Amino Acids (1998) 15:235–239

Amino
Acids

Total plasma homocysteine as part of the routine aminogram by ion-exchange chromatography

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Accepted December 12, 1997

Summary. Ion-exchange chromatography (IEC) with ninhydrin post-column derivatisation is the only technique available for the assay of total, (free plus bound), cysteine and homocysteine which also enables the routine measurement of all other commonly occurring amino acids. IEC assay of total cysteine and homocysteine typically involves incubating buffered plasma for 60 minutes at 37°C with dithiothreitol (DTT), but these assay conditions significantly extend total analysis time and compromise other amino acid values, notably glutamine and glutamate. However, it is possible to carry out the DTT reduction in plasma virtually instantaneously and without additional buffering, thus preserving the integrity of other diagnostically important amino acids. Assay precision is adequate for cardiovascular risk assessment.

Keywords: Amino acids – Homocysteine – Ion-exchange chromatography – Analysis

Introduction

Diagnosis and management of inborn errors of transulphuration (cystathionine synthase deficiency), by use of IEC has traditionally measured free homocystine (Hcy_2), cystine (Cys_2) and possibly the Cys-Hcy mixed disulphide. These compounds are notoriously subject to variable recovery according to sample history and this may be significant where values are low, e.g. following treatment. In mild conditions where homocysteine (Hcy) concentrations are below the protein binding capacity of about 140 μmol/L (Bonham et al., 1997; Ueland et al., 1996), then the lack of free Hcy_2 in the plasma not only makes the urinary nitroprusside screening test insensitive but may result in erroneous conclusions from the apparently zero plasma concentration (Kang et al., 1979). Clinically, the measurement of total homocysteine (tHcy) is the most sensitive indicator for disorders of homocysteine metabolism (Bonham et al., 1997) and the measurement of the free aminothiols, Cys_2,

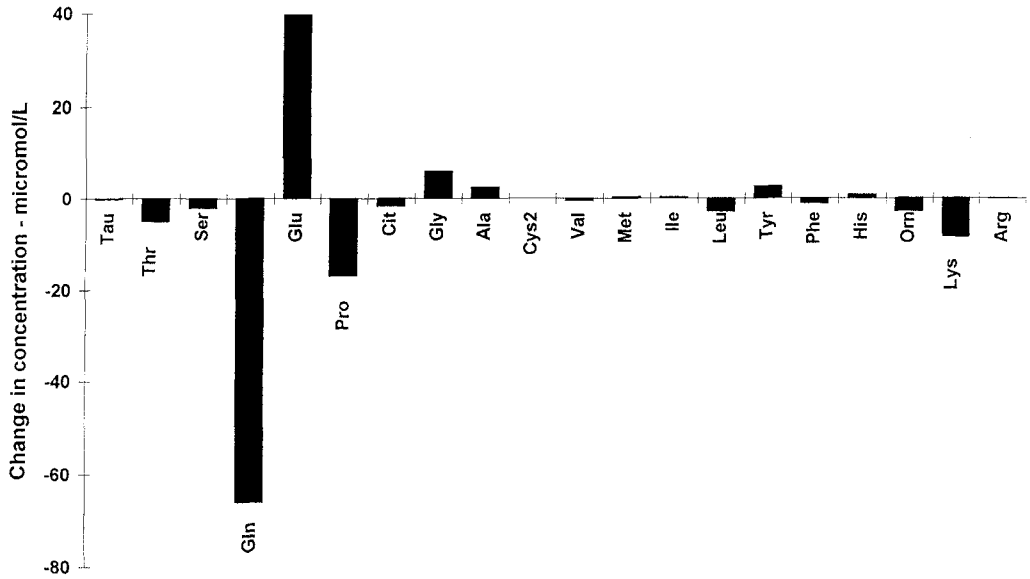

Fig. 1. Mean change in amino acid concentration during 60 minute incubation at 37°C with DTT, (n = 5)

Hcy_2, and Cys-Hcy, should be unnecessary since they can be regarded as artefacts produced by oxidation.

The acknowledged importance of homocysteine as a risk factor for vascular thrombosis (Malinow, 1994; Boers, 1994) and as an indicator of marginal vitamin B12 and folate status (Brasttrom, 1996; Ueland et al., 1993) has resulted in the introduction of a number of specific assays for this amino acid. Available methods and associated advantages and disadvantages have been comprehensively reviewed by Ueland et al. (1993). The only technique capable of producing a complete amino acid profile under the conditions necessary to assay tHcy is IEC by conventional amino acid analyser.

Mechanistic investigation of aminothiol metabolism involving the measurement of both free and bound forms and derivatives requires reduction following acid precipitation of proteins (Kang et al., 1979). Under these acid conditions DTT reduction is not effective, a pH of 7–8 being necessary (Dawson, 1990) and additional sample buffering is required. This also applies to acidic aqueous preparations. In previous methods for analysis of tHcy buffered plasma is incubated with DTT or other reducing agent for 60 minutes or more at 37°C before precipitation of proteins (Kang et al., 1979; Ueland et al., 1993). However, comparison of results obtained from plasma pre-treated with DTT with those from plasma analysed conventionally shows that some amino acids, notably glutamine and glutamate are unstable under these conditions (Fig. 1). Individual data (not shown) comprising Fig. 1 show losses of glutamine from 12% to 20%. DTT is a very efficient reducing agent for the conversion of -S-S- to -SH with reactions proceeding to completion within minutes at pH7–8 (Dawson, 1990), consequently, the physiological pH of neat plasma should permit thiol reduction to occur without additional

buffering and prolonged incubation. This possibility has been investigated here.

Methods

Analyses were carried out on a Biochrom 20 amino acid analyser, (Pharmacia Biotech, Cambridge, UK), using a high resolution lithium column and standard physiological separation programme used without modification. Cysteine and homocysteine are discretely separated from other amino acids. Plasma samples were deproteinised by the addition of an equal volume of 10% aqueous sulphosalicylic acid (SSA) containing 200 μmol/L norleucine as internal standard. Complete protein precipitation was assured by standing at room temperature for 60 minutes before centrifugation, 70 μl of supernatant, (≡ 35 μl plasma), was then applied to the column. Aqueous preparations of Cys_2 and Hcy_2 used for calibration and recovery experiments were prepared with the addition of HCl to aid solubility followed by adjustment to pH 7.0 with NaOH before dilution to the required final volume. DTT reduction of these solutions was carried out without further buffering. Recovery factors and colour equivalents for Cys and Hcy were calculated from aqueous solutions of known concentrations of Cys_2 and Hcy_2 subjected to DTT reduction. Completeness of reduction was checked by assaying on the amino acid analyser for residual Hcy_2 and Cys_2.

The validity of a rapid reduction was investigated as follows. To 1 volume of heparinised plasma was added 0.1 volume of fresh aqueous 0.8 M (12%) DTT. After intervals of 1, 2, 5, 10, 30 and 60 minutes post DTT addition at room temperature, (22 ± 2°C), protein was precipitated with 10% SSA as above and a full amino acid profile obtained. These results were compared to baseline values and also to results obtained by an established method (Andersson et al., 1989).

To assess recovery, small volumes of strong neutral aqueous Cys_2 and Hcy_2 were added to previously assayed plasma and allowed to stand at room temperature for 12 hours to obtain maximum protein binding before analysis. No residual free Hcy_2 was detected. Inter-assay CVs at two levels were obtained from plasma prepared similarly.

Results and discussion

Results (Table 1) confirm that reduction of neat plasma by the addition of 0.1 volumes of 12% DTT is complete within minutes at room temperature without the need for additional buffering and that values for other amino acids remain viable. Inter-assay CV and recoveries of tHcy at low and elevated concentrations, (Table 2), are similar to those previously reported for IEC (Candito, 1997). A CV of 8%, achievable under routine analytical conditions at low concentrations (Table 1), should be adequate for cardiovascular risk assessment. Although the shortened procedure here should give better analytical sensitivity at low values due to use of undiluted sample it is unlikely that these parameters can be improved upon without reporting sub-integer concentrations.

Specific assay methods do not enable the simultaneous measurement of other amino acids which is desirable when carrying out broader metabolic investigations. IEC is the only method which enables this but typical assay conditions significantly extend analysis time and, as shown here, compromise the stability of other amino acids, especially glutamine and glutamate. These drawbacks are overcome by use of a rapid reduction step. The acid condition

Table 1. Amino acid values during DTT reduction at 22 deg. compared to buffered plasma for 60 min. at 37 deg

Analyte	0 min	1 min	2 min	5 min	10 min	30 min	60 min	SD	CV%	60′ at 37 deg.
Tau	47	50	44	49	50	47	51	2.25	4.66	44
Thr	118	115	109	114	116	117	114	2.71	2.36	104
Ser	123	119	114	120	120	126	122	3.46	2.87	112
Gln	639	627	601	602	591	617	612	15.29	2.50	564
Glu	19	18	18	19	29	30	31	5.73	24.45	32
Pro	151	151	135	126	131	139	147	9.21	6.58	130
Cit	14	12	15	14	12	13	15	1.18	8.68	10
Gly	243	250	222	244	238	249	250	9.24	3.81	236
Ala	263	269	258	261	270	267	262	4.13	1.56	246
Cys2	17	ND	ND	ND	ND	ND	ND			
Val	191	194	184	188	190	194	198	4.23	2.21	186
Met	22	22	19	21	21	20	23	1.25	5.89	20
Ile	70	71	65	68	70	70	70	1.88	2.73	68
Leu	112	110	105	105	113	115	114	3.81	3.45	106
Tyr	38	37	39	34	32	37	34	2.36	6.57	30
Phe	62	58	55	59	59	63	61	2.50	4.19	58
His	68	64	64	67	65	68	67	1.64	2.48	58
Orn	45	43	42	44	45	46	44	1.25	2.82	42
Lys	147	148	137	141	140	144	147	3.89	2.71	136
Arg	66	64	62	65	67	67	67	1.76	2.69	60
Cys	ND	230	241	255	253	253	266	11.40	4.56	224
Hcy	ND	8	7	8	8	7	9	0.69	8.77	8

Table 2. Inter-assay precision and recoveries. All concentration expressed as reduced cysteine or homocysteine

	Inter-assay precision			
Analyte	Mean value (n = 6)	Range	Std.dev.	CV%
Cysteine	244	219–265	18	7.5
Homocysteine	7.1	6.5–7.9	0.5	7.3

	Recovery of homocysteine			
Baseline	Hcy added	Total	Measured	% recovery
20	12	32	31	97
11	110	121	128	106
32	285	317	316	100

of samples prepared for IEC also has the advantage of providing a stable environment for DTT reduced aminothiols. Cysteine and homocysteine are discretely separated from other amino acids, homocysteine eluting just before methionine and cysteine just before alpha aminoadipic acid.

Awareness of the role of total homocysteine as a sensitive indicator, not only of inherited defects of homocysteine metabolism, but also of compromised remethylation, including co-factor deficiency, has increased greatly in the last few years. Many laboratories have already added the assay of total homocysteine to their routine diagnostic repertoire, and with the advent of specific automated immunoassays is set to increase. Total homocysteine is more reliable diagnostically than measurement of free homocystine, cystine and their mixed disulphide, which vary according to specimen history, and these may be irrelevant in the diagnosis and monitoring of inborn errors of homocysteine metabolism when total homocysteine can be measured.

The ability to easily and reliably measure tHcy, in addition to other amino acids, with no time penalty compared to conventional IEC analysis, should encourage the measurement of tHcy as part of the routine amino acid profile.

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Received October 4, 1997