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Short communication

Adaptation of a high-performance liquid chromatographic method for quantitative determination of homocysteine in urine

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Abstract

A modification of the Bio-Rad® total homocysteine HPLC-test is presented in order to enable not only plasma homocysteine measurements but also the quantification of homocysteine in urine samples using the same principle of measurement. Coelution of the internal standard provided in the test kit with an endogenous compound in urine demands for an alternative analytical procedure. Therefore, we introduced 3-mercaptopropionic acid as a substitute for the internal standard. The analytical method validation was performed for the matrix of urine specimens. The applicability of this method was demonstrated in a clinical study with volunteers after homocysteine thiolactone hydrochloride loading.

Keywords: Homocysteine; Urine; HPLC

1. Introduction

The aim of our study was to evaluate pharmacokinetic data of homocysteine (tHcy) in different matrices such as whole blood, plasma and urine after homocysteine thiolactone hydrochloride loading using one and the same analytical system for all matrices to avoid intermethod variations. Several varieties of analytical methods for the determination of total homocysteine have been published in recent years [1–21], but there are only few papers published reporting quantification of tHcy in urine samples [22–26]. For routine tHcy monitoring these methods are not appropriate, because highly specialized and expensive equipment is necessary.

Bio-Rad® Laboratories provide a ready-to-use HPLC kit for determination of tHcy in plasma, serum and capillary whole blood with fluorometric detection after trialkylphosphine reduction of disulfides, mixed disulfides, and protein-bound thiols and 4-(aminosulfonyl)-7-fluoro-2,1,3-benzooxadiazole (ABD-F) derivatization. However, for urinary analysis the kit can not be applied due to an unknown endogenous compound interferring with the internal standard (IS) provided with the kit. Therefore, we had to modify the existing method by replacing the IS with 3-mercaptopropionic acid.

2. Experimental

2.1. Reagents and chemicals

L-Homocystine, 3-mercaptopropionic acid and L-homocysteine thiolactone hydrochloride were purchased from Sigma–Aldrich Chemie (Taufkirchen, Germany). Chemicals used for derivatization and mobile phase were taken from a commercially available test kit (Homocysteine by HPLC Cat. No. 195-4075, Bio-Rad® Laboratories, Munich, Germany).

2.2. Instrumentation

HPLC analyses were performed with a Sykam Chromatography System (Gilching, Germany) equipped with an autosampler

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S 5200, a column thermo controller S 4011, a solvent delivery system S 1121 and a fluorescence detector S 3305. For instrument control, data acquisition and data analysis, a Peak Simple Chromatography Data System Model 203 (SRI Instruments, Torrance CA, USA) including the Peak Simple NT software was used.

Reduction and derivatization of the samples were carried out in a programmable Multicycler PTC 200 (Biozym, Oldendorf, Germany).

2.3. Samples

Informed consent was obtained from 14 male volunteers prior to study. They received a single oral dose of L-homocysteine thiolactone hydrochloride (10 mg/kg equivalent to 65.1 μmol/kg body weight). Urine specimens were collected before and at 0, 0.5, 1, 2, 3, 4, 6, 8, and 12 h post-dosing. The total volume of urine was determined and aliquots were immediately frozen and stored at −20 °C.

2.4. Standard solutions and quality control samples

Since homocyst(e)ine is an endogenous compound excreted in urine, calibration samples in distilled water were used for quantitation. Fifty milligrams powdered L-homocystine were dissolved in 1 ml 1 N HCl and diluted with water to obtain a stock solution of 200 μmol/l. Standard working solutions of homocystine (0–50 μmol/l) were prepared prior to use by appropriate dilution with water. Quality control samples of homocystine were prepared as calibration samples but with different concentrations.

As an IS 3-mercaptopropionic acid was used. A stock solution was prepared with 10.61 μl in 10 ml water. A further dilution of 1:100 with assay reconstitution buffer yielded the working concentration of 100 μmol/l.

All stock solutions could be stored in aliquots at −20 °C until analysis.

Calibration was performed with a 50 μmol/l calibrator according to the manufacturer's manual, whereas the peak area ratios of homocystine over internal standard were plotted against the known concentration. Correlation coefficients were calculated by least-squares linear regression analysis.

2.5. Derivatization procedure

Ten microliters of each calibrator, controls or samples were transferred to a 0.5 ml polypropylene microcentrifuge vial. Reduction reagent (10 μl), IS (20 μl) and derivatization reagent (20 μl) were added and vortex-mixed. After reduction and derivatization in a thermo cycler at 50 °C for 5 min following 4 °C for 5 min, 20 μl of precipitation reagent were added to each vial and thoroughly mixed on a vortex mixer. Precipitated proteins were removed by centrifugation at 10,000 × *g* for 5 min. Fifty microliters of the supernatant were mixed with the equal amount of mobile phase and transferred to an autosampler vial. Twenty microliters of the resulting fluorescent compounds were injected into the chromatograph.

2.6. Chromatographic conditions

The analysis was performed according to the manufacturer's instruction.

2.7. Assay validation

2.7.1. Linearity

Homocystine concentrations (0–50 μmol/l) in urine and dilution linearity were assessed. Urine samples containing high tHcy concentrations were diluted serially with Bio-Rad® Assay reconstitution buffer up to eight-fold.

2.7.2. Accuracy, precision and reproducibility

Accuracy and precision of this assay were evaluated by calculating the intra- and inter-day coefficient of variance. To measure the intra-day variance, 10 replicates of control samples with two different concentrations were derivatized and measured within 1 day. The inter-day variance was evaluated at two different concentrations on 10 different non-consecutive days.

2.7.3. Recovery

Total recovery of defined amounts of homocystine added to three different samples with known concentrations was determined. Water, a predose and a 3 h-urine sample were spiked with 20 μmol homocystine and analyzed as described. Recovery was calculated as the sum of basal concentration and spiked amount of homocystine.

2.7.4. Stability

Stability was tested in urine samples at −20 °C (1 week, 1, 2 and 3 months). For thaw-freeze stability testing, the concentration of a 3 h-urine sample was tested in eight freeze–thaw cycles. In addition, series of derivatized samples were stored in autosampler vials at −20 °C until analysis.

2.7.5. Sensitivity

The lower limit of quantification was determined for homocystine based on the criteria that the analyte can be determined with sufficient accuracy and precision. The limit of detection was determined as the lowest concentration, which gives a signal-to-noise ratio of 3 for homocystine.

3. Results and discussion

3.1. Chromatography

The chromatographic separation of the thiol-containing compounds was performed isocratically within 8 min, resolution of tHcy and IS resulted in retention times of 2.86 and 5.28 min, respectively. No interferences of the IS were observed with endogenous components present in urine samples (Fig. 1). The analytes were identified on the basis of their retention times compared to standard solutions. Homocysteine showed the same retention time in urine as described for plasma and whole blood in the Bio-Rad® instruction manual.

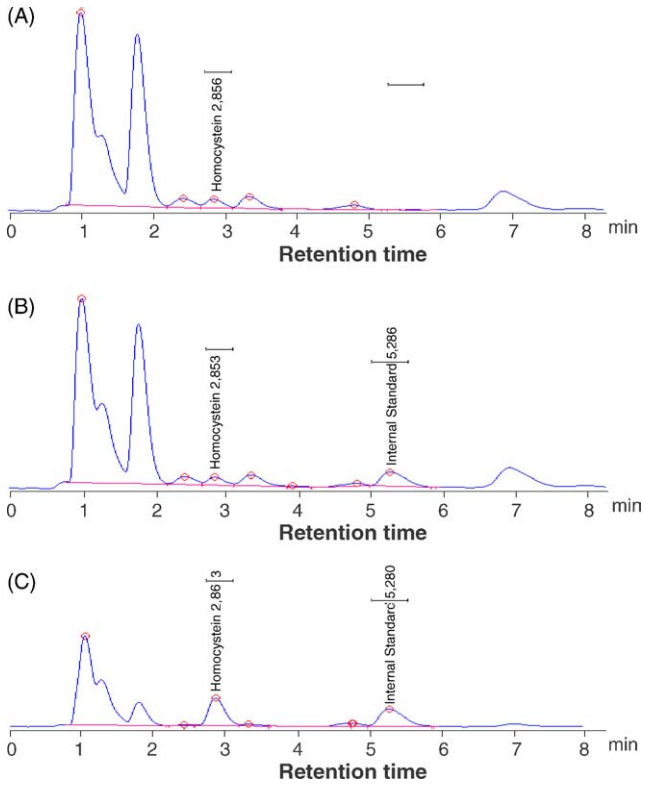

Fig. 1. HPLC chromatogramms of homocysteine in human urine. (A) predose urine sample without internal standard; (B) predose urine sample with internal standard; and (C) urine sample collected 3 h after an oral dose of 65.1 μmol/kg body weight L-homocysteine thiolactone hydrochroride.

3.2. Validation

Since 2 mol of homocysteine are generated by reduction of 1 mol homocystine, all results are expressed in equivalents of homocysteine.

A six point standard curve showed linear response up to 100 μmol/l with a coefficient of correlation $r^2 = 0.9977$. Serial dilution of homocysteine concentrations >100 μmol/l resulted in a linear relationship between measured and calculated values ($r^2 = 0.9995$; regression line $y = 0.9975x - 0.5342$).

The intra-day coefficient ($n = 10$) of variation was 2.93% (±1.15) for 40 μmol/l, 1.71% (±1.72) for 100 μmol/l homocysteine and 5.05% (±2.02) for internal standard areas, respectively. The inter-day coefficient ($n = 10$) was 2.88% (±0.18) for 6.25 μmol/l and 2.5% (±2.53) for 100 μmol/l homocysteine, respectively.

The system showed analytical recoveries for homocysteine ranging from 96.5 to 99.3%, when samples were spiked with known amounts of the analyte to give final concentrations of 40 μmol/l in water, 49.9 μmol/l in a predose urine sample and 99.3 μmol/l in a 3-h urine sample.

Homocysteine was found to be stable in urine when samples were stored at −20 °C up to 3 months. Repeated freeze–thaw cycles showed no degradation effect on homocysteine in urine. Derivatized samples stored at −20 °C showed no significant changes in chromatograms or homocysteine content.

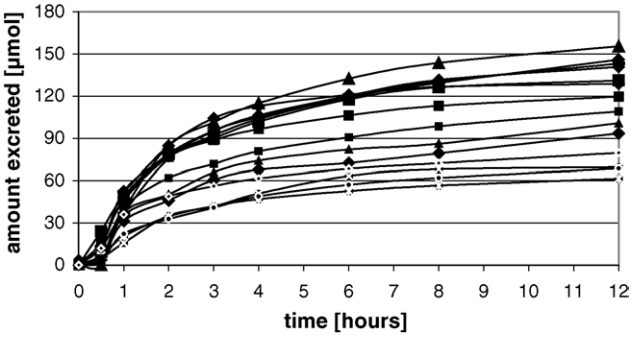

Fig. 2. Cumulative tHcy urinary excretion of 14 volunteers after a single oral intake of 65.1 μmol/kg body weight L-homocysteine thiolactone hydrochloride.

As can be seen, all parameters such as linearity, precision, accuracy, recovery and stability satisfy the requirements for bioanalytical method validation. The successful application of the method was demonstrated in a clinical study with 14 volunteers. Cumulative urinary excretion could be determined and accounted for 2.26% (±0.67) of the administered dose (Fig. 2) [27].

Due to the fact that we found about 50 different publications for homocysteine analysis with many proposals for improvements and technical innovations, none of the published methods seem to provide an absolute satisfactory analytical procedure regarding accuracy, practicability, sample throughput, expense factor and suitability for different matrices. Based on our own experience, the Bio-Rad® HPLC kit offers a ready-to-use kit, which can be easily established. The basic analytical procedure remains unaffected by introducing 3-mercaptopropionic as an IS. Although the IS elutes later than homocysteine with a retention time of 5.28 min, the chromatographic separation of all compounds could be completed within 8 min. None of the chromatograms of the derivatized urine specimens showed overlapping peaks of late eluting compounds. Another advantage concerning the expense factor is the reduction of the proposed volumes of the reaction mixture to one fifths, which showed no adverse effect to reduction and derivatization of the samples.

4. Conclusion

In conclusion, we have adapted an existing method with the benefit of a ready-to-use method to enable quantification of tHcy in urine samples. Having one and the same method at hand that is applicable to different matrices such as plasma, whole blood and urine, it is possible to compensate inherent analytical variations between methods.

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