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Journal of Chromatography A, 828 (1998) 397–400

JOURNAL OF CHROMATOGRAPHY A

Short communication

High-performance liquid chromatographic determination of total plasma homocysteine with or without internal standards

Roberto Accinni[a,*], Jonica Campolo[a], Silvia Bartesaghi[b], Giuseppe De Leo[b], Claudio Lucarelli[c], Cristina F. Cursano[a], Oberdan Parodi[a]

[a]*C.N.R Institute of Clinical Physiology, Medical and Surgical Cardio-Thoracic Department A, De Gasperis, Niguarda Hospital, Milan, Italy*

[b]*Laboratory of Clinical Biochemistry, Fatebenefratelli Hospital, Erba (CO), Italy*

[c]*Instituto Superiore di Sanità, Laboratory of Clinical Biochemistry, Rome, Italy*

Abstract

Hyperhomocysteinemia is an independent risk factor for atherosclerosis and vascular occlusive disease. Assessment of total plasma concentration of homocysteine (tHcys) requires accurate and reproducible measurements. The aim of this study was to test a rapid isocratic HPLC method for tHcys analysis with an internal standard (I.S.) of α-mercaptopropionylglycine (MPG), 2-mercaptoethylamine (ME), or *N*-acetylcysteine (NAC) or without I.S., and to verify whether the use of an I.S. improves the precision. The method without I.S. showed an excellent linearity ($y=1.59x-0.15$, $r=1$), recovery (100%) and a within-assay relative standard deviation (R.S.D.) of 1.2%. Instead, in our hands, the presence of I.S.s decreased the reproducibility (within-assay R.S.D. ranged from 4.5 to 6.5%) and lengthened the chromatogram by up to four to five times. In conclusion, HPLC measurement of plasma tHcys without I.S. improves accuracy with respect to determination with I.S.; moreover, this approach allows to routinely process larger amounts of plasma samples.

Keywords: Internal standards; Homocysteine; α-Mercaptopropionylglycine; Acetylcysteine; 2-Mercaptoethylamine

1. Introduction

Over the last years, a high plasma total homocysteine (tHcys) concentration has been recognized as a novel and potentially important risk factor for atherosclerotic and thromboembolic vascular disease. This non-essential, sulfur-containing amino acid is formed as an intermediate, during the essential amino acid methionine metabolism, which is at the intersection of two main metabolic pathways: remethylation and transulfuration, regulated by methylenetetrahydrofolate reductase (MTFHR) and cystathionine β-synthase (CBS), respectively.

The known reasons for an increased level of plasma tHcys are inherited genetic defects of CBS and MTFHR or acquired causes, such as deficiency of folic acid, vitamins B_6 and B_{12}.

Various methods to measure tHcys have been described during the last decade with high-performance liquid chromatography (HPLC) methods with fluorescence detection are the most commonly used [1–3].

The aim of our study, using a method adapted from that of Ubbink et al. [2], was to assess the reproducibility of the method with internal standards (I.S.s) of α-mercaptopropionylglycine (MPG), 2-

*Corresponding author. Corresponding address: C.N.R. Institute of Clinical Physiology – Section of Milan, Niguarda Cà Granda Hospital, Piazza Ospedale Maggiore 3, 20162 Milan, Italy.

0021-9673/98/$ – see front matter
PII: S0021-9673(98)00661-X

mercaptoethylamine (ME) and *N*-acetylcysteine (NAC) [4–7] or without I.S., and to compare this method with a commercially available assay for plasma tHcys determination.

2. Experimental

2.1. Chemicals

Homocysteine, MPG, ME, NAC, tri-*n*-butylphosphine (NTB), ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F), acetonitrile were purchased from Fluka (Sigma–Aldrich, Milan, Italy).

All other chemicals were of analytical-reagent grade.

2.2. Apparatus and chromatographic conditions

Two Model 422 SV HPLC pumps, coupled to a 465 HPLC autosampler (Kontron Instruments, Milan, Italy) were fitted with a Supelcosil LC 18 DB analytical column (250 mm×4.6 mm I.D. Supelco, Sigma–Aldrich). Fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm, using a SFM25 fluorescence spectrophotometer coupled to a Kroma system (Kontron Instruments).

The following isocratic conditions were used without I.S.: 0.1 mol l^{-1} potassium dihydrogenphosphate buffer, pH 2.1 containing 80 ml l^{-1} acetonitrile as mobile phase with a flow-rate of 1 ml min^{-1}. Two different mobile phases were used with I.S.: 0.1 mol l^{-1} potassium dihydrogenphosphate buffer, pH 2.1 (A) and A+50% acetonitrile (B) at a flow-rate of 1 ml min^{-1}. The percentage of mobile phase B ranges from 7 to 30%, according to I.S. used (Table 1).

2.3. Sample and standard preparation

Plasma in EDTA (50 μl) mixed with I.S. (50 μl) or 0.14 mol l^{-1} NaCl (50 μl) in the method without I.S., was treated with a 10% NTB in dimethylformamide solution (10 μl) for 30 min at 4°C in order to reduce thiols and release them from plasma proteins.

Subsequently, 100 μl of 10% trichloroacetic acid containing 1 mmol l^{-1} EDTA, was added, mixed and centrifuged (21 000 *g*, 2 min). The clear supernatant (100 μl) was mixed with 20 μl of 1.55 mol l^{-1} sodium hydroxide, 250 μl of 0.125 mol l^{-1} borate buffer, pH 9.5, containing 4 mmol l^{-1} EDTA and 10 μl of a 10 mg ml^{-1} thiol-derivatizing reagent, SBD-F, in 0.125 mol l^{-1} borate buffer, pH 9.5. Complete derivatization was performed at 60°C for 60 min. After cooling at 4°C for 15 min, 10 μl were analysed by HPLC.

A stock solution of standard homocysteine (600 μmol l^{-1}) was diluted to yield appropriate concentrations (1–60 μmol l^{-1}). The concentration of I.S.s ranged from 10 to 100 mmol l^{-1}.

3. Results and discussion

A typical plasma chromatogram without I.S., obtained by isocratic HPLC assay is shown in Fig. 1. The tHcys peak was well resolved from cysteine and cysteinyl–glycine without interfering compounds and its elution was very rapid (4.5 min), an important condition for routine use.

The linearity of the method without I.S. was evaluated by addition of homocysteine standard ($Hcys_{st}$) increasing concentration (1–60 μmol l^{-1})

Table 1
Chromatographic gradient conditions of different internal standards

MPG		NAC		ME	
Time (min)	Buffer B (%)	Time (min)	Buffer B (%)	Time (min)	Buffer B (%)
0	8	0	8	0	7
7	8	7	8	7	7
11	30	11	30	11	10
20	30	20	30	20	10
21	8	21	8	21	7
25	8	25	8	25	7

Buffer A=0.1 mol l^{-1} potassium dihydrogenphosphate buffer pH 2.1%; buffer B=A+50% of acetonitrile.

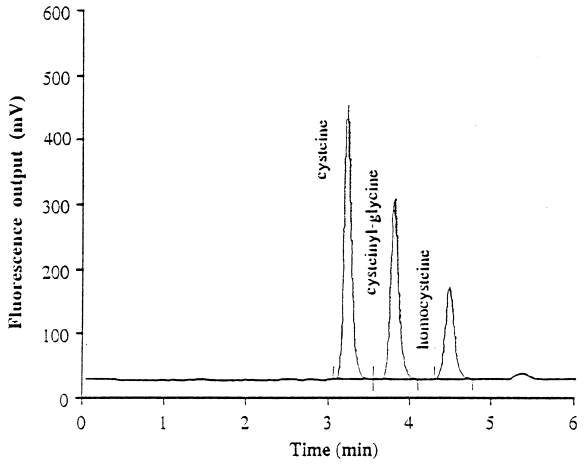

Fig. 1. HPLC determination of plasma homocysteine. Mobile phase=0.1 mol l^{-1} potassium dihydrogenphosphate buffer, pH 2.1, containing 80 ml l^{-1} of acetonitrile. The analysis was carried out on Supelcosil LC 18 DB (5 μm) column (250×4.6 mm I.D.). Flow-rate 1 ml min^{-1}; excitation at 385 nm and emission at 515 nm.

to a 0.14 mol l^{-1} NaCl solution. A linear regression analysis yielded $y=1.59x-0.15$ ($r=1$), where y is the peak area and x the concentration of SBD–$Hcys_{st}$ (μmol l^{-1}). The within-assay precision of plasma tHcys (R.S.D.=1.2%) was determined by injecting 10 aliquots of the same plasma; the day-to-day precision (R.S.D.=2.5%) was estimated from repeated analysis of the same plasma samples on 10 different days over one month. The recovery test was performed adding known $Hcys_{st}$ concentrations (10 μmol l^{-1}) to the same plasma sample ($n=10$). The concentrations in plasma sample (tHcys) and in plasma samples with added $Hcys_{st}$ (tHcys+$Hcys_{st}$) were determined in 10 replicates; analytical recovery, calculated as recovery%=100×(tHcys+$Hcys_{st}$)−tHcys/$Hcys_{st}$, was 100%.

Three different low-molecular-mass sulfhydryl compounds have been used as I.S. (MPG, NAC and ME). The chromatographic conditions, shown in Table 1, allowed a good resolution of these I.S.s from the other thiols (data not shown). The retention times of MPG, NAC and ME were 13.2, 12.3 and 4.8 min, respectively. To elute these I.S.s we had to apply different chromatographic conditions that lengthened the analysis by up to four to five times with respect to the isocratic condition without I.S.

Table 2
Within-assay R.S.D. values of total plasma homocysteine, obtained with or without I.S.

	Within-assay R.S.D. (%)
tHcys	1.2
tHcys+MPG	6.5
tHcys+NAC	5.1
tHcys+ME	4.5

The within-assay precisions of single I.S., tested on 10 replicates for each, were 3.7, 4.5 and 2.5% for MPG, NAC and ME, respectively. The within-precision assay of plasma tHcys using MPG, NAC and ME as internal recovery standards (10 replicates of each I.S.), was higher than without I.S. (Table 2). These results indicate that the method with I.S. is less reproducible than the one without I.S.

The different light sensitivity and chemical behaviour of the SBD adducts of sulfhydryl compounds could further worsen the reproducibility (Fig. 2).

The method without I.S. was applied in two different laboratories (Milan and Erba-Como) on two different HPLC systems equipped with different fluorescence detectors. The results obtained on 50 assays showed a good agreement ($r=0.998$). Moreover, 50 samples with low and high tHcys concentrations were tested also with a different HPLC method (homocysteine HPLC, Bio-Rad Labs.). The correlation coefficient was 0.997.

The method without I.S. ensures an accurate,

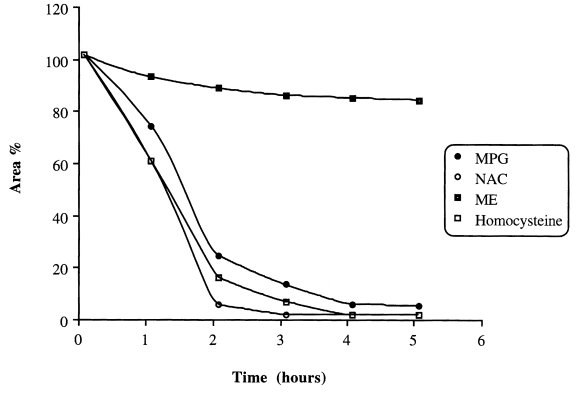

Fig. 2. Decay of SBD aminothiol derivatives (homocysteine, MPG, NAC and ME) exposed to full daylight. The rapid decrease and different behaviour of each at light were determined with periodic HPLC injection at every hour for 5 h.

linear and rapid determination of plasma tHcys, an important condition for routine use. These results are consistent also when comparing data among different laboratories and with other HPLC methods. Using this method without I.S. in a control group ($n=100$) we obtained tHcys levels (11.8 ± 3.2 μmol l^{-1}) similar to those from other reports [8–10]. The presence of I.S. instead, lengthened the chromatograms by up to four to five times and decreased the tHcys precision. In fact, the variation of tHcys in plasma sample with I.S. was higher than without I.S. (6.5, 5.1 and 4.5% for MPG, NAC, ME, respectively versus 1.2% without I.S.) while the within-assay R.S.D.s of single I.S.s (MPG, NAC, ME) were 3.8, 4.5 and 2.6%, respectively.

4. Conclusions

Although the important function of I.S. to evaluate recovery and precision of methods is recognized, our results indicate that the presence of I.S. is unnecessary in this analytical condition; in fact, the introduction of I.S. decreases method precision. The precision of the method is critical, since it has been recently shown that small difference of plasma tHcys (1–2 μmol l^{-1}) could identify different levels of hyperhomocysteinemia, graduating the risk.

Therefore, a correct stratification of homocysteinemic risk requires a high accuracy and interlaboratory reproducibility of the method; removal of the intrinsic variability of I.S.s appears to improve, rather than decrease, accuracy of tHcys determination.

Acknowledgements

We thank Cinzia Dellanoce for technical assistance, Elisabetta Spagnolo for secretarial assistance and Marina Parolini for statistical consultation.

References

[1] A. Araki, Y. Sako, J. Chromatogr. 422 (1987) 43.
[2] J.B. Ubbink, W.J.H. Veermaak, S. Bissbort, J. Chromatogr. 565 (1991) 441.
[3] T. Fiskerstrand, H. Refsum, G. Kvalheim, P.M. Ueland, Clin. Chem. 39 (1993) 263.
[4] I. Fermo, C. Arcelloni, G. Mazzola, R. Paroni, Biochim. Clin. 21 (1997) 386.
[5] N.P.B. Dudman, X.W. Guo, R. Crooks, L. Xie, J.S. Silberberg, Clin. Chem. 42 (1996) 2028.
[6] P. Durand, L.J. Fortin, S. Lussier-Cacan, J. Davignon, D. Blache, Clin. Chim. Acta 252 (1996) 83.
[7] K. Kuo, R. Still, S. Cale, I. McDowell, Clin. Chem. 43 (1997) 1653.
[8] G. Montalescot, A. Anriki, B. Chadefaux-Vekemans, J. Blacher, F. Philippe, G. Drobinski, R. Benzidia, P. Kamoun, D. Thomas, Int. J. Cardiol. 60 (1997) 295.
[9] M.L. Zighetti, M. Cattaneo, C.R. Falcon, R. Lombardi, S. Harari, S. Savonitto, P.M. Mannucci, Thrombosis Res. 85 (1997) 279.
[10] I. Fermo, S. D'Angelo Viganò, R. Paroni, G. Mazzola, G. Calori, A. D'Angelo, Ann. Intern. Med. 123 (1995) 747.