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Short Communication

Modification of the analysis of amino acids in pig plasma

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Abstract

Determination of amino acids in pig plasma with the classical ninhydrin system is influenced by the excessive amount of protein and lipophilic compounds in the sample, leading to a decline in resolution. This problem was eliminated by using 80 mg of sulphosalicylic acid per ml of plasma, and solid-phase extraction with a C_{18} cartridge as an additional clean up step. The latter resulted in significantly higher quantities of threonine, asparagine, glutamic acid, glutamine, glycine, alanine, valine and lysine, and lower levels of phenylalanine and tryptophan ($P < 0.05$). The use of a C_{18} cartridge had a minor effect on the analytical error.

1. Introduction

Determination of free amino acids in blood plasma with the classical ion-exchange method is widely used in biochemical and clinical research [1–3]. In general the analysis is preceded by deproteinization with 50 mg sulphosalicylic acid (SSA) per ml of plasma [4–13]. Following this procedure with pig plasma we observed an unacceptable decrease in resolution between asparagine, glutamic acid and glutamine probably due to contamination of the resin with white coloured residues. As a consequence, the column had to be regenerated after analysis of a few samples. Only one report on a similar problem was found in the literature, describing the removal of the lipophilic material from pig plasma by extraction with isooctan was mentioned [14].

Hypothesizing that the contamination we observed, was caused either by an ineffective deproteinization or by lipophilic compounds, we examined the effect of using a larger amount of SSA and of solid-phase extraction with a C_{18} cartridge. Furthermore, we investigated the influence of implementation of the C_{18} cartridge on the amount of amino acids in plasma of pigs and the analytical error of the determination.

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). Hydrochloric acid, analytical grade, was from

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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 with addition of a small volume of hydrochloric acid and stored at − 80°C. Before use, this solution was thawed and diluted with loading buffer, *i.e.* lithium buffer of pH 2.2.

Water used for the preparation of these standard solutions was deionized (Milli-Q water purification system, Millipore, Bedford, MA, USA).

2.2. Apparatus

The amino acid analyzer was an Alpha Plus 4151, with a pre-wash column as 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). The connection between the amino acid analyzer and the personal computer was an A/D converter Model 406 from Beckman.

Separation of the amino acids and urea was performed on a lithium high resolution column Series I Ultropac 8 resin (27 cm × 4.6 mm I.D.) from Pharmacia.

2.3. Chromatographic conditions and quantification

The elution programme (Table 1) resulted in a good separation of all amino acids and urea. Detection was performed by a colour reaction with ninhydrin which was 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 autosampler at 4°C.

Quantitation took place with the standard amino acid solution and internal standard correction. L-Norleucine was used as the internal standard.

2.4. Samples

Arterial and venous blood was obtained from individual pigs fitted with permanent catheters (vena porta, vena mesenterica and arteria mesenterica). The samples were collected into heparinized tubes and placed in melting ice until further treatment.

2.5. Sample pretreatment

Plasma was removed after centrifuging for 30 min at 2500 *g* and 4°C. Four methods of plasma pretreatment were investigated:

(1) The plasma with the internal standard passed through an activated C_{18} cartridge and

Table 1
Elution programme

Step	Time (min)	Buffer	Temperature (°C)	pH	Lithium concentration (*M*)
1	17.00	1	34	2.80	0.20
2	47.00	2	34	3.00	0.30
3	24.00	3	34	3.02	0.60
4	9.00	3	67	3.02	0.60
5	35.00	4	67	3.45	0.90
6	64.00	5	72	3.55	1.65
7	15.00	LiOH	85	-	0.30
8	5.00	1	72	2.80	0.20
9	56.00	1	35	2.80	0.20

was deproteinized with 80 mg of SSA per ml of plasma;
(2) The plasma with the internal standard passed through an activated C_{18} cartridge and was deproteinized with 40 mg of SSA per ml of plasma;
(3) The plasma was exclusively deproteinized with 80 mg of SSA per ml of plasma.
The plasma was centrifuged for 30 min at 2500 *g* and 4°C and 1.00 ml of supernatant was taken for further treatment.
(4) The plasma was deproteinized with 80 mg of SSA per ml of plasma, centrifuged as above and, with the internal standard, forced through an activated C_{18} cartridge to collect 1.00 ml of the solution.

Subsequently, irrespective of the method, 100 μl of lithium hydroxide was added to 1.00 ml of sample. In method 3, the internal standard was also added to the sample. The pH of the solution was adjusted to 2.2 with lithium hydroxide or sulphosalicylic acid. The sample 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 in a 80-μl sample vial with the loading buffer (pH 2.2).

2.6. Sample clean up

The C_{18} cartridges were Adsorbex 100 mg (Cat. No. 19864) from Merck. The cartridges were activated by rinsing with 0.70 ml methanol and 0.70 ml water and pre-washed with 0.50 ml sample solution. After this procedure the sample was forced through the cartridge.

2.7. Statistics

The resolution between two peaks was calculated as follows:

$$R_S = dt_R/2(W_1^{1/2}/2.354 + W_2^{1/2}/2.354)$$

where, R_S is resolution, dt_R is the difference in retention time between two peaks, and $W^{1/2}$ is the width of the peak on half height.

The statistical significance between the means from two methods and the variation of the two methods were evaluated by *t*-tests and *F*-test for small numbers of replicates [15].

3. Results

3.1. Effect of the sample pretreatment on the separation

Methods 1 and 4 still gave a good resolution after 10 samples (Fig. 1 A and D). By using methods 2 and 3 the resolution between glutamic acid and glutamine and between glycine and alanine decreased after 6 or 7 samples to such an extent that it was unacceptable for the analytical purpose, *i.e.* $R_S < 1$ (Fig. 1B and C).

3.2. Effect of pretreatment with the C_{18} cartridge on the levels of amino acids

The effect of the use of the C_{18} cartridge on the amount of amino acids detected was studied using the chemical standard solution and pig plasma samples. For the chemical standard solution, the use of the C_{18} cartridge tended to increase the level of amino acids (Table 2). For aspartic acid and arginine the increase was statistically significant ($P < 0.05$). In the case of plasma samples, significantly higher levels ($P < 0.05$) of threonine, asparagine, glutamic acid, glutamine, glycine, alanine, valine and lysine were found when the C_{18} cartridge was applied. The difference between the mean values obtained from both methods was less than 5%. The concentration of phenylalanine and tryptophan decreased significantly ($P < 0.05$) when the C_{18} cartridge was applied. In several samples the level of tryptophan decreased more than 50%.

No relationship was found between the degree of difference, the concentrations of the amino acids, the pig used to obtain plasma samples or the kind of blood vessel from which the samples were taken.

3.3. Effect of pretreatment with the C_{18} cartridge on the analytical error

The deviation in the level of most amino acids increased by using the C_{18} cartridge, especially

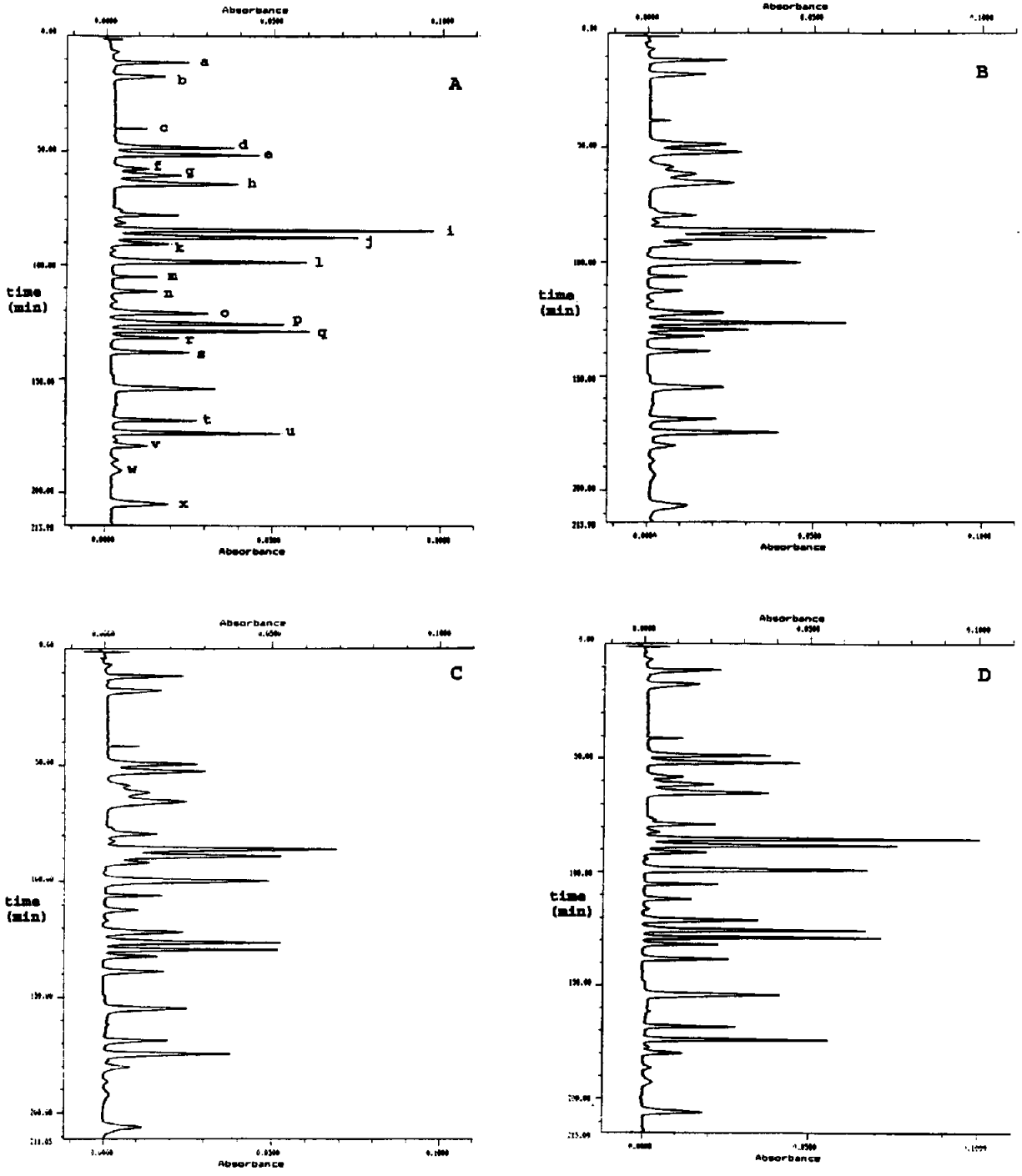

Fig. 1. Chromatograms of the tenth samples of series which were treated conform method 1 (A), 2 (B), 3 (C) or 4 (D). Amount injected 80 μl. Peak identification code: a, taurine (566 ng); b, urea (6215 ng); c, aspartic acid (96.8); d, threonine (782); e, serine (850); f, asparagine (369); g, glutamic acid (744); h, glutamine (1662); i, glycine (1195); j, alanine (1201); k, citrulline (590); l, valine (1633); m, cystine (279); n, methionine (352); o, isoleucine (916); p, leucine (1551); q, norleucine (internal standard) (1010); r, tyrosine (603); s, phenylalanine (705); t, ornithine (452); u, lysine (1293); v, histidine (349); w, tryptophan (348); x, arginine (944). Values in brackets give the amount of the compounds injected in ng.

Table 2
Mean levels of amino acids in a standard solution and plasma samples prepared with or without implementation of a C_{18} cartridge

Amino acid	Concentration (μmol/l)			
	Chemical standard ($n = 5$)		Samples ($n = 10$)	
	without C_{18}	with C_{18}	without C_{18}	with C_{18}
Taurine	95.8	96.5	54.5	54.8
Urea	991	1010	3408	3459
Aspartic acid	99.2[a]	100[b]	21.0	22.6
Threonine	97.6	99.0	420[a]	435[b]
Serine	98.8	100	183	192
Asparagine	99.0	98.1	95.8[a]	99.8[b]
Glutamic acid	99.7	99.9	184[a]	195[b]
Glutamine	98.9	100	358[a]	373[b]
Glycine	99.2	99.9	1022[a]	1070[b]
Alanine	98.4	97.9	323[a]	334[b]
Citrulline	98.2	98.7	81.4	81.6
Valine	99.5	99.6	498[a]	520[b]
Cystine	98.8	99.1	48.0	51.1
Methionine	98.4	98.3	39.8	42.1
Isoleucine	100	99.8	199	202
Leucine	101	100	292	296
Tyrosine	99.6	100	135	141
Phenylalanine	98.8	99.9	110[a]	100[b]
Ornithine	100	101	116	118
Lysine	79.9	80.1	271[a]	281[b]
Histidine	99.1	98.9	77.0	80.7
Tryptophan	32.7	33.6	51.1[a]	30.5[b]
Arginine	100[a]	103[b]	126	132
Hydroxyproline	104	104	34.5	35.9
Proline	76.8	75.7	220	228

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

with the plasma sample (Table 3). This increase appeared to be statistically significant ($P < 0.05$) for ornithine in both the standard and the sample and for glycine and urea in the sample. For cystine, hydroxyproline and proline, the use of the cartridge led to a remarkable decrease of the relative standard deviation.

4. Discussion

Our problem of column contamination was solved by using a higher dose of the deproteinizing agent (SSA) in combination with an apolar extraction (C_{18} cartridge). Since the implementation of the C_{18} cartridge requires larger volumes of plasma (3 ml), it was preferred to first deproteinize the samples, leading to an increased sample volume (method 4).

The use of the C_{18} cartridge led to statistically significant higher levels of some amino acids ($P < 0.05$), particularly those with a high concentration in the sample (*i.e.* glycine, valine, glutamine and threonine). The levels of phenylalanine and tryptophan were significantly lowered ($P < 0.05$) because of their hydrophobic character, caused by the free phenyl group.

The low recovery of tryptophan was the only major disadvantage of this method. Thus, an alternative method for the determination of free tryptophan in plasma samples would be needed. Additionally, another deproteinizing agent ought to be implemented because SSA frees tryptophan bound to albumin [16,17].

Table 3
Relative standard deviation of the analysis of a standard solution and a plasma sample prepared with or without implementation of a C_{18} cartridge

Amino acid	Relative standard deviation ($n = 5$) (%)			
	Chemical standard		Sample	
	without C_{18}	with C_{18}	without C_{18}	with C_{18}
Taurine	2.4	1.7	1.2	3.3
Urea	2.4	10	0.6[a]	3.5[b]
Aspartic acid	0.6	0.4	11	15
Threonine	3.0	2.3	4.9	3.8
Serine	1.7	2.0	2.9	5.5
Asparagine	1.6	1.0	7.5	6.3
Glutamic acid	1.0	1.2	2.0	4.3
Glutamine	1.5	1.0	1.1	3.0
Glycine	0.7	2.1	1.0[a]	3.2[b]
Alanine	1.1	2.3	2.0	3.0
Citrulline	1.0	0.7	5.7	2.3
Valine	0.6	1.4	1.2	3.5
Cystine	0.9	0.7	4.0	1.9
Methionine	1.0	1.3	1.8	3.0
Isoleucine	0.7	1.1	1.1	1.6
Leucine	1.4	1.6	1.2	0.7
Tyrosine	1.0	0.5	2.0	2.2
Phenylalanine	0.6	1.0	2.6	4.5
Ornithine	0.3[a]	2.0[b]	1.1[a]	4.8[b]
Lysine	0.5	1.4	1.8	2.8
Histidine	0.8	1.1	3.3	5.6
Tryptophan	5.1	4.1	10	10
Arginine	0.7[a]	2.5[b]	3.7	2.9
Hydroxyproline	4.3	4.5	18	9.2
Proline	4.4	3.3	7.3	4.2

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

Application of the C_{18} cartridge led to a larger analytical error, but the relative standard deviation did not exceed 5%, which compares well with the accuracy found in other studies on these analytes [18–21].

It seems that the analytical problem depends on the composition of the plasma and, therefore, on physiological (live weight, sex, age) and nutritional status of the pig and the time between feeding and sampling. Notably dietary fat and protein and the age of the pig effect the level of plasma cholesterol [22–25]. Until these relationships are clearly established, it is advisable to implement the above described method 4 to avoid column contamination. The cost and time of the analysis are not remarkably increased by the introduction of the C_{18} cartridge used to clean up the pig plasma samples.

5. References

[1] D.H. Spackman, W.H. Stein and S. Moore, *Anal. Chem.*, 30 (1958) 1190.
[2] A.P. Williams, *J. Chromatogr.*, 373 (1986) 175–190.
[3] I.M. Moodie, G.S. Shephard and D. Labadarios, *J. High. Res. Chromatogr.*, 12 (1989) 509–516.
[4] A. Monidini, G. Bongiovannie, S. Fumero and L. Rossi, *J. Chromatogr.*, 74 (1972) 255–263.
[5] R.W. Hubbard, J.G. Chambers and A. Sanchez, *J. Chromatogr.*, 431 (1988) 163–169.
[6] M.S. Edmonds and D.H. Baker, *J. Nutr.*, 117 (1987) 1396–1401.

[7] N.E.P Deutz, P.L.M. Reijven, M.C.F. Bost, C.L.H. van Berlo and P.B. Soeters, *Gastroenterol.*, 101 (1991) 1613–1620.
[8] H. Hagemeister, K.E. Scholz-Ahrens, H. Schulte-Coerne and C.A. Barth, *J. Nutr.*, 120 (1990) 1305–1311.
[9] E.R. Chavez and H.S. Bayley, *Br. J. Nutr.*, 36 (1976) 189–198.
[10] R.H. Wilson and J. Leibholz, *Br. J. Nutr.*, 45 (1981) 301–319.
[11] T.T. Daabees, D.W. Andersen, W.L. Zike, L.J. Filer and L.D. Stegink, *J. Nutr.*, 114 (1984) 1541–1547.
[12] M.S. Edmonds and D.H. Baker, *J. Animal Sci.*, 64 (1987) 1664–1671.
[13] J.H.G. Holmes, H.S. Bayley, P.A. Leadbeater and F.D. Horney, *Br. J. Nutr.*, 32 (1974) 479–489.
[14] L.A.P. Hoogenboom, A. Pijpers, P.W. van de Steeg, M.M.L. Aerts, J.H.M. Verheijden and H.A. Kuiper, *Tijdschrift voor Diergeneeskunde*, 114 (1989) 1054–1057.
[15] D.L. Massart, B.G.M. Vandeginste, S.N. Deming, Y. Milchotte and L. Kaufman, *Chemometrics*, Elsevier, Amsterdam, 1989, pp. 33–57.
[16] T.L. Perry, S. Hansen and J. Kennedy, *J. Neurochem.*, 24 (1975) 587–589.
[17] F. Pohlandt, *J. Pediatr.*, 92 (1978) 617–623.
[18] A. Tizianello, G. De Ferrari, G. Garibotto, G. Gurreri and C. Robaudo, *J. Clin. Invest.*, 65 (1980) 1162–1173.
[19] B.S. Lindblad, G. Alfvén and R. Zetterstrom, *Acta Paediatr. Scand.*, 67 (1978) 659–663.
[20] J.P. Milsom, M.Y. Morgan and S. Sherlock, *Metabolism*, 28 (1979) 313–319.
[21] D.M. Gregory, D. Sovetts, C.L. Clow and C.R. Shriver, *Metabolism*, 35 (1986) 967–969.
[22] M.H. Jurgens and E.R. Peo, *J. Animal Sci.*, 30 (1970) 894.
[23] R.H. Barnes, E. Kwong, G. Fiala, M. Rechcigl, R.N. Lutz and J.K. Loosli, *J. Nutr.*, 69 (1959) 261.
[24] G.L. Allee, D.H. Baker and G.A. Leveille, *J. Nutr.*, 101 (1971) 1415–1422.
[25] H.J. Mersmann, W.G. Pond and J.T. Yen, *Growth*, 46 (1982) 189–192.