A Novel Method To Analyze Betaine in Chicken Liver: Effect of Dietary Betaine and Choline Supplementation on the Hepatic Betaine Concentration in Broiler Chicks

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Betaine was measured from liver tissue by a high-performance liquid chromatographic (HPLC) method developed for this study. The method involves homogenization of liver in acetate buffer at pH 6 and precipitation of protein with trichloroacetic acid, which was removed by diethyl ether extraction. Betaine was separated using a cation exchange column in Ca^{2+} form and detected with a refractive index detector. This method also allows the determination of *S*-adenosylmethionine (S-AM) from the same liver extract but with different HPLC conditions. Broiler chicks were fed with experimental diets supplemented with four different doses of betaine or choline ranging from 0 to 5 mol equiv. After a 3 week feeding period, the livers were analyzed for betaine and S-AM. Dietary betaine was twice as efficient in increasing the hepatic betaine concentration as dietary choline. The hepatic S-AM concentrations were similar in all dietary treatments.

Keywords: *Betaine; choline; S-adenosylmethionine; chicken; liver; HPLC*

INTRODUCTION

Betaine (glycine betaine, trimethylglycine) is a naturally occurring, nontoxic amino acid derivative that is commonly found in a large number of plant and animal species. It functions as a dietary source of methyl groups and also helps in cell volume regulation under osmotic stress (*1*−*6*). Choline is a structural component of cell membrane phospholipids and is also an indispensable precursor of neurotransmitter acetylcholine.

In farm animal diets, methionine, choline, and betaine are the most important sources of methyl groups. Methyl groups are essential in the diet as vertebrates lack the ability to synthesize these groups (*6*). Methyl groups from *S*-adenosylmethionine (S-AM) are used in numerous metabolic reactions, for example, the methylation of nucleic acids, the biosynthesis of creatine, carnitine, and choline, and many functions of the immune system (*7*, *8*). Dietary methyl group donors are used to ensure a ready supply of S-AM, which is the primary methyl group donor used within the body. S-AM is formed from methionine and adenosine triphosphate (*8*). The demethylated product of S-AM is *S*-adenosylhomocysteine, which is further cleaved to adenosine and homocysteine. Betaine donates one methyl group to homocysteine via activity of betaine-homocysteine methyltransferase that results in the regeneration of methionine. This pathway is called the transmethylation cycle and has been reviewed by Finkelstein (*9*) and Kidd et al. (*6*). The transmethylation reaction allows the body to conserve methionine and to minimize the concentration of homocysteine in tissues as elevated levels of this amino acid have been linked to the occurrence of several diseases (*10*−*14*). Betaine has been shown to alleviate the growth depression caused by excessive levels of homocysteine in chicken (*15*). The methyl groups of choline become available for transmethylation only after oxidation of choline to betaine in a two-step reaction that occurs in the mitochondria of liver cells (*6*, *8*). Betaine found in the broiler chick tissues is a sum of betaine originated from the diet and that converted from choline.

In the present trial betaine was analyzed from the liver because it is the site of the conversion of betaine from choline. S-AM was analyzed because the dietary methyl group sources might affect the hepatic S-AM levels. Traditional methods for measuring betaine and S-AM require separate tissue preparations, so the same tissue cannot be used for both assays conveniently. Also, not all assay components are readily available. The method of Barak and Tuma (*16*) involves a reaction with potassium periodide to form betaine periodide for subsequent spectrophotometric determination. Another method is based on the isotope dilution principle and requires addition of betaine-homocysteine methyltransferase prior to measurement using gas chromatographic−mass spectrometry (*17*). However, this methyltransferase is not commercially available and must be separated from liver prior to the assay. Another assay using a reaction with 4′-bromophenacyl triflate has also been used to form an ultraviolet-absorbing derivative of betaine that is then run using high-performance liquid chromatography (HPLC) separation (*18*, *19*). The benefits of this method are high sensitivity and application to various sample types. Unfortunately, 4′-bro-

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mophenacyl triflate is not commercially available. Thus, the objective of this work was to develop a method of measuring both betaine and S-AM using the same liver tissue and commercially available materials.

In the subsequent feeding trial with broiler chicks, the impact of dietary betaine and choline on the hepatic concentration of betaine and S-AM was measured. Betaine and choline were added at four different levels to a practical diet containing sufficient native choline to support phospholipid and acetylcholine synthesis (*20*). The reason to add choline and betaine to a diet already adequate in choline was that the need for methyl groups may exceed the level needed to achieve an optimal growth rate. In the trials of Swain and Johri (*21*), supplemental methionine and choline improved the immune response of healthy broiler chicks, and they suggested that methionine and choline levels in the diets of broiler chicks should be increased beyond the recommended level for better health and production. The methyl group reserves, for example, the hepatic betaine stores, may thus help the birds to tolerate environmental or physiological stress. Dietary betaine supplementation has been reported to have beneficial effects on poultry, especially for coccidiosis-infected chicks (*22*−*25*) and for turkeys with a flushing syndrome (*26*).

It is highly likely that when choline is added to a diet already adequate in choline, only a part of the surplus choline is converted into betaine. The aim of the present trial was to estimate how much more choline chloride will have to be added to the diet of broiler chicks to achieve the same rise in the liver betaine concentration as when the diet is supplemented with pure betaine. We also wanted to study the effect of the betaine or choline supplementation on the liver concentration of S-AM and the possible correlation between the hepatic concentrations of betaine and S-AM.

MATERIALS AND METHODS

Reagents and Standards. All chemicals were of analytical grade. Betaine monohydrate was obtained from Fluka (Buchs, Switzerland) and *S*-adenosyl-L-methionine *p*-toluenesulfonate salt from Sigma (St. Louis, MO).

HPLC Analysis. The HPLC system consisted of a Merck 655A-12 liquid chromatograph pump (Darmstadt, Germany), a Merck L-5000 LC controller, a Waters 717 plus autosampler (Milford, MA), and a Hewlett-Packard 1047A RI detector (Palo Alto, CA). Turbochrom software (Perkin-Elmer) was used for data acquisition.

Betaine separation was performed using a cation exchange column of Ca^{2+} type (300 × 7.8 mm, Aminex HPX-87C, Bio-Rad, Hercules, CA). The column temperature was 85 °C. A 2 μm frit and C18 Bondapak precolumn (Guard-Pak, Waters, Milford, MA) were placed before the analytical column. The mobile phase was 0.004 M $Ca(NO_3)_2$, and the flow rate was 0.6 mL/min. The injection volume was 50 μL. The run time was 45 min. Similar chromatographic conditions have been used to analyze betaine in molasses (*27*).

S-AM was analyzed using a modification of the method of Wise and Fullerton (*28*). The HPLC column was a Symmetry C18 (150 × 3 mm, Waters). The mobile phase consisted of 50 mM sodium acetate and 10 mM heptanesulfonate in acetonitrile/water (7:93, v/v) solution. The pH of the solution was adjusted to 4.4 with acetic acid. The flow rate was set at 0.4 mL/min, and the UV detection was at 260 nm. The calibration standard was dissolved in water and then processed in the same way as the liver samples.

Sample Preparation. The livers were stored at −20 °C until analysis could be done. One gram of liver was homogenized in 2 mL of 0.1 M sodium acetate buffer, pH 6, using a Thomas homogenizer. Protein was precipitated by adding 1.5 mL of 40% (w/v) trichloroacetic acid. The solution was mixed on vortex and then placed on ice for 30 min. The sample was then centrifuged at 5000*g* for 10 min. The supernatant was extracted twice with an equal volume of diethyl ether to remove trichloroacetic acid and to adjust the pH to ∼3. The sample was then diluted to 5 mL with water, filtered through a 0.2 μm PVDF filter, and stored at −20 °C.

Table 1. Percentage Composition of the Basal Diet in the Feeding Trial

ingredient	percentage
corn	56.9
soybean meal	40.6
dicalcium phosphate	1.8
limestone	0.8
salt	0.4
trace mineral premix[a]	0.2
vitamin premix[b]	0.2
methionine	0.17
calculated contents	
crude protein	22.0
metabolizable energy (MJ/kg)	11.6
methionine	0.53
methionine + cysteine	0.90
lysine	1.14
choline (g/kg)	1.35
calcium	0. 92
non-phytate P, %	0.43

[a] The trace mineral premix supplied the following per kilogram of diet: 0.64 g of calcium, 29.2 mg of iron, 5.1 mg of copper, 50.3 mg of magnesium, 65.1 mg of zinc, 0.5 mg of iodium, and 0.2 mg of selenium. [b] The vitamin premix supplied the following per kilogram of diet: 1.4 mg of calcium, 5.9 mg of phosphorus, 12500 IU of vitamin A, 3100 IU of vitamin D_3, 40.0 mg of vitamin E, 36.0 mg of tocopherol, 5.0 mg of phylloquinone, 3.0 mg of thiamin, 6.0 mg of riboflavin, 4.0 mg of pyridoxine, 0.03 mg of cyanocobalamin, 0.30 mg of biotin, 1.0 mg of folic acid, 40.0 mg of niacin, 15.0 mg of panthotenic acid, and 0.61 mg of antioxidants.

Betaine Calibration. Betaine calibration was accomplished according to the standard addition method. A stock solution of betaine monohydrate was prepared in water to give a final concentration of 85 μmol/mL betaine. One gram of pooled, homogenized liver sample was spiked with 0.1 mL of the betaine stock solution and/or diluted with water to give a concentration range of 400−8500 nmol/g for the five different levels. The blank sample was prepared by adding 0.1 mL of water to the pooled liver sample. The calibration samples were then prepared as described previously.

Feeding Trial with Broiler Chicks. Day-old, mixed sex Ross 208 chicks were divided into seven dietary treatments, 25 chicks per treatment. The birds were housed in battery cages, five birds per cage, in a temperature-controlled room. The pelleted corn soybean meal based diet (Table 1) was supplemented with 0, 1.7, 3.3, or 5.0 mol equiv of betaine or choline to generate seven dietary treatments. The mole equivalent additions are equal to the addition of 200, 400, or 600 g/ton of Betafin (97% betaine, Finnfeeds Finland Ltd., Espoo, Finland) and 462, 924, or 1386 g/ton of choline chloride 50% (Table 2). The unsupplemented control diet was analyzed for total choline (1.34 mg/g), total methionine (4.5 mg/g), free methionine (1.53 mg/g), and total betaine (<0.1 mg/g). The choline and betaine levels in the other treatment feeds were also analyzed, and they fell within the 5% range from the calculated contents. The chicks were weighed on days 0, 7, 14, and 21 to check that they were healthy and growing at a normal rate. Feed and water were available ad libitum. The birds were sacrificed and sampled for liver tissue on day 21 for later determination of betaine and S-AM. The animal use and care protocol was approved by the Experimental Animal Committee of the Department of Biosciences, University of Helsinki, Finland.

Statistical Methods. The effect of dietary betaine or choline in elevating the liver betaine was analyzed by multiple linear regression using a model

Table 2. Liver Betaine and S-AM Contents and Bird Weight Data of the Feeding Trial[a]

		diets													
		control		Bet 200 (1.7)[b]		Bet 400 (3.3)[b]		Bet 600 (5.0)[b]		Chol 462 (1.7)[c]		Chol 924 (3.3)[c]		Chol 1386 (5.0)[c]	
		mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
betain (nmol/g of tissue)	M[d] ($n = 7$)	512	194	644	104	1185	442	1096	284	530	210	922	235	933	430
	F[d] (n = 7)	562	262	883	303	1056	259	1197	221	711	183	969	267	840	325
	mean ($n = 14$)	537	223	763	250	1120	354	1147	250	620	211	945	243	886	369
S-AM (nmol/g of tissue)	M ($n = 7$)	42[a]	2	43[a]	5	43[a]	4	45	7	39	6	46	6	44	7
	F ($n = 7$)	52[b]	5	51[b]	4	57[b]	3	52	6	48	8	51	6	52	6
	mean ($n = 14$)	47	7	47	6	52	6	49	7	43	8	49	6	48	8
weight (g)	M ($n = 11-14$)	921	37	864	97	966	54	902	65	939	69	888	72	966	41
	F ($n = 11-14$)	815	37	868	27	860	57	838	97	836	52	822	41	812	32
	mean ($n = 25$)	868	66	866	67	913	76	870	85	887	79	855	66	889	32

[a] A different letter as a superscript indicates a statistically significant difference ($P < 0.05$) between females and males. The liver metabolites are given against tissue fresh weight. [b] Betafin (97% betaine, Finnfeeds Finland Ltd., Espoo, Finland) was added at, for example, 200 g/ton of feed (or 1.7 mol/ton of feed). [c] Choline chloride was added at, for example, 462 g/ton of feed (or 1.7 mol/ton of feed). [d] M, males; F, females.

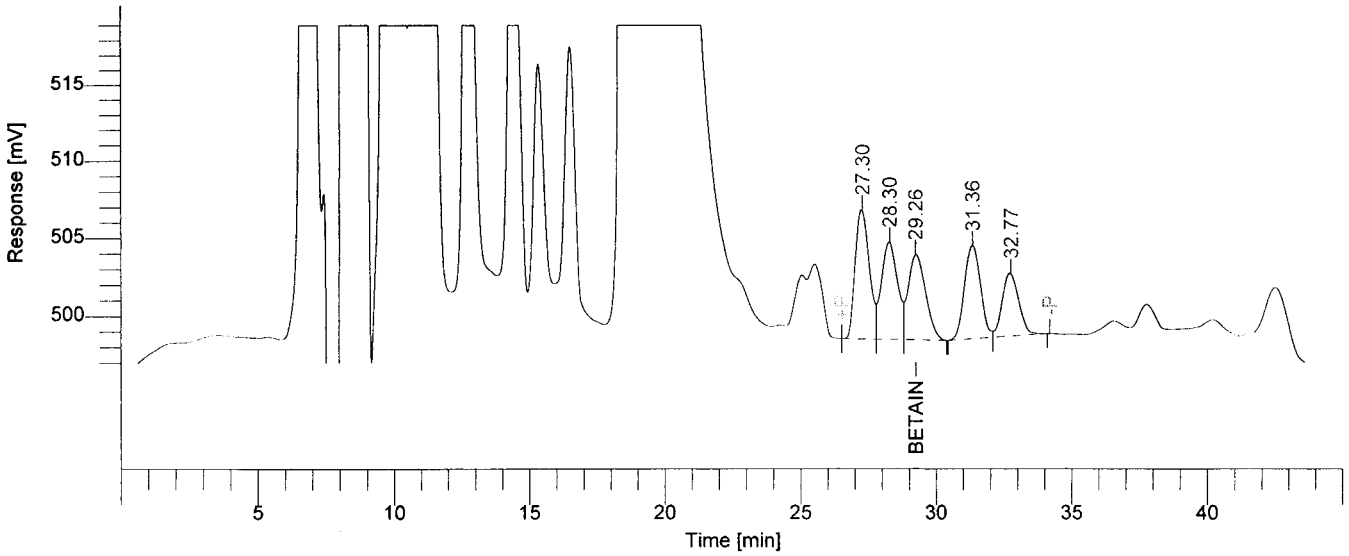

Figure 1. HPLC separation of betaine in a chicken liver sample.

$$\text{betaine in liver} = a_0 + a_1 \times \text{betaine in feed} + a_2 \times \text{choline in feed} \quad (1)$$

where a_1 and a_2 are the coefficients for the liver effect of betaine and choline and a_0 represents the background level of betaine in liver.

The relative efficacy of dietary choline to increase hepatic betaine in comparison with dietary betaine is given as the ratio of the two coefficiences:

$$(a_2/a_1) \times 100\% \quad (2)$$

The correlations between liver betaine and S-AM levels were calculated with regression analysis. The effects of dietary treatments and bird sex on the liver betaine and S-AM were analyzed with two-way ANOVA (SYSTAT 7.0).

RESULTS

Specificity and Column Stability. Figure 1 shows a typical chromatogram of a chicken liver sample analyzed for betaine. The retention time of betaine was 29.3 min. The retention times of several common amino acids and dimethylglycine, a metabolite of betaine, were also studied on the same system, and none overlapped with betaine. After many injections, however, alanine began to shift toward the betaine peak and after ~400 sample runs finally coeluted. The column could be regenerated with 0.1 M $Ca(NO_3)_2$ to solve this problem. The frit and the precolumn were replaced after 100 sample runs. The concentration of $Ca(NO_3)_2$ in the mobile phase, ranging from 0 to 0.006 M, had no effect on the retention time of betaine, but column stability was better with $Ca(NO_3)_2$ in the eluent than with pure water.

Calibration, Linearity, and Recovery. In the course of recovery experiments, we observed that the recoveries of betaine were ~80% when the calibration was done with standards prepared in water. However, the recoveries were linear ($r^2 = 0.9994$) in the concentration range of at least 400−8500 nmol/g. The calibration was thereafter carried out by spiking a pooled liver sample at five different concentrations.

Intra-assay and Inter-assay Precisions. The intra-assay precision for betaine was carried out by processing the same liver sample six times on the same day. The coefficient of variation (CV) was 5.3% for a mean value of 5323 nmol/g. The inter-assay precision for betaine was determined by processing the same liver sample on three days. The CV was 5.2% for a mean value of 5189 nmol/g ($n = 14$).

Stability of Livers. The stability of livers stored at −20 °C was studied by dividing livers from four chickens into four separate parts. One part of each liver was prepared and analyzed immediately. The three other

parts were stored at −20 °C for 1, 2, and 3 months in plastic vials. No betaine degradation was observed during the storage.

Feeding Trial. According to the regression model, the hepatic betaine concentration was increased more by dietary supplementation of betaine than by choline ($P < 0.001$). The efficiency of dietary choline to elevate the hepatic betaine concentration was on average 55% and according to the linear regression model 62% of that of the dietary betaine. The dietary treatments did not affect the hepatic S-AM concentration (two-way ANOVA). The liver betaine concentration did not differ statistically between sexes, but the S-AM concentration was higher in females than in males ($P < 0.001$, two-way ANOVA; Table 2). The dietary treatments did not affect the weight gain of the chicks. There was a weak positive correlation with the liver betaine and S-AM concentrations in female chicks ($P < 0.05$, $r^2 = 0.08$).

DISCUSSION

Betaine Assay. The sensitivity of the assay was adequate to determine the concentration of betaine in the liver samples, and all values fell within the linear range of the method. Elution of some unknown compound close to betaine (Figure 1) and coprecipitation with proteins in sample preparation probably caused betaine recoveries to be low when the quantitation was done with standards prepared in water. The losses in recovery and the effect of close-eluting compounds were corrected for by using liver as the calibration matrix. The intra-assay deviation for liver betaine (CV = 5.3%) was considerably smaller than the deviation between different individuals within the same treatment (CV = 16−42%, Table 2). The inter-assay precision (CV = 5.2%) and stability of betaine in a stored liver (at −20 °C) allow the method to be used on successive days if a large number of samples are to be analyzed.

Feeding Trial. Dietary betaine can reduce the need for dietary methionine inclusion in poultry feed due to its methyl donor role (*29*, *30*). A wealth of methyl groups in the diet of broiler chicks may enhance their immune response (*21*), a factor that can become important in areas of the world that have plans on banning or restricting the use of feed antibiotics. According to Stekol et al. (*31*), dietary betaine methylates homocysteine to methionine ∼3 times more efficiently than dietary choline in the chicken. The present study shows that dietary betaine is nearly twice as efficient as the same mole equivalent level of choline for increasing liver betaine levels in broiler chicks. The difference between the effect of choline and betaine may not have been due to the conversion efficiency only, as it is possible that the dietary choline supplementation had decreased the rate of choline absorption from the intestinal contents.

The hepatic S-AM concentration was unaffected by the dietary supplementation of betaine or choline. However, there was a weak correlation between the hepatic concentrations of S-AM and betaine in female chicks. Considering the role of betaine in the transmethylation cycle, the effect of liver betaine level on the regulation of the liver S-AM levels should perhaps also be studied in situations when the dietary availability of methyl groups is limiting, which was not the case in the present study. In research conducted with rats, betaine has been shown to be protective against liver damage associated with decreased S-AM levels caused by ethanol feeding (*32*) or by exposure to carbon tetrachloride (*33*).

The body weight data did not indicate any effect of the dietary supplements on the growth rate of the chicks, which is in agreement with the findings of Swain and Johri (*21*) with regard to growth and FCR of birds given increased levels of methionine and choline. It is likely that the number of chicks in the present trial was too small to detect possible treatment effects on growth.

In conclusion, this novel HPLC method allowed convenient analyses of both betaine and S-AM from the same tissue sample. The method could also detect differences in liver betaine in birds fed different dietary levels of choline or betaine.

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