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Age Related Reference Values for Urinary Free Amino Acids: A Simple Method of Evaluation

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Summary: The urinary excretion of free amino acids has been studied on 339 normal subjects, using ion exchange chromatography. Age related reference values were obtained. A simple method for their evaluation is discussed, and the application of the method to the detection of increased glycinuria, resulting from *n*-dipropyl acetate therapy, is described.

Altersbezogene Referenzwerte für freie Aminosäuren im Harn: Eine einfache Methode für ihre Bewertung

Zusammenfassung: Die Ausscheidung freier Aminosäuren im Harn wurde bei 339 normalen Probanden mit Hilfe der Ionenaustauschchromatographie untersucht. Altersbezogene Referenzwerte wurden erhalten. Eine einfache Methode für ihre Bewertung und deren Anwendung zum Nachweis einer erhöhten Glycinurie bei Therapie mit *n*-Dipropylacetat wird diskutiert.

Introduction

Aminoaciduria has been the subject of numerous reports, but there are relatively few reference values for normal subjects, obtained by column chromatography (1, 2). The present work describes a simple procedure for the determination of these values.

Materials and Methods

Subjects studied were hospitalized children and adults. Patients with enzymatic defects and premature infants (3), and patients with renal failure or with other diseases for which variations of aminoaciduria have been described: muscular (4), bone (5), skin (6, 7) and eye (8) diseases, were all excluded from the study. We also omitted patients whose therapy is known to induce either methodological interferences in amino acid chromatography (9) or aminoaciduria variations (10–15). All subjects studied (191 males and 148 females) were in good nutritional state and their protein intake was normal. Physical activity was judged to be about the same for all subjects. Aminoaciduria was also studied on one subject during 5 days, and on 10 subjects receiving *n*-dipropyl acetate therapy.

The single morning urine sample was collected in a bottle containing a thymol crystal. The samples were either immediatly prepared for analysis or kept at – 24 °C. This storage does not significantly modify aminoaciduria measurements (16). Creatinine was assayed by the *Jaffe*'s reaction (17). 50 mg of crystallized sulfosalicylic acid were added to 1 ml of urine and the sample was then centrifuged 10 min at 500 *g*. A part of the supernatant (corresponding to 50 µg of creatinine) was used for the chromatography, and norleucine (62.5 nmoles) was added as internal standard. The column chromatography was performed on a Technicon TSM Amino Acid Analyzer. The total analysis employed only one column (18) length 35 cm, internal diameter 0.63 cm with chromobeads Type C3 resin. The column was eluted by a discontinuous gradient (19) of 0.1 mol/l citric acid – sodium citrate buffers: for

70 min with pH 3.25 buffer containing per liter 60 ml of methycellosolve and 5 ml of thiodiethanol; then for

40 min with pH 4.25 buffer; followed by

110 min with pH 6.0 buffer containing NaCl 0.55 mol/l.

Elution was performed at 57.5 °C with a 0.55 ml/min flow. This method separated: phospho-ethanolamine, taurine, hydroxyproline, aspartic acid, threonine, serine, glutamic acid + glutamine, citrulline, proline, glycine, alanine, cystine, valine, cystathionine, methionine, alloisoleucine, isoleucine, leucine, tyrosine, phenylalanine, hydroxylysine, histidine, lysine and arginine. When the pH of the buffer was shifted from 4.25 to 6.0, homocystine, β-alanine, β-aminoisobutyric acid and γ-aminoisobutyric acid were eluted together, after the peak of phenylalanine. Every tenth sample through the analyzer was a mixture of the 17 commonest amino acids and the internal standard (62.5 nmoles of each). Only the fourteen aminoacids representing more of 90% of the whole aminoaciduria (1) were currently measured: threonine, serine, glutamic acid (+ glutamine), proline, glycine, alanine, cystine, valine, leucine, tyrosine, phenylalanine, histidine, lysine. The results were expressed either as µmol/g creatinine or as a fraction of the sum of all 14 amino acids measured.

Results

Results obtained from the single morning urine samples of one subject are gathered in table 1. The results expressed in µmol/g creatinine show a relative stability, the largest variations being those of lysine, threonine,

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Tab. 1. Urinary excretion of free amino acids in one subject during 5 days in μmol/g creatinine and as a fraction of the sum of amino acids (see Results).

Day	1	2	3	4	5	Mean ± S. D.	Coefficients of variation (%)
Threonine							
(μmol/g creatinine)	155	114	107	90	101	113.4 ± 28.7	25.3
(fraction of the sum)	0.062	0.056	0.058	0.049	0.052	0.055 ± 0.006	10.9
Serine							
(μmol/g creatinine)	163	126	125	139	153	141.2 ± 19.3	13.7
(fraction of the sum)	0.066	0.061	0.067	0.075	0.078	0.069 ± 0.008	11.6
Glutamic acid + glutamine							
(μmol/g creatinine)	163	144	127	168	180	156.4 ± 24.2	15.5
(fraction of the sum)	0.066	0.071	0.068	0.091	0.093	0.078 ± 0.015	19.2
Proline	traces	traces	traces	traces	traces		
Glycine							
(μmol/g creatinine)	535	466	418	417	465	460.2 ± 55.7	12.1
(fraction of the sum)	0.216	0.227	0.225	0.225	0.239	0.226 ± 0.009	4.0
Alanine							
(μmol/g creatinine)	119	76	82	76	80	86.6 ± 21.1	24.4
(fraction of the sum)	0.048	0.037	0.044	0.041	0.041	0.042 ± 0.005	11.9
$^1/_2$ Cystine							
(μmol/g creatinine)	73	47	49	55	62	57.2 ± 12.2	21.3
(fraction of the sum)	0.029	0.023	0.026	0.029	0.032	0.028 ± 0.004	14.3
Valine							
(μmol/g creatinine)	28	25	25	27	23	25.6 ± 2.3	9.0
(fraction of the sum)	0.011	0.012	0.013	0.015	0.012	0.013 ± 0.001	13.1
Leucine							
(μmol/g creatinine)	39	33	33	26	29	32.0 ± 5.7	17.0
(fraction of the sum)	0.016	0.016	0.018	0.014	0.015	0.016 ± 0.002	10.6
Tyrosine							
(μmol/g creatinine)	107	100	86	98	78	93.8 ± 13.4	14.3
(fraction of the sum)	0.043	0.049	0.046	0.053	0.040	0.046 ± 0.005	10.9
Phenylalanine							
(μmol/g creatinine)	49	38	38	40	34	39.8 ± 6.4	16.1
(fraction of the sum)	0.020	0.019	0.020	0.021	0.017	0.019 ± 0.002	8.9
Histidine							
(μmol/g creatinine)	654	542	480	493	500	533.8 ± 82.1	15.4
(fraction of the sum)	0.264	0.264	0.264	0.266	0.257	0.263 ± 0.004	1.5
Lysine							
(μmol/g creatinine)	395	343	286	225	238	297.4 ± 82.7	27.8
(fraction of the sum)	0.159	0.167	0.154	0.121	0.122	0.145 ± 0.025	17.2

alanine and cystine. The coefficients of variation become smaller when the concentration of each amino acid is expressed as a fraction of the sum of the 14 amino acids measured (paired *Student*'s test: $p < 0.01$).

The results expressed in μmol/g creatinine for 339 subjects are shown in tables 2 and 3. All subjects over 13 years old have been gathered in the same group. The aminoaciduria decreases with the increasing age of the subjects, especially for those amino acids that show the highest excretion. The large variance observed for each amino acid seems to be related to individual variations, both of aminoaciduria and of creatininuria. In table 4, each amino acid concentration is expressed as a fraction of the sum of currently measured amino acids. The variance observed is especially large in the first and second groups (less than 6 months of life). Significant increases of glutamic acid + glutamine ($p < 10^{-3}$), tyrosine ($p < 10^{-7}$), phenylalanine ($p < 10^{-9}$), histidine ($p < 10^{-4}$), valine ($p < 10^{-3}$) and a significant decrease of glycinuria ($p < 10^{-3}$) are observed between the first and the second groups (table 3). In contrast, all fractions are almost constant over the age of 6 months, except for serine and lysine. The coefficients of variation observed in table 3 and 4 are compared by paired *Student*'s test (table 5). For all amino acids except leucine, these coefficients are smaller when the results are expressed as a fraction of the sum of amino acids measured.

We had previously shown that glycinuria was increased by *n*-dipropyl acetate therapy (12, 15); this increase

Tab. 2. Normal range observed for urinary excretion of free amino acids (in μmol/g creatinine).

	0 to 1 month	1 to 6 months	6 to 12 months	1 to 2 years	2 to 4 years	4 to 7 years	7 to 10 years	10 to 13 years	over 13 years
Threonine	218 – 1486	254 – 1379	206 – 1146	170 – 976	149 – 810	107 – 655	108 – 479	107 – 378	105 – 549
Serine	287 – 3844	382 – 2778	342 – 2117	379 – 1565	338 – 1202	225 – 901	183 – 894	141 – 705	125 – 631
Glutamic acid + glutamine	325 – 1994	531 – 2059	190 – 2250	322 – 1631	260 – 1380	213 – 1212	131 – 591	105 – 784	103 – 586
Proline	70 – 2300	0 – 600	0 – 300	0 – 270	0 – 220	0 – 120	0 – 60	0 – 60	0 – 60
Glycine	1017 – 10417	1315 – 8804	1422 – 5754	1025 – 4596	1026 – 4310	761 – 3119	497 – 1713	256 – 2105	422 – 2063
Alanine	554 – 2957	613 – 2874	428 – 2064	389 – 1497	255 – 1726	212 – 970	144 – 594	135 – 899	119 – 574
$^1/_2$ Cystine	127 – 901	131 – 750	139 – 631	106 – 488	124 – 492	117 – 448	84 – 348	103 – 301	82 – 392
Valine	38 – 230	63 – 237	61 – 331	40 – 183	59 – 191	41 – 95	26 – 110	21 – 113	22 – 74
Leucine	41 – 220	26 – 209	31 – 183	28 – 136	34 – 217	20 – 105	23 – 88	17 – 90	20 – 59
Tyrosine	83 – 401	103 – 690	103 – 944	96 – 542	111 – 591	113 – 480	114 – 342	96 – 383	77 – 239
Phenylalanine	62 – 220	49 – 391	107 – 367	57 – 314	80 – 306	43 – 260	35 – 159	28 – 146	27 – 120
Histidine	365 – 2857	727 – 3167	877 – 3346	850 – 3005	1009 – 2524	585 – 2256	277 – 1651	346 – 1825	168 – 1422
Lysine	454 – 2313	284 – 1507	391 – 1661	352 – 1083	279 – 1017	144 – 782	295 – 963	106 – 819	200 – 887
Total	6437 – 26238	5488 – 19893	6707 – 19191	4492 – 13097	3964 – 12752	2408 – 9821	2234 – 10928	1143 – 7885	1966 – 8256

Tab. 3. Means (± SD) of urinary excretion of free amino acids (in μmol/g creatinine).

	0 to 1 month	1 to 6 months	6 to 12 months	1 to 2 years	2 to 4 years	4 to 7 years	7 to 10 years	10 to 13 years	Over 13 years
Threonine	670 ± 273	705 ± 303	511 ± 247	441 ± 187	449 ± 187	295 ± 145	222 ± 103	246 ± 82	216 ± 103
Serine	1486 ± 730	1501 ± 603	1140 ± 484	828 ± 320	757 ± 250	524 ± 180	417 ± 206	413 ± 152	306 ± 139
Glutamic acid + glutamine	931 ± 446	1180 ± 453	1097 ± 504	835 ± 367	661 ± 290	524 ± 254	368 ± 142	370 ± 164	265 ± 121
Proline	70→2300	< 600	< 300	< 270	< 220	< 120	< 60	< 60	< 60
Glycine	4831 ± 2325	3646 ± 1704	2777 ± 1137	2138 ± 853	2364 ± 964	1620 ± 745	977 ± 402	1194 ± 501	931 ± 414
Alanine	1677 ± 691	1521 ± 551	1147 ± 420	796 ± 295	728 ± 348	463 ± 217	353 ± 145	417 ± 193	294 ± 126
$^1/_2$ Cystine	458 ± 223	374 ± 165	359 ± 131	303 ± 117	260 ± 101	236 ± 91	175 ± 69	188 ± 57	179 ± 80
Valine	111 ± 50	132 ± 40	141 ± 61	104 ± 40	104 ± 30	68 ± 17	57 ± 23	56 ± 20	39 ± 13
Leucine	106 ± 42	102 ± 48	101 ± 44	79 ± 26	79 ± 39	58 ± 23	49 ± 18	44 ± 16	33 ± 9
Tyrosine	241 ± 81	390 ± 140	384 ± 174	320 ± 107	271 ± 121	264 ± 93	218 ± 78	189 ± 71	142 ± 43
Phenylalanine	122 ± 46	183 ± 78	196 ± 72	154 ± 55	164 ± 68	107 ± 49	87 ± 38	76 ± 25	60 ± 21
Histidine	1594 ± 595	1800 ± 595	1903 ± 652	1688 ± 653	1527 ± 369	1217 ± 459	878 ± 381	896 ± 409	688 ± 316
Lysine	941 ± 416	736 ± 289	769 ± 278	619 ± 200	530 ± 184	467 ± 184	511 ± 216	424 ± 199	410 ± 176
Total	15403 ± 5327	12097 ± 3641	11053 ± 3856	7574 ± 2191	7960 ± 2511	5351 ± 2148	4713 ± 2030	4609 ± 1727	4023 ± 176
Number of subjects	48	35	33	45	34	37	24	32	51

was subsequently found by others (13, 14). In 10 subjects the increase of glycinuria (table 6) was more significant when expressed as a fraction ($p < 10^{-3}$) than as μmol/g creatinine ($p < 10^{-2}$).

Discussion

Not all amino acids were measured in this investigation. Some (taurine, β-aminoisobutyric acid, tryptophan) were omitted for technical reasons, the elution of the resin being adapted to a screening program. Others were omitted because they are poorly excreted in normal subjects (hydroxyproline, cystathionine, methionine, isoleucine, ornithine, arginine), but these are all separated and can be measured if their concentrations are increased.

A single morning urine sample is normally used for the determination of reference values for aminoaciduria (21), and the amino aciduria is normally related to creatinine excretion. Creatinine concentration was claimed to be relatively constant in the single morning urine sample (20, 21). This assumption is certainly true for any one subject, the amino acid excretion showing little variation during several days (table 1). But the variation of muscular weight with age and sex causes the results for different individuals to be more scattered (tables 2 and 3). Moreover the immaturity of renal amino acid transport systems (22, 23) may explain the differences observed between the first group of subjects (less than 1 month old) and the others. The use of the sum of predominant amino acids in the excretion pattern as a criterion significantly decreases the variances of results (table 5). The amino acid excretion related to the creatininuria is a useful index for the diagnosis of aminoacidopathies and amino-aciduria, but it is inadequate for the appraisal of small changes in one or several amino acids. Thus the effect of *n*-dipropyl acetate therapy on glycinuria appears more dramatic when expressed as a fraction change. When results are expressed in μmol/g creatinine (table 6), glycinuria overlaps the limit of two standard deviations in 6 cases only; it stays between $\bar{x}$ + 1 S. D. and $\bar{x}$ + 2 S. D. in one case; it remains between $\bar{x}$ and $\bar{x}$ + 1 S. D. in two cases and is even inferior to $\bar{x}$ in one case. When results are expressed as a fraction glycinuria is always over $\bar{x}$ + 1 S. D. and in 8 cases, overlaps $\bar{x}$ + 2 S. D. This gives a better statistical significance in a t-test with paired series $p < 0.001$ ($t = 5.53$), whereas $p < 0.01$ ($t = 3.54$) for the former method.

The method of expression described here makes it possible to study moderate variations in the excretion of only some amino acids; such changes may be spontaneous or due to drug administration. It also removes the need to assay aminoaciduria and creatininuria simultaneously on a total 24 hour urine.

Tab. 4. Urinary excretion of free amino acids expressed as a fraction of the sum of 14 amino acids (see Methods).

	0 to 1 month	1 to 6 months	6 to 12 months	1 to 2 years	2 to 4 years	4 to 7 years	7 to 10 years	10 to 13 years	Over 13 years
Threonine	0.057 ± 0.020	0.058 ± 0.015	0.053 ± 0.021	0.054 ± 0.018	0.048 ± 0.013	0.046 ± 0.013	0.048 ± 0.007	0.053 ± 0.007	0.050 ± 0.012
Serine	0.106 ± 0.025	0.115 ± 0.025	0.113 ± 0.027	0.099 ± 0.023	0.100 ± 0.017	0.094 ± 0.020	0.086 ± 0.013	0.093 ± 0.011	0.081 ± 0.018
Glutamic acid + glutamine	0.067 ± 0.029	0.097 ± 0.033	0.103 ± 0.036	0.101 ± 0.024	0.087 ± 0.027	0.088 ± 0.023	0.084 ± 0.020	0.090 ± 0.017	0.084 ± 0.019
Proline	0.038 ± 0.016	< 0.045	< 0.025	< 0.017	< 0.015	< 0.012	< 0.014	< 0.012	< 0.014
Glycine	0.343 ± 0.069	0.280 ± 0.056	0.230 ± 0.069	0.249 ± 0.050	0.245 ± 0.041	0.240 ± 0.040	0.213 ± 0.053	0.255 ± 0.036	0.238 ± 0.051
Alanine	0.119 ± 0.027	0.123 ± 0.028	0.102 ± 0.038	0.098 ± 0.030	0.098 ± 0.026	0.084 ± 0.021	0.085 ± 0.022	0.079 ± 0.023	0.078 ± 0.018
½ Cystine	0.034 ± 0.015	0.030 ± 0.012	0.038 ± 0.013	0.039 ± 0.012	0.033 ± 0.007	0.038 ± 0.009	0.033 ± 0.012	0.032 ± 0.012	0.039 ± 0.012
Valine	0.008 ± 0.003	0.011 ± 0.003	0.013 ± 0.004	0.012 ± 0.004	0.012 ± 0.003	0.013 ± 0.003	0.013 ± 0.005	0.012 ± 0.003	0.013 ± 0.005
Leucine	0.008 ± 0.003	0.008 ± 0.003	0.009 ± 0.004	0.010 ± 0.003	0.009 ± 0.004	0.011 ± 0.004	0.012 ± 0.003	0.010 ± 0.003	0.010 ± 0.004
Tyrosine	0.019 ± 0.007	0.031 ± 0.009	0.039 ± 0.008	0.041 ± 0.008	0.041 ± 0.010	0.047 ± 0.012	0.047 ± 0.017	0.037 ± 0.011	0.041 ± 0.010
Phenylalanine	0.009 ± 0.004	0.015 ± 0.006	0.019 ± 0.006	0.018 ± 0.005	0.018 ± 0.005	0.017 ± 0.005	0.019 ± 0.004	0.017 ± 0.005	0.018 ± 0.006
Histidine	0.111 ± 0.033	0.147 ± 0.033	0.195 ± 0.044	0.190 ± 0.033	0.183 ± 0.044	0.202 ± 0.040	0.197 ± 0.028	0.202 ± 0.030	0.194 ± 0.040
Lysine	0.075 ± 0.030	0.061 ± 0.019	0.087 ± 0.032	0.073 ± 0.014	0.079 ± 0.028	0.093 ± 0.025	0.094 ± 0.034	0.093 ± 0.023	0.106 ± 0.027
Number of subjects	34	31	25	35	22	29	14	14	37

Tab. 5. Comparison of coefficients of variation observed in tables 3 and 4.

	CV for giving the results in μmol/g creatinine (%)	as a fraction of the sum of amino acids (%)	Paired *Student*'s test	P
Threonine	43.6 ± 5.0	26.8 ± 8.8	6.05	< 0.001
Serine	41.0 ± 6.0	20.0 ± 4.3	9.62	< 0.001
Glutamic acid + glutamine	44.1 ± 3.6	28.7 ± 7.7	5.91	< 0.001
Glycine	43.3 ± 3.0	20.4 ± 4.7	11.17	< 0.001
Alanine	41.8 ± 4.5	27.6 ± 4.5	5.94	< 0.001
$^1/_2$ Cystine	40.0 ± 5.3	33.2 ± 7.4	2.64	< 0.02
Valine	35.6 ± 6.8	31.0 ± 6.2	2.65	< 0.02
Leucine	39.2 ± 6.9	36.1 ± 6.7	1.30	NS
Tyrosine	36.9 ± 5.0	27.3 ± 6.2	3.18	< 0.01
Phenylalanine	39.1 ± 4.4	31.6 ± 6.9	3.36	< 0.001
Histidine	37.8 ± 6.9	20.6 ± 4.8	4.98	< 0.001
Lysine	39.8 ± 4.8	30.6 ± 6.9	3.58	< 0.01

Tab. 6. Effect of *n*-dipropyl acetate therapy on glycinuria: Controls were the same age as the corresponding patients; results are expressed by mean ± SD.

Age	Glycine expressed in μmol/g creatinine		as a fraction of the sum of 14 amino acids (see results)		Total aminoaciduria (μmol/g creatinine)	
	Subjects	Controls	Subjects	Controls	Subjects	Controls
5 years	905	1620 ± 745	0.570	0.240 ± 0.040	1594	5351 ± 2148
3 years	4630	2364 ± 964	0.420	0.245 ± 0.041	11023	7960 ± 2511
6 years	3750	1620 ± 745	0.590	0.240 ± 0.040	6378	5351 ± 2148
9 years	1364	977 ± 402	0.550	0.213 ± 0.053	2463	4713 ± 2030
5 years	3125	1620 ± 745	0.450	0.240 ± 0.040	6925	5351 ± 2148
3 years	6800	2364 ± 964	0.390	0.245 ± 0.041	17435	7960 ± 2511
14 months	7412	2138 ± 853	0.370	0.249 ± 0.050	20032	7574 ± 2191
11 years	2604	1194 ± 501	0.320	0.255 ± 0.036	8137	4609 ± 1727
6 years	3077	1620 ± 745	0.368	0.240 ± 0.040	8362	5351 ± 2148
3 years	3912	2364 ± 964	0.300	0.245 ± 0.041	13040	7960 ± 2511

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