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Evaluation of systematic errors due to deproteinization, calibration and storage of plasma for amino acid assay by ion-exchange chromatography

Leon H. de Jonge*, Michel Breuer

DLO Institute for Animal Science and Health, P.O. Box 65, 8200 AB Lelystad, Netherlands

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Abstract

Three factors contributing to inter-laboratory variation in the determination of amino acids in plasma, i.e. deproteinization, calibration and storage conditions, were evaluated in this study. Deproteinization clearly enlarged the coefficient of variation in the determination of cystine, aspartic acid and tryptophan. During this process losses of hydrophobic amino acids occurred, in particular, when the volume of the supernatant was small. Correction for this effect, using an internal standard, was not possible. Delaying the removal of the supernatant for 1 h decreased the concentration of tryptophan. Correction for this effect, using an internal standard, was not possible. The use of different commercial standards also led to systematic errors during the calibration of samples. The amino acid concentrations in deproteinized plasma remained stable for at least 1 year when stored at a temperature of −40°C or lower. Above this temperature, glutamine and asparagine were found to be degraded. This degradation could be minimized by neutralizing the samples before storage. The concentration of cystine decreased considerably during storage of non-deproteinized plasma. Correction for these changes due to storage is not advised and, in most cases, is impossible.

Keywords: Amino acids

1. Introduction

Determination of free amino acids in blood plasma by ion-exchange chromatography (IEC) is one of the most commonly used analytical methods in clinical and nutritional research. Despite the technical and theoretical developments in this respect, results still vary widely between laboratories as documented in numerous collaborative studies [1–5]. It seems that besides random errors arising during chromatographic analyses, systematic errors also exist in data obtained from various laboratories.

The aim of this research was to evaluate systematic errors associated with (1) plasma deproteinization using sulphosalicylic acid, (2) calibration and (3) storage of samples. Particular attention was paid to the deproteinization because this step is often mentioned as a serious source of variation. By

*Corresponding author.

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defining the significance of all these errors, the intra-laboratory precision should be improved.

2. Experimental

2.1. Chemicals and reagents

Individual crystalline salts of L-amino acids (Kit no. 21, L-ornithine art. 0-2375, L-taurine art. T-0625, L-norleucine art. N-6877) were obtained from Sigma (St. Louis, MO, USA). 5-Sulphosalicylic acid dihydrate (SSA), urea, lithium hydroxide monohydrate, and methanol were obtained from Merck (Darmstadt, Germany). Analytical-grade hydrochloric was obtained from Baker (Deventer, Netherlands). Lithium buffers, ninhydrin, and lithium hydroxide were obtained from Pharmacia LKB Biochrom (Cambridge, UK).

A standard stock solution of amino acids and urea was prepared by dissolving these compounds in deionized water to which a small volume of hydrochloric acid was added, and was then stored at −80°C. The solution was thawed and diluted with loading buffer (0.2 *M* lithium citrate, pH 2.2) before use.

Commercial standard solutions were obtained from Sigma art. A-9906, Pierce no. 20077, and Pharmacia LKB Biochrom no. 80-2037-62. Water used for the preparation of these standard solutions was deionized (Milli-Q water purification system, Millipore, MA, USA).

2.2. Apparatus

The amino acid analyzer was an Alpha plus 4151, with a pre-wash column as an ammonia-trap, from Pharmacia LKB Biochrom. Data processing was performed on a PS/2-55 from IBM (Portsmouth, UK) with System Gold software, revision 5.10 from Beckman (San Ramon, CA, USA). Connection between the amino acid analyzer and the personal computer was via an A/D converter Model 406 from Beckman.

Amino acids and urea were separated on a 27 cm-lithium high resolution column series I Ultropac 8 resin (ϕ 4.6 mm) from Pharmacia.

2.3. Chromatographic conditions and quantification

The elution programme was identical to that reported previously [6]. Amino acids were detected following a colour reaction with ninhydrin and measured spectrophotometrically at 570 nm and at 440 nm. The flow-rate of the buffers and the ninhydrin was 20 ml/h. The samples were kept in an autoloader at 4°C.

Amino acids were quantified with the standard solution, and an internal standard correction. L-Norleucine was used as the internal standard and was added to each individual sample.

2.4. Samples

Venous blood was obtained from individual cows by venipuncture (*vena jugularis*). The samples were collected in heparinized tubes and placed in melting ice. Plasma was removed after centrifuging for 30 min at 2500 *g* at 4°C.

2.5. Deproteinization and sample preparation

Plasma was deproteinized by the addition of 80 mg of sulphosalicylic acid (SSA) per ml of plasma and immediately centrifuged for 30 min at 2500 *g* and 4°C. The supernatant was removed and, after addition of the internal standard, was stored at −80°C.

The solution was thawed and the pH was adjusted to 2.2 with lithium hydroxide or SSA. It was filtered through a 0.20 μm filter membrane type Acrodisc LC 13 PVDF (No. 4455) from Gelman Sciences (Ann Arbor, MI, USA) and put into a 100 μl sample vial with the loading buffer (pH 2.2).

2.6. Experimental design and statistics

2.6.1. Deproteinization

Contribution of deproteinization to the analytical error. Plasma was divided into five batches that were deproteinized separately. For each batch four samples were taken and analyzed after adding the internal standard to each sample. Results between

and within the batches were compared using the ANOVA procedure [7]. For amino acids for which deproteinization made a significant contribution ($P < 0.05$) to the analytical error, the coefficients of variation within and between batches were calculated [8].

Effect of length of delay before removing the supernatant following the addition of SSA. Plasma was divided into three batches to be deproteinized separately. The supernatants were removed after 0, 30 and 60 min, respectively. Statistical significance of the differences between the means of the batches was distinguished using Student's *t*-test for small numbers of replicates [7].

The effects of volume and the internal standard using different methods of deproteinization. Plasma was divided into two batches, that were treated as follows:

1. 1 ml of plasma was deproteinized with 80 mg of solid SSA.
2. 1 ml of plasma was deproteinized with 1 ml of SSA solution (80 mg/ml).

In both methods the internal standard was added before and after deproteinization.

Calibration. Influence of the standard solution on the calibration was evaluated by using three commercial standard solutions. They were calibrated and differences between the means determined and those given by the manufacturers were compared using Student's *t*-test.

2.6.2. Storage

Effect of temperature and neutralization of the plasma sample during storage on the concentration of the amino acid. For this purpose, plasma was deproteinized and divided into batches that were stored at 20, 4, −20, −40 and −80°C, respectively. Amino acid concentrations were monitored over 12 months and the changes were described as a first order curve ($C_t = C_0\ e^{kt}$, where C_t = the concentration at time t, C_0 = the initial concentration and t = time) as reported in the literature [9,10]. Correlation coefficients were used to judge the precision of the model. Also, the effects of neutralization of the sample solution before storage, on variations in amino acid concentrations, were examined at −20, −40 and at −80°C.

Storage of non-deproteinized plasma. Samples were stored at −20, −40 and −80°C and, just before analysis thawed and deproteinized. The results were compared with those obtained from the analysis of plasma deproteinized before storage.

3. Results

3.1. Deproteinization

3.1.1. Contribution to the analytical error

Deproteinization had a significant effect on the variation in the concentration of aspartic acid, serine, glutamine, citrulline, cystine and tryptophan (Table 1). Comparing the coefficient of variation (C.V.) between and within the batches showed that the largest increase of the C.V. due to deproteinization was for aspartic acid, cystine and tryptophan. However, the C.V. was greater than 10% only in the case of aspartic acid which was present in low concentrations.

3.1.2. Effect of the length of delay before removing the supernatant following the addition of SSA

Increasing the length of the delay before removing the supernatant had no significant influence on the concentrations of most amino acids (Table 2). Only the concentration of tryptophan decreased considerably (by approximately 16%) due to a delay of 30 min, and remained unchanged after this time.

3.1.3. Volume and internal standard effects when using different methods of deproteinization

Irrespective of the deproteinization method, approximately 0.1 g (weight after freeze-drying) from 1 ml of plasma precipitated . Therefore, the calculated volume of the supernatant was approximately 0.9 ml (Method 1) and approximately 1.9 ml (Method 2). The concentrations of the amino acids were greater in the larger volume of the supernatant than in the

Table 1
Effect of deproteinization on the variation coefficient (C.V.) and the C.V. within and between batches (n[batch] =5, n[analysis] =20). Only amino acids for which this effect was significant are shown

Amino acid	Concentration (μmol/l)	Significance[a]	C.V.(%)	
			Within-batch	Between-batch
Asp	9.13	***	7.2	12.7
Ser	65.1	*	4.2	5.9
Gln	246	*	2.2	2.7
Cit	58.0	*	2.2	3.3
Cyss	17.7	**	3.4	5.6
Trp	39.6	*	5.9	7.8

[a]Significance of the effect of deproteinization on the total analytical variation:
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

smaller volume (Table 3). Although the differences were founded to be significant ($P < 0.05$) for several amino acids, they were below 5% in most cases. For the hydrophobic amino acids, i.e., phenylalanine, tyrosine, leucine and tryptophan, these differences were remarkably high (up to 27%).

Addition of the internal standard (norleucine)

Table 2
Concentration of amino acids as a function of the delay time (0, 30 and 60 min) of removing the supernatant after addition of SSA (n=3)

Amino acid	Concentration (μmol/l)		
	0 min	30 min	60 min
Tau	28.0	27.5	28.5
Asp	7.8[a]	8.1[b]	8.2[ab]
Thr	158	151	156
Ser	113	112	113
Asn	63.7	61.0	62.5
Glu	56.1	54.2	55.2
Gln	214	204	207
Pro	78.6	77.6	78.4
Gly	398	398	394
Ala	249	249	246
Cit	81.9	80.8	77.6
Val	261	261	258
Cyss	4.8[a]	5.0[b]	4.9[b]
Met	27.9	27.0	27.2
Ile	141	140	144
Leu	151	150	150
Tyr	66.7	66.2	66.1
Phe	58.8	59.0	60.3
Orn	57.0[a]	58.6[b]	57.7[b]
Lys	104[a]	102[b]	102[b]
His	50.9	49.8	50.6
Trp	51.3[a]	43.9[b]	42.8[b]
Arg	94.2	94.3	90.3

[a,b] Means with different superscripts differ significantly ($P < 0.05$).

Table 3
Concentration of amino acids obtained using two different methods of deproteinization and the relative differences between them (internal standard was added after deproteinization)

Amino acid	Concentration (μmol/l)		II − I (%)
	I	II	
Tau	27.2	28.2	3
Asp	7.8	8.5	9
Thr	152	154	1
Ser	112	113	1
Asn	60.2[a]	62.5[b]	4
Glu	54.4	54.2	0
Gln	200	204	2
Pro	78.5	79.4	1
Gly	375[a]	390[b]	4
Ala	235[a]	245[b]	4
Cit	73.3[a]	77.3[b]	5
Val	248[a]	259[b]	4
Cyss	3.9	3.8	0
Met	25.2	26.3	3
Ile	133[a]	140[b]	5
Leu	141[a]	151[b]	7
Tyr	61.2[a]	65.8[b]	7
Phe	54.6[a]	60.5[b]	11
Orn	56.0	58.2	3
Lys	99.0[a]	104[b]	5
His	48.5[a]	51.2[b]	5
Trp	31.9[a]	40.2[b]	26
Arg	86.1	88.7	3

Method 1: 1 ml of plasma with 80 mg of solid SSA.
Method 2: 1 ml of plasma with 1 ml of SSA solution (80 mg/ml).
[a,b] Means with different superscript differ significantly ($P < 0.05$).

before deproteinization reversed the previously observed effects (Table 4). In this case, the amino acid concentrations in the smaller supernatant volume were greater. Differences in the concentrations of most amino acids between both methods were larger than 5% and, thus, greater than when the internal standard was added after deproteinization (Table 3). For the hydrophobic amino acids these differences were smaller compared to those obtained from samples where the internal standard was added after deproteinization.

Exclusively, when using Method 1 (and not Method 2), the results were clearly influenced by the presence of the internal standard added either before or after deproteinization.

Table 4
Concentration of amino acids using two different methods of deproteinization and differences between them (internal standard added before deproteinization)

Amino acid	Concentration (μmol/l)		I − II (%)
	I	II	
Tau	30.0[a]	27.1[b]	11
Asp	8.1	8.6	6
Thr	163[a]	152[b]	7
Ser	118[a]	113[b]	5
Asn	65.5[a]	60.9[b]	7
Glu	59.2[a]	54.2[b]	9
Gln	216[a]	198[b]	9
Pro	85.7[a]	78.4[b]	9
Gly	406[a]	383[b]	6
Ala	256[a]	242[b]	5
Cit	80.7[a]	75.7[b]	6
Val	270[a]	254[b]	6
Cyss	4.2	3.9	10
Met	27.8	26.7	4
Ile	144[a]	137[b]	5
Leu	155[a]	149[b]	4
Tyr	66.9[a]	64.6[b]	4
Phe	60.7	59.2	3
Orn	61.1	57.7	6
Lys	108.4[a]	101[b]	7
His	52.3	51.0	3
Trp	36.3[a]	39.9[b]	−9
Arg	91.7[a]	86.3[b]	6

$n = 3$ for both methods.
Treatment 1: 1 ml of plasma with 80 mg of solid SSA.
Treatment 2: 1 ml of plasma with 1 ml of SSA solution (80 mg/ml).
[a,b] Means with different superscripts differ significantly ($P < 0.05$).

3.2. Calibration

3.2.1. Influence of the standard solution on the calibration

Calibration of the three commercial standard solutions compared to a standard prepared by us revealed that the concentrations of several amino acids were significantly different ($P < 0.05$) from those given by the manufacturers (Table 5). Significant differences in the content of aspartic acid and asparagine contents in the LKB-standard were probably caused by degradation of asparagine. The results imply that there were clear differences between the commercial standards. In the case of histidine, calibration of samples with the Sigma standard or with the LKB standard led to systematic differences of approximately 10%.

3.3. Storage

3.3.1. Influence of temperature and neutralization of the solution on the concentration of amino acids

The concentration of most amino acids in deproteinized plasma showed no clear trend as a result of storage time, except for glutamine, asparagine, glutamic acid and aspartic acid, which were affected when stored at a temperature above −80°C. The rate of concentration change (dC) diminished by lowering the storage temperature (Table 6).

The most substantial degradation of glutamine to glutamic acid was observed in plasma stored above −40°C. This degradation was a first order reaction ($r > 0.98$). At −40°C glutamine was stable for at least 6 months. Storage at −80°C diminished the degradation to such an extent that the concentration of glutamine was stable for at least 1 year. The increase in the concentration of glutamic acid caused by the degradation of glutamine, was not of the first order ($r < 0.98$). In general, the increase in the concentration of glutamic acid was less then the decrease in the concentration of glutamine.

Asparagine was clearly less susceptible to degradation than glutamine. Only at 20°C, was the degradation of asparagine, and consequently, the increase in the concentration of aspartic acid of the first order ($r > 0.98$). Overall, the increase of aspartic acid was less then the decrease of asparagine.

Table 5
Concentration of amino acids in the standard solution obtained from Pierce, Sigma and LKB Pharmacia (n = 6)

Amino acid	Concentration (μmol/l)[a]		
	Pierce	Sigma	LKB
Tau	101.1	96.8 *	100.1
Asp	98.8	97.1 *	113.3 **
Hypro	100.1	101.5	101.2
Thr	101.3	99.8	100.7
Ser	104.2	101.3	104.7
Asn	-	-	85.2 *
Glu	98.5 *	94.7 *	96.1
Pro	91.8 *	99.2	99.0
Gly	104.4 *	100.7	102.9
Ala	99.5	97.8	99.0
Cit	99.0	97.2 *	97.8
Val	105.8 **	102.8 **	103.8
Cyss	99.5	97.6 **	49.7
Met	102.3 **	103.6 **	104.4
Ile	99.6	97.3 *	99.1
Leu	101.6 *	99.6	101.0
Tyr	102.4 **	99.8	101.1
Phe	102.5 **	100.3	100.3
Orn	101.7	97.9 *	99.8
Lys	99.1	96.1 **	103.6
His	100.8	98.4 *	109.4 **
Arg	92.7 **	100.0	99.6

All declared concentrations should be equal to 100 μmol/l, except for cystine in the standard of LKB, which should be 50 μmol/l.
[a]* Differences between the quantities declared by the manufacturers and those found by assay are significant at $P < 0.05$.
** Differences between the quantities declared by the manufacturers and those found by assay are significant at $P < 0.01$.

Neutralization of the samples before storage remarkably reduced the degradation of glutamine at −20°C (Table 7)

After 6 months small decreases of cystine and methionine were observed at storage temperatures above −40°C, which led to approximately 10% lower values.

3.3.2. Storage of non-deproteinized samples

Changes of amino acid concentrations in non-deproteinized plasma during storage, were very similar to those obtained for deproteinated plasma except for cystine (Table 8). After 17 days of storage at −20°C and at −40°C, cystine almost completely disappeared. The concentration of cystine was also lower than in deproteinized plasma and decreased rapidly after 6 months when stored at −80°C.

Table 6
Relative change (dC) of unstable amino acids in deproteinized plasma at several temperatures and the correlation coefficient (r) of the first order curve of the change.

Amino acid		Storage temperature (°C)				
		20	4	−20	−40	−80
Asp	dC[a]	0.60	0.1	0.08	−0.02	−0.02
	r	0.96	0.71	0.73	0.28	0.36
Asn	dC	−0.50	−0.07	−0.04	–[b]	–[b]
	r	0.99	0.93	0.89	–[c]	–[c]
Glu	dC	0.40	0.13	0.22	0.03	–[b]
	r	0.87	0.82	0.91	0.94	–[c]
Gln	dC	−16.0	−1.80	−0.22	−0.02	–[b]
	r	0.99	0.99	0.99	0.86	–[c]

First order curve in the form of $C_t = C_o\, e^{kt}$ where C_t is the concentration at time t; C_o is the initial concentration; and t is the storage time.
[a] dC in %/day.
[b] $|dC| < 0.01$.
[c] Correlation coefficient not calculated.

After 6 months at −20°C, the concentrations of all amino acids started to decrease, which led to approximately 15% lower values for most amino acids after 1 year.

4. Discussion

This study showed that systematic errors occur during blood plasma deproteinization, calibration,

Table 7
Relative change (dC) of unstable amino acids in deproteinized plasma after neutralization at several temperatures and the correlation coefficient (r) of the first order curve of the change

Amino acid		Storage temperature (°C)		
		−20	−40	−80
Asp	dC[a]	−0.09	−0.06	−0.05
	r	0.58	0.58	0.76
Asn	dC	–[b]	–[b]	–[b]
	r	–[c]	–[c]	–[c]
Glu	dC	–[b]	–[b]	–[b]
	r	–[c]	–[c]	–[c]
Gln	dC	–[b]	–[b]	–[b]
	r	–[c]	–[c]	–[c]

For an explanation see Table 6.

Table 8
Relative change of unstable amino acids in non-deproteinized plasma at −20°C, −40°C and −80°C and the correlation coefficient of the first order curve of the change

Amino acid		Storage temperature (°C)		
		−20	−40	−80
Asp	dC[a]	0.11	0.03	− 0.09
	r	0.78	0.35	0.70
Asn	dC	− 0.14	–[b]	–[b]
	r	0.99	–[c]	–[c]
Glu	dC	0.26	0.03	0.01
	r	0.64	0.44	0.43
Gln	dC	−0.06	–[b]	–[b]
	r	0.96	–[c]	–[c]
Cys	dC	− 14	−8.5	− 0.05
	r	0.98	0.88	0.93

For an explanation see Table 6.

and sample storage which can negatively influence comparisons of data from collaborative inter-laboratory studies.

In general, deproteinization with SSA is a very reproducible process. Only in the case of aspartic acid, cystine and tryptophan, does it lead to a clearly greater coefficient of variation. This is presumably due to a specific interaction of these amino acids with protein, especially with albumin, during deproteination, as mentioned for cystine and tryptophan [11–14]. Delay in the removal of the supernatant led to a decrease of the concentration of tryptophan, while the concentrations of the other amino acids remained unchanged.

Comparing different deproteinization methods showed that a small volume of the supernatant decreases the concentration of amino acids, probably due to a relative large inclusion or adsorption. Hydrophobic amino acids were more sensitive to losses than other amino acids. Deproteinization with acetone or acetonitrile, which increase the hydrophobicity of the matrix, leads to larger losses of hydrophilic amino acids [17]. Therefore, the losses may depend also on the hydrophobicity of the sample. Because of the different behaviour of amino acids, it is difficult to find an internal standard to correct for these losses, as was shown by the use of norleucine. Because of its large hydrophobicity, the use of norleucine led to an overcorrection for most amino acids. Therefore, it is better to use a deproteinization method that yields a larger supernatant volume, because the results are not influenced by the time of addition of the internal standard.

In the commercial standard solutions, the concentration of several amino acids were significantly different from the stated concentrations. Although they were within the confidence limits given by the manufacturers (4% for Sigma), they influenced the calibration of the samples. Therefore, the use of different commercial standard solutions causes systematic errors in the analysis of samples. This fact is also reported by another study [5]. Preparation of a standard by dissolving salts of the individual amino acids is a good approach to solving this problem.

Overall, the concentrations of the amino acids in deproteinated plasma were stable for at least 1 year, when stored at a temperature of −40°C or −80°C. Above this temperature, glutamine and asparagine degraded to glutamic acid and asparatic acid, respectively. This degradation was reduced by neutralization of the samples before storage. The degradation of glutamine that occurred above −40°C and that of asparagine that occurred at 20°C, was of the first order, but the rates were different from those reported by others [9,10]. Therefore, correction of the concentrations of glutamine and asparagine due to the length of storage is not advised. No changes were found for other amino acids, although indications that such changes occur are available from other studies [15,16]. Possible reasons for these differences can be ascribed to the use of plasma instead of serum, or of animal plasma instead of human plasma.

Storage of plasma in non-deproteinized or deproteinized form exerted no influence on amino acid concentration, except for cystine. Cystine concentration decreases very rapidly at temperatures above −80°C. Similar observations were also reported elsewhere [10,16]. This decrease was so rapid that after 17 days no cystine could be detected, so that a correction for this amino acid was impossible. In contrast to an earlier report [16], no significant increase in the concentration of most amino acids was found when non-deproteinized plasma was stored at a temperature of −20°C.

From our study, it can be concluded that systematic errors can be caused by using different methods of deproteinization and calibration, which

affect the concentrations determined for all amino acids. Particularly, differences in deproteinization and addition of the internal standards should be taken into consideration. A relatively large variation in the concentration of hydrophobic amino acids may indicate that there are systematic differences between the deproteinization methods. Accumulation of these errors may lead to systematic differences exceeding 10% between laboratories. This systematic difference may be even greater for the unstable amino acids under suboptimal storage conditions, and for cystine, if deproteinization and removal of the supernatant is delayed. Avoiding these errors should help to improve the intra-laboratory precision.

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