

Plasma Dependent and Independent Accumulation of Betaine in Male and Female Rat Tissues

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Summary

Tissue betaine is an intracellular osmolyte that also provides a store of labile methyl groups. Despite these important biological roles, there are few data regarding tissue betaine content. We measured the betaine concentration of plasma and various tissues (brain, heart, lungs, liver, kidney, spleen, intestine, reproductive tissues, skeletal muscle and skin) in male and female rats and assessed whether there were any gender-specific differences in betaine content or distribution and whether there was any relationship between tissue accumulation and plasma levels. Betaine was highest in the liver and kidney with values ranging from 1.6 to 9.5 mmol/l and 2.0 to 5.4 mmol/l, respectively. Plasma betaine concentrations were significantly lower than tissue levels except in the brain ($\approx 25\%$ of plasma) and skeletal muscle (similar to plasma). Regression analysis of the combined male and female data revealed a significant plasma-related accumulation of betaine in the heart, skin and skeletal muscle, while the lung, liver, kidney, spleen, and intestine showed significant plasma-related and plasma-independent accumulations of betaine. The betaine content of the skin, liver and kidney was not significantly different between males and females, but in plasma and all tissues analyzed it was significantly higher in males ($P < 0.01$).

Key words

Betaine • Osmolyte • Methyl donor • Tissue content • Rats

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Introduction

Betaine (glycine betaine or *N,N,N*-trimethylglycine) is a bodily requirement. It is actively accumulated in many mammalian tissues where it plays a vital role as an intracellular osmolyte, regulating cell volume by countering changes in extracellular tonicity and stabilizing macromolecules against a variety of physiological perturbations (Häussinger 1996, Schliess and Häussinger 2002, Wehner *et al.* 2003). In addition, betaine is an important methyl donor in one-carbon metabolism, where betaine-homocysteine methyltransferase (BHMT; E.C. 2.1.1.5), the only known enzyme which uses betaine as a substrate, mediates the transfer of a methyl group from betaine to homocysteine, forming methionine and *N,N*-dimethylglycine (Millian and Garrow 1998, Sunden *et al.* 1997).

Betaine is found at high levels in certain foods (Zeisel *et al.* 2003, deZwart *et al.* 2003) and is absorbed directly from the diet *via* non-specific porters in the gut. In humans, depending on habitual diet, daily betaine intake varies and has previously been estimated to range between 300-2500 mg (Craig 2004, Olthof *et al.* 2003). However, recent reports suggest that normal dietary intake is much lower (Slow *et al.* 2005, Cho *et al.* 2006, Detopoulou *et al.* 2008) and the maximum daily betaine intake is unlikely to exceed 400-500 mg. Betaine can also be endogenously produced in the liver and kidney cortex from its metabolic precursor choline (Zhang *et al.* 1992, Zeisel and Blusztajn 1994, Fischer *et al.* 2005). The concentration of betaine in human plasma and the amount

excreted in the urine is under homeostatic control (Lever *et al.* 2004, 2007). It is freely filtered in the kidney and almost all (>98 %) is resorbed in the proximal tubules. However, plasma levels are lower in patients with renal disease (Lever *et al.* 1994) and urinary excretion is elevated in some patients with diabetes mellitus (>25 %; Dellow *et al.* 1999) and in 10 % of patients with dyslipidemia (Lever *et al.* 2005). BHMT expression and activity also has a crucial role in regulating circulating and liver betaine concentrations (Finkelstein *et al.* 1982) and it is likely that it is also a key determinant of extra-hepatic tissue betaine content.

Despite its two important biological functions there are few data on the tissue concentrations of betaine. It is not known if there are gender-specific differences in tissue betaine content even though it has been shown that BHMT expression and activity is influenced by various hormones with gender-specific profiles (Finkelstein *et al.* 1971), and there is a gender difference in plasma levels in humans (Lever *et al.* 1994). Similarly, whether there is any relationship between plasma and tissue betaine concentrations or any relationship between tissue betaine and plasma homocysteine concentrations remains to be investigated. Our hypothesis is that the betaine that is accumulated as a cellular osmolyte is an important metabolically available store of methyl groups. Our aim was to quantify tissue betaine concentrations, establish the variation in tissue content that is likely to be found, and determine whether there is any relationship between plasma levels and tissue accumulation or gender-specific differences. To assess this we measured the betaine content of plasma, brain, heart, lungs, liver, kidneys, spleen, intestine, reproductive tissues (testes – males; uterus, fallopian tubes and ovaries – females), skeletal muscle and skin in adult male and female rats aged between 4-12 months.

Methods

Animal protocol

The experimental protocol was approved by the Canterbury Animal Ethics Committee in compliance with the New Zealand Animal Welfare Act 1999.

Thirty adult Sprague Dawley rats (15 males; 15 females) aged between 4-12 months (400-800 g) were obtained from the Christchurch Animal Research Facility (University of Otago, Christchurch School of Medicine and Health Sciences, Christchurch, New Zealand). Animals were housed in standard caging, with a

maximum of 3 animals per cage on a 12 h light cycle with free access to food and water. Animals were maintained on standard rat chow, which contained 902.1 ± 34.1 µg betaine/g diet (mean \pm S.D. of 3 measures) on analysis.

A blood sample was collected *via* cardiac puncture into EDTA-containing tubes and stored on ice immediately following euthanasia *via* CO₂ inhalation. Tissue samples – heart, lung, liver, kidney, spleen, intestine, skeletal muscle, skin (with fur attached), reproductive tissues (testes in males; uterus with attached fallopian tubes and ovaries in females) and brain – were rapidly dissected from each animal without exsanguination, individually wrapped in aluminium foil and flash frozen in liquid nitrogen. Prior to flash freezing the intestine, any residual food was flushed out using normal saline solution and the skeletal muscle samples were obtained from the right hind leg for all animals. Plasma was separated within 2 h of blood collection by centrifugation at 3000 x g for 10 min. All tissue and plasma samples were stored frozen at –80 °C until analysis.

Betaine extraction, derivatization and HPLC

The whole heart, brain, spleen, one kidney, the reproductive tissues (either the complete female reproductive tract or one testicle) and a portion of liver, lung, skeletal muscle, skin and intestine (\approx 1 g of each) was weighed and utilized for each animal. Tissues were immersed in liquid nitrogen and pulverized into a fine powder using a mortar and pestle. Betaine was extracted from the tissues using a modified procedure for solid food analysis, as described previously (deZwart *et al.* 2003). Briefly, a homogeneous paste was obtained by adding a known amount of water and further homogenizing using an Ultra-Turrax T25 homogenizer (IKA Labor Technik, Staufen, Germany). The homogenates were shaken for a minimum of 1 hour then centrifuged for 10 min at 11 000 x g. The aqueous supernatant was removed and extracted with an equal volume of dichloromethane and shaken for 5 min before centrifuging at 11 000 x g for 10 min. The aqueous phase containing the betaine was removed and stored frozen at –20 °C until analysis.

Betaine was measured in the plasma and tissue extracts by high performance liquid chromatography (HPLC) after derivatization with 2-naphthacyl triflate and UV detection as previously described (Storer *et al.* 2006, Storer and Lever 2006). The concentration of betaine

($\mu\text{mol/l}$ tissue water) was calculated by comparing to external standards and correcting for tissue weight, water volume added and the % tissue water content as reported by Reinoso *et al.* (1997) and Long (1961), while plasma concentrations were corrected for water content as reported by Miller (1942).

Plasma total homocysteine

Plasma total homocysteine was measured by fluorescence polarization on an Abbott IM_X analyzer (Abbott Diagnostic Division, Abbott Laboratories, USA).

Statistics

The mean betaine concentration (\pm S.D.) for plasma and tissue samples for the combined male and female data ($n=30$) and within each gender ($n=15$ each) were calculated. A normality test showed that the betaine concentrations were not normally distributed in all tissues, and therefore comparisons were carried out on log transformed data, which were acceptably normal in all cases. Two-way analysis of variance was used to detect gender differences, and for comparing the betaine content of each tissue with plasma betaine, with *post-hoc* Bonferroni t-test comparisons. This was corroborated by one-way repeated measures analysis of variance of the data for each gender. Linear regression analysis was used to determine the significance of plasma betaine concentration as a predictor of tissue betaine concentrations. Data were analyzed using SigmaStat version 3.1 (Systat Software Inc., San Jose, CA). Statistical significance was defined as $P<0.05$ unless otherwise stated.

Results

Tissue betaine content

The betaine content of the male and female rat tissues is shown in Table 1. Of the tissues analyzed, the liver contained the highest concentrations of betaine (1.6 to 9.5 mmol/l tissue water), followed by those of the kidney (2.0 to 5.4 mmol/l). In contrast, the brain was found to contain the lowest betaine concentrations (undetectable to 190 $\mu\text{mol/l}$).

Associations between plasma and tissue betaine concentrations

In both sexes the concentrations of betaine in all tissues, except muscle and brain, were higher than the plasma concentrations ($P<0.001$), while the concentration in the brain was significantly lower ($P<0.001$; Table 1).

Analysis within each gender confirmed these findings and also showed that in both the female reproductive tract (uterus, fallopian tubes and ovaries) and in the testes, betaine concentrations were higher than the circulation. Although an important determinant of tissue betaine appears to be the plasma betaine concentration, the strength of this relationship varies from tissue to tissue (Table 2). Many tissues also accumulate significant amounts of betaine that are not dependent on plasma levels (the constant in Table 2). For example, the plasma betaine concentration accounts for less than 20 % of the variance in liver betaine concentrations, where the accumulation is the highest. Similarly in the testes, another tissue particularly rich in betaine (2.4 to 3.4 mmol/l) most accumulation is independent of the plasma betaine (Table 2). Furthermore, the tissues that show a strong dependence on plasma betaine (combined male and female data, $r^2>0.5$; heart, lung, spleen and intestine, Table 2) show a stronger correlation with each other (Pearson coefficients, $r = 0.74$ to 0.85) than with liver ($r = 0.28$ to 0.68) or kidney ($r = 0.43$ to 0.53). Similar relationships are observed when each gender is analyzed separately but are less significant.

Gender differences in tissue betaine content and associations with plasma homocysteine

The betaine content in the skin, liver and kidney was not significantly different between males and females, however in plasma and in all other tissues analyzed, it was significantly higher in males ($P<0.01$). In the study animals the mean male plasma total homocysteine (\pm SD) was significantly higher than the females (4.2 ± 0.8 $\mu\text{mol/l}$ vs 3.1 ± 0.5 $\mu\text{mol/l}$; $P<0.001$). In the total animal group, plasma total homocysteine correlated with the betaine content of several tissues and with plasma betaine, but when each gender was considered separately, there was no relationship between homocysteine and plasma or tissue betaine in male rats, and the trends in the females were only marginally significant ($P=0.040$ in skin, 0.058 in liver & 0.063 in spleen). Various multiple regression models with plasma betaine and homocysteine as predictor variables for tissue betaine, also failed to show any significant contribution from the homocysteine.

Discussion

Our results demonstrate that betaine is accumulated to high levels in virtually all tissues, with the highest betaine content occurring in the liver and kidney. The observed pattern of tissue betaine

Table 1. The betaine content of various tissues of the male and female rat.

Organ	Tissue Water Content ¹ %	Males Betaine content		Females Betaine content	
		µmol/l	µmol/g tissue	µmol/l	µmol/g tissue
Plasma	96	186 ± 43**	0.17 ± 0.04	101 ± 37**	0.09 ± 0.03
Heart	78	372 ± 114*	0.32 ± 0.03	224 ± 77*	0.19 ± 0.02
Lung	79	500 ± 119**	0.43 ± 0.03	290 ± 96**	0.25 ± 0.02
Liver	71	4097 ± 1115	3.22 ± 0.23	4096 ± 1988	3.21 ± 0.4
Kidney	77	3785 ± 546	3.24 ± 0.12	3319 ± 1034	2.78 ± 0.22
Spleen	77	801 ± 143*	0.67 ± 0.03	519 ± 243*	0.43 ± 0.05
Skin	65	412 ± 185	0.30 ± 0.03	305 ± 153	0.22 ± 0.03
Skeletal muscle	76	214 ± 70*	0.18 ± 0.02	124 ± 40*	0.10 ± 0.01
Brain	79	50 ± 44**	0.04 ± 0.01	26.2 ± 26.7**	0.02 ± 0.01
Intestine	75	920 ± 196**	0.75 ± 0.04	524 ± 146**	0.43 ± 0.03
Female reproductive	76	N/A	N/A	216 ± 123	0.18 ± 0.03
Testes	86	2842 ± 292	2.63 ± 0.07	N/A	N/A

Data are means ± S.D. ¹Concentrations (µmol/l) are expressed as the concentration in tissue water, which were calculated from the % tissue water as reported by Reinoso *et al.* (1997), with the exception of plasma which was based on the report of Miller (1941) and the female reproductive tissues, which was based on the report for rat ovary from Long (1961). Number of animals: 15 of each gender. Significant gender difference, * $P < 0.01$; ** $P < 0.001$, calculated from two-way ANOVA on log-transformed data. Female reproductive tissues included the uterus, fallopian tubes and ovaries.

distribution is generally similar to the previous findings of Martin and Finkelstein (1981) in male rat tissues, but compared to that report, we found lower absolute betaine concentrations in the liver, kidney (\approx 2-fold for both tissues) and spleen (\approx 3-fold), and higher levels in the heart (\approx 2.5-fold). We also detected betaine in the lungs of all animals and in the brain in 28 of our 30 animals (Table 1). Similar to our findings in the brain, Lien (1995) detected betaine in the female rat cerebrum, cerebellum, striatum, midbrain, pons and medulla oblongata (0.4 ± 0.05 to 0.9 ± 0.02 mmol/kg water), although concentrations were higher than those we observed. The discrepancies in the betaine content between the reported studies could be the result of several factors, including genetic variation in the animals and differences in the treatment of the animals before the study. Two aspects that are likely to be particularly relevant are the betaine content of the diet and the hydration status of the animals.

Plasma betaine concentrations are under homeostatic control (Lever *et al.* 2004), a process that is influenced by dietary betaine or choline intake and betaine-homocysteine methyltransferase (BHMT) activity (Holm *et al.* 2005, Schwab *et al.* 2006, Collinsova *et al.* 2006). BHMT activity is also affected by the supply of dietary

betaine, choline and methionine (Park and Garrow 1999), by hormones (Finkelstein *et al.* 1971, Ratnam *et al.* 2006) and by osmotic stress (Delgado-Reyes and Garrow 2005, Schäfer *et al.* 2007). Similarly, the betaine content of the liver has been shown to be controlled by dietary betaine, choline and protein intake and BHMT activity (Finkelstein *et al.* 1982). Thus, it is likely that the tissue betaine content of extra-hepatic tissues is regulated in the same manner.

Tissue betaine is not necessarily derived from plasma, as (for example) liver and kidney betaine may also be derived through the two-step oxidation of choline *via* the choline dehydrogenase and betaine aldehyde dehydrogenase expressed in these tissues (Grossman and Hebert 1989, Huang and Lin 2003), while the relatively high association between intestinal betaine and plasma betaine ($r^2 = 0.76$) may reflect the uptake of dietary betaine (Kettunen *et al.* 2001). Almost certainly, tissue betaine concentrations are also determined by the osmotic stress of the animals (Wehner *et al.* 2003). Indeed, most of the accumulated betaine in various tissues is likely to be because of its important role as a cellular osmolyte. The hydration state of the animals before euthanasia was not controlled in this study, thus it is likely to have contributed to at least some of the observed variation in tissue betaine concentrations. It could also explain why the correlations

Table 2. Relationships between plasma and tissue betaine.

Tissue	n	Slope	P slope	Constant	P const.	r ²
Heart	30	1.7	<0.001	58	0.12	0.65
Lung	30	2.1	<0.001	94	0.039	0.61
Liver	30	11.8	0.015	2396	0.002	0.19
Kidney	30	8.1	<0.001	2388	0.001	0.32
Spleen	30	3.1	<0.001	220	0.012	0.55
Skin	30	1.5	0.005	144	0.068	0.25
Muscle	30	0.8	<0.001	57	0.052	0.41
Brain	30	0.1	0.46	25	0.19	0.02
Intestine	30	3.9	<0.001	159	0.019	0.76
Female reproductive tissues	15	1.7	0.043	41	0.63	0.28
Testes	15	2.5	0.17	2369	<0.001	0.38

Predictions of tissue betaine contents ($\mu\text{mol/l}$ tissue water, y) from plasma betaine concentrations (x , $\mu\text{mol/l}$). Linear regression slope and intercept (constant) parameters and coefficient of determination (r^2). Significant parameters ($P < 0.05$) are in bold. Female reproductive tissues included the uterus, fallopian tubes and ovaries.

between various tissues are sometimes stronger than the correlations with the plasma betaine concentration. Similarly, another aspect that may contribute to some of the variation observed in the tissue betaine content in the present study is the difference in the age of the animals utilized (4-12 months). It is not known whether tissue betaine content varies with age, but a wide range of ages was chosen for the purposes of this study so that the variability within a representative rat population could be adequately assessed.

To our knowledge this is the first time that betaine has been systematically measured in the tissues of female animals. Generally for most tissues analyzed, betaine content was higher in males compared to females, which is likely, at least in part, to be a function of BHMT activity. BHMT is the only known enzyme that utilizes betaine as a substrate and mediates the methylation of homocysteine to methionine. Plasma homocysteine concentration may be of clinical significance in humans because elevations increase the risk of all forms of vascular disease (coronary, cerebral and peripheral) (Wald *et al.* 2002). BHMT expression and activity plays a key role in regulating betaine concentrations and determines the fate of betaine between its two competing biological functions; stored to control cellular osmolarity or metabolized to provide a methyl group for homocysteine methylation. As well as diet, BHMT expression is influenced by various hormones, including corticosteroids, insulin, estradiol and testosterone (Finkelstein *et al.* 1971, Ratnam *et al.* 2006). This is therefore likely to cause differences in the tissue betaine

content between males and females. Another possible cause is the gender difference in homocysteine concentrations, with male animals having significantly higher plasma homocysteine concentrations than females. The apparent relationships we observed between tissue betaine and plasma homocysteine concentration in the total group of animals reflects the gender differences in both betaine and homocysteine metabolism, and underlies the need for studies to be conducted on animals of each gender separately as in humans (Lever *et al.* 2007).

The high level of betaine observed in the testes is interesting. There is evidence to suggest that betaine, acting as a methyl donor, may have an important role in maintaining male fertility (Kelly *et al.* 2005). The metabolism of betaine *via* BHMT can ultimately lead to the production of *S*-adenosyl methionine, which is crucial in the testes for the synthesis of creatine, which is thought to be important for sperm motility and function (Lee *et al.* 1998). Although rat liver is the only tissue that has significant BHMT activity (Martin and Finkelstein 1981; McKeever *et al.* 1991), it is possible that specific cell types within a tissue produce BHMT. Indeed, a search of microarray gene chip data (Edgar *et al.* 2002; <http://www.ncbi.nlm.nih.gov/geo/>) shows that Sertoli cells in the testes express BHMT mRNA. Combined, this indicates the possibility that at least some of the accumulated betaine could be utilized as a source of methyl groups, which may contribute to testicular creatine production and thus aid in maintaining sperm motility and function.

In conclusion, the present study confirms that betaine is stored in mammalian body tissues, and these stores need to be taken into account in models of one-carbon metabolism. We provide values of the tissue betaine content in male and female rats. It functions as an important cell volume regulator or provides a source of labile methyl groups for one-carbon metabolism *via* the action of BHMT. Disturbances in tissue betaine concentration have the potential to have adverse consequences for health and well-being, and as such, it would be of particular interest to further investigate the effect of dietary betaine and choline intake on tissue betaine content.

Conflict of Interest

There is no conflict of interest.

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