kg b.w.), mallard (male, 2 kg b.w.), Japanese quail (male, 100—150 g b.w.), eudgerigar (male, 25—30 g b.w.) and bengalee (male, 15—20 g b.w.) obtained from a poultry farm and a pet shop. The rodents used were male ddY mice weighing 20—24 g and male Wistar rats weighing 180—200 g. The ornithine conjugation of some carboxylic acids was carried out by using the mitochondria fraction from selected bird and rodent kidneys (the ornithine conjugation was not found in liver). The mitochondria were prepared by the method of Johnson and Lardy.[9] The isolation medium contained 250 mM sucrose, 5 mM HEPES and 0.1 mM EDTA at pH 7.6. Protein was measured by the procedure of Lowry *et al.*[10] using bovine serum albumin as a standard. The incubation mixture contained the following in a final volume of 2.0 ml: 2.5 mM $MgSO_4$, 2 mM

ornithine, 7 mM ATP, 0.1 mM carboxylic acids, 100 mM Tris–HCl buffer, pH 7.4, and mitochondria fraction. The reaction was done in a water-bath at 38 °C for 50 min. Under these conditions, the activity increased linearly until 60 min of incubation time.

When benzoic acid was used as substrate, the glycine conjugation was also determined by using the mitochondria fraction. The incubation mixture consisted of the same components as described above except for 2 mM glycine (instead of 2 mM ornithine).

Results and Discussion

Several mobile phases were examined in order to obtain the complete separation of the carboxylic acids and their ornithine conjugates. When a mixture of 10 mM ammonium acetate buffer and methanol was used as the mobile phase, each carboxylic acid and its ornithine conjugate was separated satisfactorily from the endogenous peaks of the incubation mixtures. Optimizing the pH of the mobile phase resulted in sharper peaks and shorter retention times. Thus, for quantitative determination of the various carboxylic acids and their ornithine conjugates we chose four mobile phases and wavelength as follows: (a) and (b) 40% (v/v) methanol and 10 mM ammonium acetate buffer, pH 5.5, and detector 225 nm for benzoic acid, ornithuric acid and hippuric acid, (c) 50% (v/v) methanol and 10 mM ammonium acetate buffer, pH 4.5, and detector 270 nm for *p*-nitrobenzoic acid and di(*p*-nitrobenzoyl)ornithine, (d) 25% (v/v) methanol and 10 mM ammonium acetate buffer, pH 3.0, and detector 254 nm for furancarboxylic acid and di(furancarboxy)ornithine and (e) 50% (v/v) methanol and 10 mM ammonium acetate buffer, pH 4.5, and detector 257 nm for phenylacetic acid and di(phenylacetyl)ornithine. In the mobile phase for benzoic acid and its ornithine conjugate (ornithuric acid), the glycine conjugate (hippuric acid) of benzoic acid was also well separated on the HPLC chromatogram with a retention time of 3.7 min; benzoic acid, 4.2 min; ornithuric acid, 13.0 min.

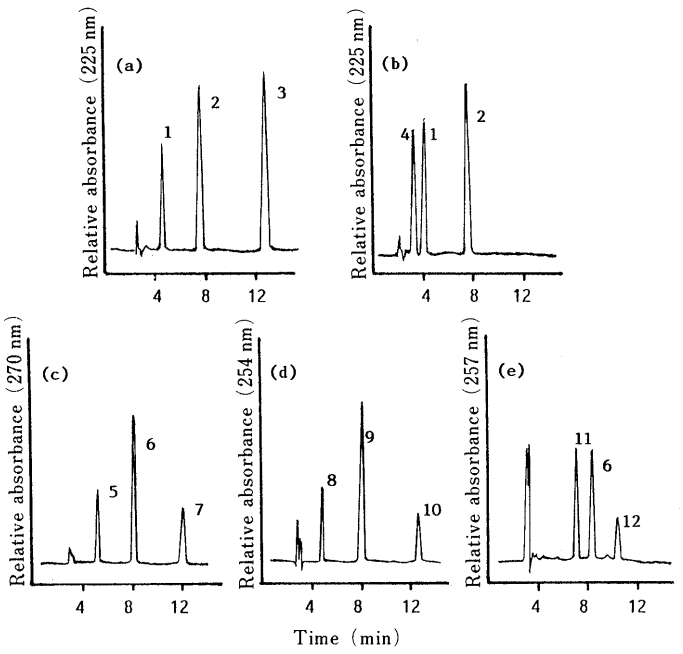

Fig. 1. Typical Chromatograms for Extracts from Incubation Mixture of Benzoic Acid and Ornithine (a) and Glycine (b), *p*-Nitrobenzoic Acid (c), Furancarboxylic Acid (d) and Phenylacetic Acid (e) and Ornithine with Chicken Kidney Mitochondria Fraction

The analytical conditions are described in the text. Peak 1: benzoic acid, 2: *m*-chlorobenzoic acid (I.S.), 3: ornithuric acid, 4: hippuric acid, 5: *p*-nitrobenzoic acid, 6: *p*-nitrophenol (I.S.), 7: di(*p*-nitrobenzoyl)ornithine, 8: furancarboxylic acid, 9: *p*-hydroxybenzoic acid (I.S.), 10: di(furancarboxyl)ornithine, 11: phenylacetic acid, 12: di(phenylacetyl)ornithine.

Typical chromatograms of the extracts from the incubation mixture are shown in Figs. 1 and 2. No peaks were observed which interfered with the detection of the carboxylic acid or their conjugates. The present method allowed us to detect each carboxylic acid and its conjugate in the incubation mixture at a concentration as low as 50 ng/ml except for phenylacetic acid and its conjugate (about 200 ng/ml) ($S/N=2$). The calibration curves for each carboxylic acid and its ornithine conjugate were prepared by adding known amounts of standard compounds to the incubation mixture, and were obtained by plotting the peak–height ratio of caboxylic acid or its conjugate to I.S. against the concentrations of carboxylic acid or its conjugate. They were linear over the concentration range studied (0.5—16 μg/ml). The correlation coefficients were better than 0.998 in all instances. The calibration curves showed little day-to-day variability in slopes and intercepts (the coefficient of variation, CV, below 5%). The recoveries were calculated by comparing the peak heights of the carboxylic acids and their ornithine conjugates extracted from incubation mixture samples with those of reference standard solutions. The mean recoveries from the samples spiked with a known concentration (3 μg/ml) of carboxylic acids and their conjugates were 91.5%—95.8% ($n=5$). In addition, the CV values ranged from 3.9% to 6.3% ($n=5$).

Using this method, we determined the ornithine conjugation of some carboxylic acids using the kidney mitochondria of selected birds and rodents *in vitro*. In Table I the formation of ornithine conjugate of carboxylic acids with chicken kidney mitochondria is presented. The formation of ornithine conjugate was observed for all four

Table I. Activity of Ornithine Conjugation for Some Acids

Acid	Ornithine conjugation (nmol/mg of protein)
Benzoic acid	14.52±0.75
Furancarboxylic acid	5.52±0.90
p-Nitrobenzoic acid	0.53±0.81
Phenylacetic acid	6.94±1.10

The reaction mixture (2 ml) containing 0.1 mM acid, 2.5 mM magnesium sulfate, 2 mM ornithine, 7 mM ATP and chicken mitochondria was incubated in 100 mM Tris–HCl buffer, pH 7.4 at 38 °C for 50 min. Each value represents the mean±S.E. from three samples.

Table II. Activity of Ornithine Conjugation and Glycine Conjugation for Benzoic Acid of Selected Birds and Rodents

Species	Ornithine conjugation	Glycine conjugation
Eudgerigar	N.D.	N.D.
Bengalee	N.D.	N.D.
Japanese quail	N.D.	N.D.
Mallard	0.99±0.2	N.D.
Chicken	14.52±0.75	N.D.
Mice (ddY)	N.D.	6.30±0.43
Rat (Wistar)	N.D.	6.71±0.95

Activity was displayed at nmol of ornithuric acid or hippuric acid/mg of protein. Each value represents the mean±S.E. from three samples. N.D.: not detectable.

carboxylic acids used as substrate. Of four carboxylic acids, benzoic acid yielded the highest degree of ornithine conjugation (14.5 nmol/mg of protein). *p*-Nitrobenzoic acid revealed the lowest degree (0.53 nmol/mg of protein). As glycine conjugation of carboxylic acid,[11] it was suggested that the extent of ornithine conjugation was chemical structure-dependent. Table II shows the formation of ornithuric acid and hippuric acid from benzoic acid using kidney mitochondria from various birds and rodents. We compared the ornithine conjugation of benzoic acid among the selected birds and rodents with the glycine conjugation. Ornithine conjugation was observed only in chicken (14.5 nmol/mg of protein) and mallard (0.99 nmol/mg of protein) and in the other species an ornithine conjugate was not detected. In addition to chicken and mallard, the ornithine conjugation of benzoic acid has been observed in turkey and goose[12,13] although we did not use these. We observed the glycine conjugation of benzoic acid only in rodents (mouse: 6.3 nmol/mg of protein, rat: 6.7 nmol/mg of protein). This result agreed with that in which the glycine conjugation was found in all mammals and the pigeon family.[12]

In conclusion, we have developed a simple, sensitive and specific method to examine the formation of the ornithine conjugate as a detoxification pathway of certain carboxylic acids. We determined the ornithine conjugation of four carboxylic acid in birds and rodents *in vitro*. As a result, we concluded that the ornithine conjugate of carboxylic acid in rodents was not present.

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