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Delayed Deproteinization Causes Methodological Errors in Amino Acid Levels in Plasma Stored at Room Temperature or -20°C

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ABSTRACT : Deproteinization has been recognized as a prerequisite for amino acid analysis of plasma samples. For plasma stored at room temperature, delaying deproteinization for 30, 60 or 120 minutes did not result in significant changes in the mean CV (coefficient of variation), which ranged from 4.4 to 5.6%. However the mean CV of aspartic acid, α-aminoadipic acid, alanine and lysine was about 10%. When the plasma was stored frozen at -20°C, the CV was increased at 0 and 120 minutes after thawing, to 12.4% (range, 4.1 to 35.3%) and 8.0% (2.5 to 30.7%), respectively. The concentrations in plasma during storage at room temperature of all the amino acids analyzed showed significant changes. In plasma stored for 30 minutes at room temperature, 17 amino acids increased in concentrations and two decreased. Extending this period to 60 or 120 minutes increased the instability as compare to the reference group. Storing plasma at -20°C for 2 weeks resulted in significantly greater changes in the amino acid concentrations than at room temperature. On extending the storage time at room temperature, after thawing, to 30, 60, and 120 minutes, 21, 20, and all 22 amino acids respectively changed significantly ($p<0.01$). The present study indicates that methodological errors occur in the concentrations determined for all amino acids when plasma is left at room temperature. The storage of frozen non-deproteinized plasma accompanied more significant changes in most amino acid concentrations and thus should be avoided. Deproteinization should be performed as soon as possible after plasma collection. (**Key Words :** Amino Acid Assay, Plasma Concentration, Deproteinization, Storage, Blood Sample)

INTRODUCTION

Plasma amino acid profiles are used in physiological, clinical (Yoneda et al., 2001), nutritional (Sarwar and Botting, 1993), and metabolic research. Significant progress has been made in amino acid analysis of physiological samples since the first commercial amino acid analyzer was introduced in 1958 (Spackman et al., 1958). Currently, automatic chromatographic equipment is widely used for amino acid analysis. The more than 40 amino acids in biological fluids can be assayed within 2 hours (Endo et al., 1992; Boucher et al., 1997). However, despite technical and theoretical developments, results still vary widely between laboratories. Several parameters can affect the results of amino acid assays, including sample collection, hemolysis or blood clotting, anticoagulants, degradation of protein due to endogenous proteolytic activity (Hulmues et al., 2004; Saleh et al., 2006), the method of deproteinization, and preservation (Parvy et al., 1991). Thus when analyzing plasma or serum amino acid concentrations, it is critical to collect, preserve, and store the sample using procedures that minimize amino acid changes.

Deproteinization has been recognized as a prerequisite for amino acid analysis of plasma samples. In many studies, the plasma is stored at -20°C, and then thawed and deproteinized just before use (Peng 1972; Veresegyhazy et al., 2001). However, no one has examined whether the timing of the deproteinization affects the results. The present study using blood samples from ten cows, determined the affects of delayed deproteinization on the concentrations of amino acids in plasma stored at room temperature (24°C) or -20°C for 2 weeks.

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MATERIALS AND METHODS

Preparation of blood plasma samples

Sample preparation : Blood samples (10 ml/head) were drawn from ten Holstein cows all on the same day at the Animal Resource Science Center, Graduate School of Agricultural and Life Sciences, the University of Tokyo (Kasama, Japan) by jugular venipuncture into heparinized

vacuum tubes (Terumo, Tokyo, Japan). The samples were placed in melting ice, and the plasma was collected immediately by centrifugation at 3,000 rpm and 4°C. The plasma was separated into two 2.0 ml centrifuge tubes (As one, Osaka, Japan). One tube was stored at room temperature. The other tube was stored at -20°C for 2 weeks.

Amino acid standard : According to the manufacturer's instructions, standard stock solutions of amino acids were prepared by diluting amino acid standard B and amino acid standard AN-2 (Wako Pure Chemicals, Osaka, Japan) with 0.02 N HCl and then subdivided into 0.5 ml centrifuge tubes (Greiner bio-one, Hanover, Germen) and stored at -80°C for later use.

Plasma deproteinization : Each 0.1-ml plasma sample was added to the same volume of 6% (w/v) trichloroacetic acid (TCA; Wako), mixed thoroughly by an automatic Lab-Mixer (NM-10H; As one), and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was filtered through 0.1-μm Ultrafree-MC (Millipore, Bedford, MA, USA) and used for the amino acid analysis.

Amino acid assay : All amino acids were assayed in triplicate and determined using an automatic amino acid analyzer (L-8500A; Hitachi, Tokyo, Japan) according to the manufacturer's instructions. About 80-100 μl of deproteinized and filtered sample was placed in a self-sealing vial on the auto-sampler rack (60 positions) maintained at 4±2°C. An accurate volume (20 μl measured with a 0.5-ml precision syringe) was injected *via* the injection valve onto the analytical column. Detection was by spectrophotometry at 570 and 440 nm with the ninhydrin reaction. To confirm the within-run precision after every 30 vials, the standard stock solution was tested. Dehydration of the sample was confirmed by testing plasma progesterone concentrations before and after freezing. It was confirmed that no changes had happened.

Amino acid assay

Contribution of deproteinization to analytical error : Plasma samples were drawn from ten Holstein cows and deproteinized separately. All amino acids were assayed in triplicate. For the amino acids for which deproteinization made a significant contribution to analytical error, coefficients of variation were calculated.

Effect of delayed deproteinization at room temperature on amino acid concentrations : The plasma samples stored at room temperature were divided into four treatment groups of ten samples each, one from each cow. Deproteinization was performed immediately after collection of the plasma in the R0min group considered the reference group. Deproteinization was carried out after 30 minutes at room temperature (24°C) in the R30min group, and after 60 and 120 minutes in the R60 min and R120 min groups, respectively. The average amino acid concentrations compared with the reference group were used to study the effect of delayed deproteinization at room temperature on concentrations of amino acids. The mean coefficient of variation (CV) was used to judge the precision of an every method.

Effect of deproteinization after 2 weeks of storage at -20°C on amino acid concentrations : The second portion of plasma was kept at -20°C for 2 weeks, and the change in amino acid concentrations was studied. The frozen plasma was thawed at 4°C for 1 h. Again, the samples were divided into four treatment groups of ten each, one from each cow. Deproteinization was performed immediately after thawing (at 4°C for 1 h) in the F0min group, and after storage at room temperature (24°C) for 30, 60 and 120 minutes in the F30 min, F60 min and F120 min groups, respectively. Average amino acid concentrations compared with the reference group were used to study the effect of delaying deproteinization on amino acid concentrations. The mean coefficient of variation (CV) was used to judge the precision of each method.

Statistical analyses : All amino acids were assayed in triplicate and the mean coefficient of variation (CV) was used to judge the precision of the method. Average amino acid concentrations were compared with those of the reference group using an analysis of variance (ANOVA) with Fisher's least significant differences test using the program Microsoft Excel Statistics IV (Abacus Concepts, Berkeley, CA, USA). Each value represents the mean±SE.

RESULTS

Coefficients of variation

CVs calculated for the 22 amino acids are presented in Table 1. Storing the plasma at room temperature prior to deproteinization, did not result in significant changes in the mean CV, which ranged from 4.4 to 5.6%. The mean CV was greater than 10% only for α-amino adipic acid and lysine. However, in the reference R0min group, the mean CV was greater than 10% for aspartic acid, α-amino adipic acid, alanine and lysine. By contrast, when the plasma was stored frozen at -20°C, there were marked differences in the CV at F0 min and F120 min; the mean CV was 12.4% (range 4.1 to 35.3%) and 8.0% (2.5 to 30.7%), respectively. The CV of 14 and 6 amino acids exceeded 10% at F0 min and F120 min, respectively. At F30 min and F60 min, the CV stabilized at 3.8 and 4.8%, respectively.

Effect of storage at room temperature prior to deproteinization on amino acid concentrations

Delaying the deproteinization significantly affected the concentration of all amino acids analyzed (Table 2). Comparing values between R0 min, and R30 min showed that the concentrations of 17 amino acids increased

Table 1. Coefficients of variation (%)

Amino acid \ Group	R0 min	R30 min	R60 min	R120 min	F0 min	F30 min	F60 min	F120 min
Phosphoserine	1.9	2.8	1.6	1.7	4.1	1.4	1.5	4.7
Taurine	1.9	3.0	3.2	2.9	5.9	3.0	2.5	4.5
Urea	2.2	2.6	2.5	3.1	6.7	3.1	2.4	2.7
Aspartic acid	13.3	8.3	9.3	8.0	17.5	10.9	7.3	8.3
Threonine	2.5	4.9	3.1	4.5	10.9	4.6	3.6	4.4
Serine	3.1	2.5	3.0	2.1	6.1	3.4	3.3	2.5
Glutamic acid	3.1	4.5	1.8	7.7	16.2	5.7	4.3	6.8
α-amino adipic acid	10.9	11.6	8.8	11.0	13.5	5.9	2.9	11.3
Alanine	11.2	12.3	3.6	4.9	35.3	4.4	6.0	30.7
Valine	1.9	1.6	2.1	3.2	9.8	7.2	2.5	4.9
Methionine	4.2	7.1	7.4	8.8	10.4	3.8	3.3	13.4
Isoleucine	1.5	3.6	1.9	4.7	11.6	3.6	3.0	4.0
Leucine	2.7	1.5	2.2	2.4	8.3	2.0	2.6	3.0
Tyrosine	4.0	3.0	2.3	4.4	10.2	3.0	3.3	4.0
Phenylalanine	2.5	2.9	3.4	4.2	6.6	2.1	2.4	8.3
Ammonia	4.8	6.7	6.2	7.6	12.4	5.7	2.5	13.4
Ornithine	5.6	4.5	5.5	9.1	16.3	5.6	1.7	12.2
Lysine	12.9	4.9	11.7	10.5	19.8	5.0	6.7	15.8
Histidine	3.9	5.0	4.6	7.1	8.6	4.3	2.7	6.0
Carnosine	3.9	11.1	3.3	4.9	13.5	4.7	4.4	4.3
Arginine	2.5	1.5	3.7	4.4	11.1	4.8	3.0	6.4
Proline	5.9	9.6	4.5	6.8	18.9	11.0	10.6	5.3
Mean	4.8	5.3	4.4	5.6	12.4	4.8	3.7	8.0
Min	1.5	1.5	1.6	1.7	4.1	1.4	1.5	2.5
Max	13.3	12.3	11.7	11.0	35.3	11.0	10.6	30.7

significantly and those of two decreased. The values for aspartic acid, leucine, and ornithine did not change significantly. Extending the storage time at room temperature to 60 or 120 minutes resulted in greater instability compared to R0 min.

Effect of delayed deproteinization after storage at -20°C on amino acid concentrations

As shown in Table 3, following storage at -20°C for 2 weeks, concentration of four amino acids were significantly decreased compare to R0 min: phosphoserine, aspartic acid, α-amino adipic acid, and alanine. Conversely, the concentrations of glutamic acid, methionine, isoleucine, leucine, tyrosine, NH_3, histidine, and carnosine increased. Levels of the other amino acids did not change dramatically. Delaying the deproteinization of frozen plasma after thawing resulted in significantly greater changes in the amino acid concentrations than storage at room temperature especially at 30 minutes after thawing. In the F30 min, F60 min, and F120 min groups, 21, 20, and all 22 amino acids respectively changed significantly ($p<0.01$). Concentrations of two amino acids, α-amino adipic acid and phosphoserine, decreased at -20°C and remained low after thawing even at room temperature until 120 minutes. Other amino acids including those that decreased while the samples were frozen increased significantly. Therefore, when deproteinization was delayed after thawing, the amino acid concentrations of frozen plasma samples changed dramatically, even some amino acids whose concentrations had decreased while frozen, like taurine, aspartic acid, α-amino adipic acid, and alanine.

DISCUSSION

The storage of plasma at room temperature prior to deproteinization did not result in significant changes in the mean CV for most of amino acids, with values ranging from 4.4 to 5.6%. However, for α-amino adipic acid and lysine, the CV was greater than 10%. Aspartic acid and alanine also had relatively high CVs. The result for aspartic acid and α-amino adipic acid may have been due to their low concentrations and are consistent with the report of Leon et al. (1996). Nevertheless, in the present study, the CVs for alanine and lysine were high despite high concentrations.

Table 2. Mean concentrations in plasma amino acids with delayed deproteinization after storage at room temperature and statistical comparisons

Group	Concentration (ng/20 μl)				Compared to the reference group (R0 min)			
Amino acid	R0 min	R30 min	R60 min	R120 min	R30 min	R60 min	R120 min	Time[1]
Phosphoserine	9.9	13.0	11.9	11.9	**	**	**	**
Taurine	44.7	50.6	47.6	47.1	**	**	**	**
Urea	2,558.5	2,984.6	2,775.6	2,704.5	**	**	**	**
Aspartic acid	10.5	9.8	9.2	9.8		**		*
Threonine	67.5	74.8	63.5	63.1	**	**	**	**
Serine	71.0	74.7	74.3	71.2	**	**		**
Glutamic acid	75.3	94.2	88.1	92.6	**	**	**	**
α-amino adipic acid	24.6	21.2	21.8	20.8	**	**	**	**
Alanine	108.3	92.5	99.9	95.2	**		**	**
Valine	303.2	320.2	302.8	295.5	**			**
Methionine	25.9	37.6	29.4	25.2	**	**		**
Isoleucine	140.7	169.3	138.1	142.8	**			**
Leucine	206.5	209.0	211.4	198.7		*	**	**
Tyrosine	97.6	112.4	90.7	104.5	**	**	**	**
Phenylalanine	67.9	76.8	65.4	61.6	**	*	**	**
Ammonia	24.0	35.3	32.9	32.7	**	**	**	**
Ornithine	91.4	86.9	79.5	85.9		**	*	**
Lysine	130.4	157.2	132.8	150.5	**		**	**
Histidine	87.0	95.7	82.2	90.4	**	*		**
Carnosine	38.3	51.2	41.4	41.7	**	*	**	**
Arginine	138.1	153.5	135.5	127.1	**		**	**
Proline	207.2	274.1	220.5	252.9	**		**	**

[1] Significance of the delay in deproteinization at room temperature.
*, ** $p<0.05$ and 0.01, respectively.

But in the Leon's report (1996), the CVs for alanine and lysine were low. This report used precipitation with sulfosalicylic acid (SSA) followed by centrifugation to remove the precipitated protein. For deproteinization with SSA the pH needs to be close to that of the standard calibration mixture. In addition, the report did not indicate when the deproteinization took place after the plasma was collected. The present results show that CV changed with time at room temperature. A major problem with the SSA method is that resolution in the region of threonine and serine and in the other areas can be adversely affected, particularly if the amount of SSA added to the column exceeds 200 mg (Adrien, 1987). Some investigators have used picric acid for deproteinization (Knipeel et al., 1969). In the present study, TCA was used for precipitation according to the manufacturer's instructions. The use of 6% TCA dose not require an adjustment of pH because it is close to that of the standard calibration mixture. Thus further studies should be conducted on the effects of deproteinization with different agents. After the thawing of non-deproteinized plasma that had been stored at -20°C, the CV was dramatically increased in the F0min and F120 min groups. The high CV values at F0 min might be caused by inadequate thawing of the samples. In the present study, plasma was thawed by leaving sample at 4°C for 1 h. Thus, the thawing procedure including temperature and duration should be studied further. Other hand, the increased CV at F120 min may due to marked changes in amino acid concentrations. Notably, the concentration of alanine increased 268.88% from F0 min to F120 min.

Since the development of an automatic chromatographic system for analyzing amino acids in 1958 (Spackman et al., 1958), significant progress has been made in study of physiological samples. Despite recent technological developments that have improved precision and sensitivity, amino acid assays remain a fastidious procedure requiring careful attention to detail. The preparation of plasma samples is usually unavoidable especially the deproteinization procedure. We observed that delaying deproteinization caused a systematic error in the determination of amino acid concentration in blood plasma regardless of whether the sample was stored at room temperature or -20°C. Even 30 minutes at room temperature significantly affected the measurements. The concentrations

Table 3. Mean concentrations in plasma of amino acids with delayed deproteinization after storage at -20°C and statistical comparisons

Group / Amino acid	Concentration (ng/20 μl)					Compared to the reference group (R0 min)				
	R0 min	F0 min	F30 min	F60 min	F120 min	F0 min	F30 min	F60 min	F120 min	Time[1]
Phosphoserine	9.9	9.1	8.7	9.2	9.4	**	**	**	**	**
Taurine	44.7	43.8	47.6	47.4	47.4		**	**	**	**
Urea	2,558.5	2,592.7	2,930.1	2,900.8	2,902.2		**	**	**	**
Aspartic acid	10.5	9.2	11.8	15.0	13.6	*	**	**	**	**
Threonine	67.5	66.8	84.8	90.3	79.5		**	**	**	**
Serine	71.0	71.7	75.5	80.9	78.7		**	**	**	**
Glutamic acid	75.3	97.5	124.7	141.9	156.9	**	**	**	**	**
α-amino adipic acid	24.6	19.2	20.0	19.7	19.1	**	**	**	**	**
Alanine	108.3	70.7	107.0	101.3	190.1	**			**	**
Valine	303.2	317.8	369.4	426.6	372.3		**	**	**	**
Methionine	25.9	39.4	36.1	35.8	41.0	**	**	**	**	**
Isoleucine	140.7	166.9	216.1	223.8	195.0	**	**	**	**	**
Leucine	206.5	224.9	249.8	241.2	222.6	**	**	**	**	**
Tyrosine	97.6	116.4	133.4	149.2	127.8	**	**	**	**	**
Phenylalanine	67.9	68.6	74.2	71.1	75.3		**		**	**
Ammonia	24.0	44.7	46.1	52.1	41.1	**	**	**	**	**
Ornithine	91.4	94.9	132.2	137.6	108.8		**	**	**	**
Lysine	130.4	128.0	168.4	209.7	173.2		**	**	**	**
Histidine	87.0	92.9	109.4	115.7	103.7	*	**	**	**	**
Carnosine	38.3	45.8	57.8	54.0	48.2	**	**	**	**	**
Arginine	138.1	141.7	191.9	208.9	187.1		**	**	**	**
Proline	207.2	231.1	287.9	292.7	305.7		**	**	**	**

[1] Significance of the delay in deproteinization after storage at -20°C for 2 weeks.

*, ** $p<0.05$ and 0.01, respectively.

of amino acids increased with time both at room temperature and at -20°C. Adrien et al. (1987) reported significant changes in levels of 19 of 23 amino acids when plasma was stored at -18°C for 5-6 months: 18 amino acids increased in concentration and one decreased. This is consistent with the present findings. The phenomenon could be explained by a further hydrolysis of proteins in the plasma samples. Plasma contains numerous proteases. Many of the proteases are released from activated, dying, or lysed neutrophils (Weiss, 1989; Faurschou, 2003) or mononuclear phagocytes (Robbie, 2001). Plasma also contains an abundance of protease inhibitors whose function is to arrest the activity of proteolytic enzymes. However, because of the large quantities and variety of proteases that can be released into the blood, the protease inhibitors normally found in plasma are not completely effective. Jeffrey et al. (2004) reported that the inclusion of a protease inhibitor cocktail in the sample tube, provided stable and reliable human plasma samples that yielded reproducible results in a proteomic analysis. Leon et al. (1996) reported that the substantial degradation of asparagine to aspartic acid was clearly less susceptible than the degradation of glutamine to glutamic acid. This is consisting with the present study (Tables 1 and 2). Suma et al. (1985) indicated that when deproteinization was conducted after plasma was kept in an ice bath for 1 h, there was no significant difference except for glutamine and 3-methyl-histidine. Perry and Hansen (1969) described how allowing blood or plasma to stand at room temperature for 1 h or storing plasma in a freezer before deproteinization can lead to major errors in the quantification of disulfide amino acids. Again, this is consisting with the present results.

Overall, the present study indicated that methodological errors in concentrations determined for all amino acids arose from different methods of storage and delays in deproteinization. With delay in deproteinization, the concentrations of most amino acids changed significantly whether the cattle plasma was stored at room temperature or -20°C. Storage of frozen non-deproteinized plasma resulted in more significant changes and thus should be avoid. This might arise from the further hydrolysis of proteins in the plasma samples or the incomplete elimination of small peptides in the deproteinized samples. Therefore, deproteinization should be performed as soon as possible after plasma collection.

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