



The Analysis of Total Homocysteine in Plasma Using a Quattro Micro Tandem Mass Spectrometer

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Abstract

This application note describes the analysis of total homocysteine in plasma using a Quattro micro Tandem Mass Spectrometer.

Benefits

This method has been shown to be rapid, precise, and accurate for the measurement of total homocysteine

Introduction

Total homocysteine (tHcy) is currently regarded as a risk factor for cardiovascular disease.¹ This increased interest has led to a multitude of studies requiring the determination of total homocysteine in conjunction with other factors. There are various methods for measuring tHcy², including HPLC, enzyme immunoassay (EIA), GC-MS, and LC-MS/MS. A recent review³ suggests that GC-MS should be considered as the reference method. A comparison of GC-MS with other laboratory methods concluded that the methods were not interchangeable and reference material was urgently required.⁴ The most recent method for measuring homocysteine uses a deuterium-labelled internal standard and tandem mass spectrometry.⁵ This development requires no derivatization and therefore leads to an increase in sample throughput³ compared to existing techniques. We have developed an LC-MS/MS method that requires minimal sample volume, no centrifugation or transfers and therefore allows the possibility of automated sample preparation together with high throughput capability.

Experimental

A Quattro micro Tandem Benchtop Mass Spectrometer fitted with a ZSpray ion source was used for all analyses. The instrument was operated in electrospray positive ionization mode and was coupled to a Waters Alliance HT HPLC System. All aspects of system operation and data acquisition were controlled using MassLynx NT v3.5 Software.

Maximum sensitivity and selectivity for homocysteine can be achieved by measuring product ions from

the fragmentation of the protonated molecule $[M+H]^+$. A solution of homocysteine (10 pmol/ μ L) was infused into the mass spectrometer and the cone voltage optimized to maximize the intensity of the $[M+H]^+$ precursor (parent) ion (m/z 136). The collision energy was then adjusted to optimize the signal for the most abundant product ion (m/z 90), using argon as the collision gas at a pressure 5.0×10^{-3} mbar. The product ion spectrum of homocysteine is shown in Figure 1. The process was repeated for the d_4 -homocysteine analogue, which was used as an internal standard for quantification and to correct for losses during sample processing.

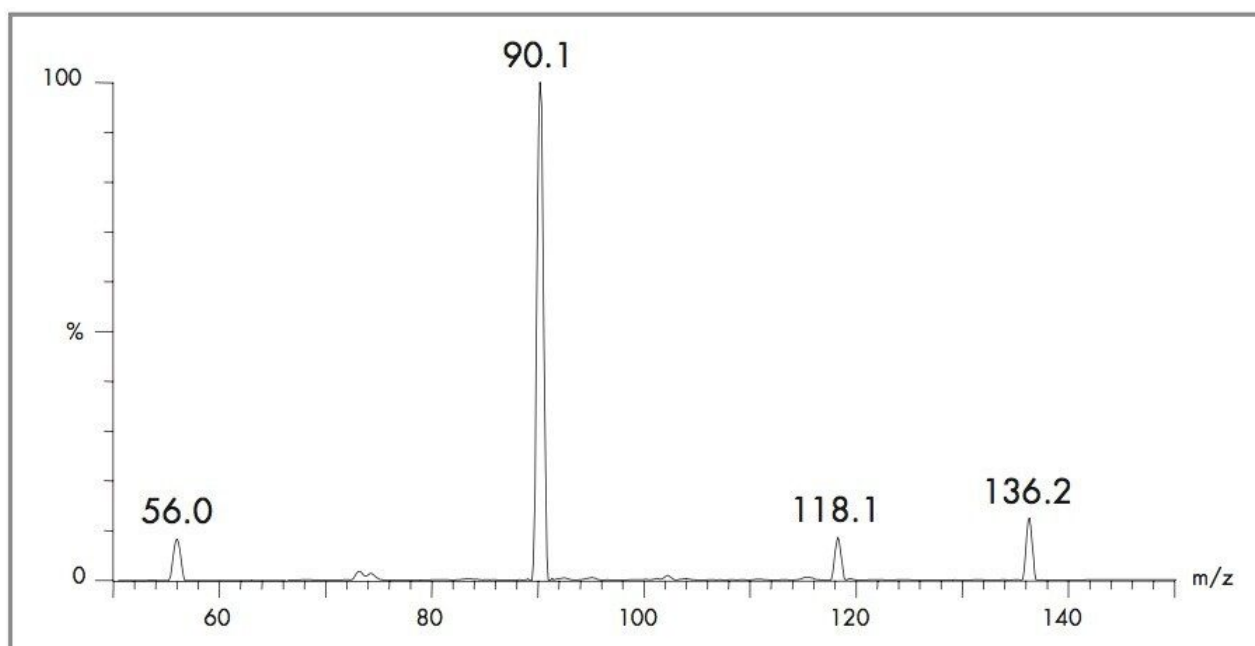


Figure 1. Product ion spectrum of homocysteine.

Plasma (10 μ L) was aliquoted into a 96-well plate and d_8 -homocystine, 10 μ M, (10 μ L) added and mixed for 1 minute. Dithiothreitol (DTT) 500 mM (20 μ L) was added and mixed for 10 minutes at room temperature.

Finally, water/0.1% formic acid/0.025% trifluoroacetic acid (100 μ L) was added as a diluent and mixed for 1 minute. The resultant solution was then sampled (4 μ L) directly from the microtitre well.

The method uses an isocratic elution using a Waters Symmetry C₈ Column (2.1 x 100 mm, 3.5 μ m) with aqueous 30% methanol/0.1% formic acid, at 250 μ L/min. The cycle time (injection-to-injection) is ~2 minutes.

Results and Discussion

The MRM chromatograms for homocysteine and d₄-homocysteine are shown in Figure 2.

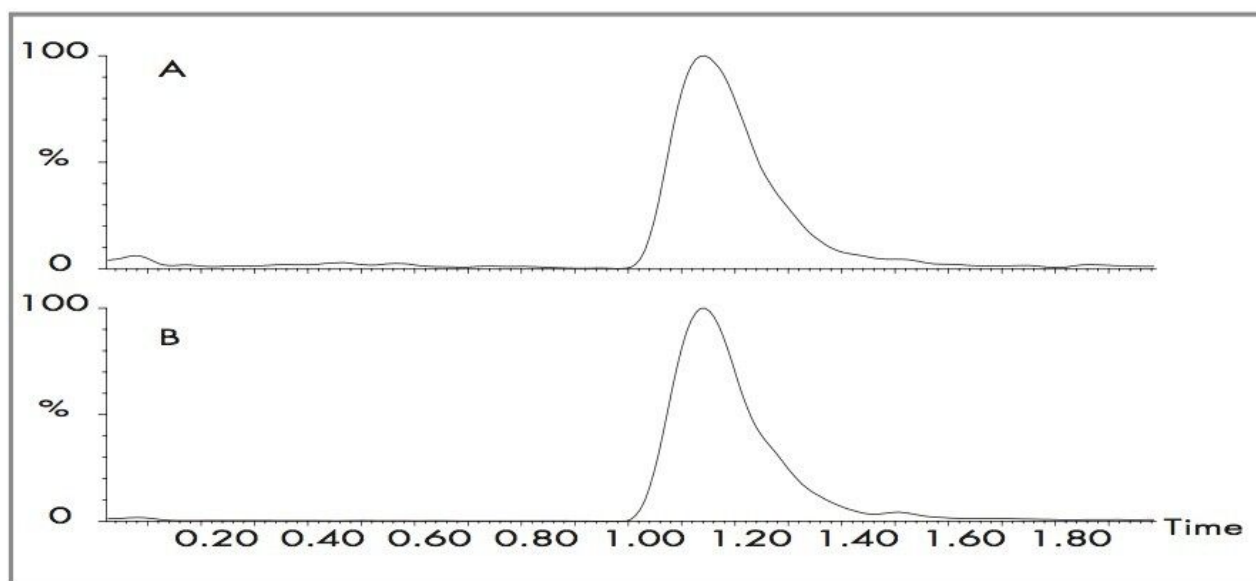


Figure 2. MRM chromatograms of d₄-homocysteine (A) and homocysteine (B) at a concentration of (10 µM) using the appropriate transition(s).

Plasma containing endogenous Hcy was spiked with a range of known Hcy concentrations (0.5, 10, 15, 25, and 50 µmol/L). The slope and positive intercept, calculated in QuanLynx Software, are used to calculate the endogenous value (7.96 µmol/L), as shown in Figure 3. The total homocysteine corrected for endogenous content is shown in Figure 4.

Analyte	Cone Voltage (V)	Collision Energy (eV)	MRM Transition
Homocysteine	15	15	136 > 90
d ₄ - Homocysteine	15	15	140 > 94

Table 1. Tune parameters for both homocysteine and d₄-homocysteine.

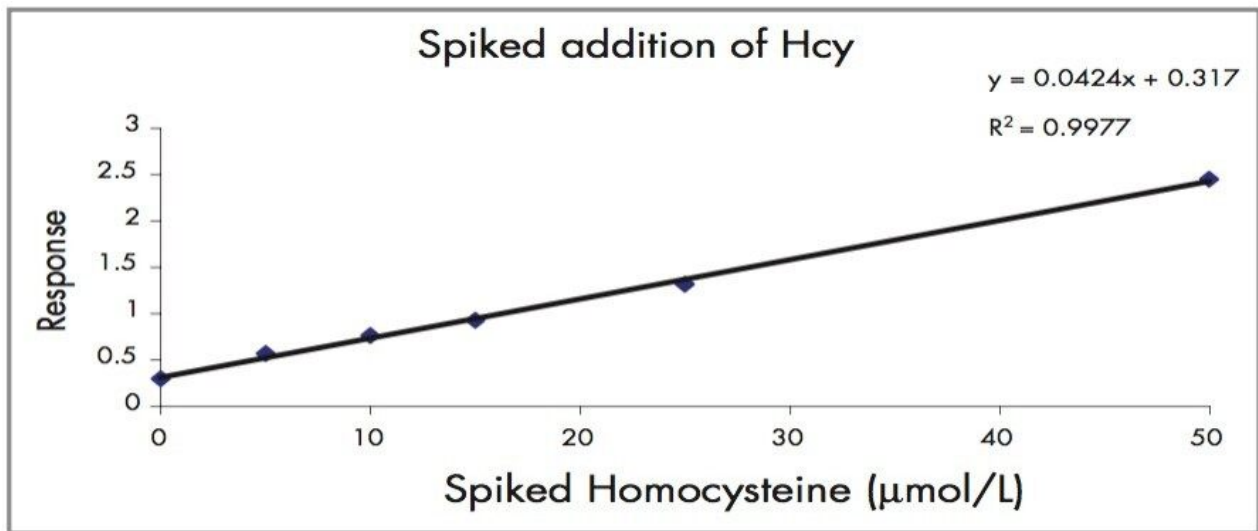


Figure 3. Calibration curve for the spiked addition of Hcy in plasma..

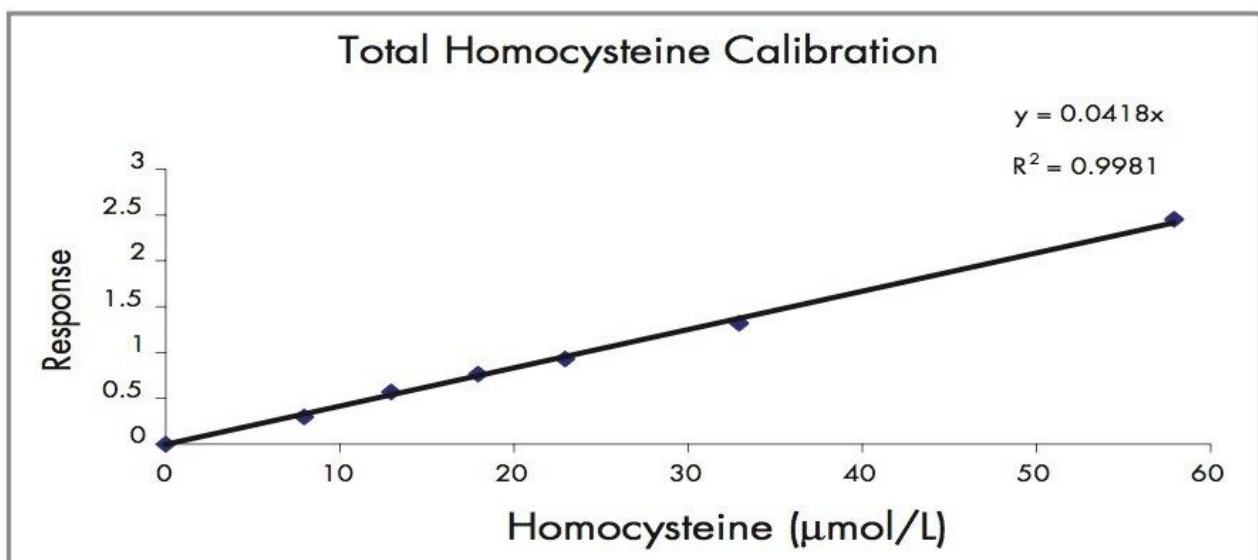


Figure 4. Corrected calibration curve for tHcy in plasma.

The performance characteristics of the assay were examined using two samples with homocysteine concentrations of 14.6 and 37.7 mM. Inter-assay results gave CV's of 5% and 8%, respectively (n=5) and the intra-assay (n=10) CV's for both samples was <2%. Validation of the method was performed using patient samples (n=50) kindly provided by Wythenshawe Hospital, Manchester, which were analyzed using both LC-MS/MS and an Abbot TDx Total Homocysteine kit. The LC-MS/MS method showed excellent correlation ($r^2 = 0.9149$) and slight positive bias (0.44) towards to LC-MS/MS

method as shown in Figure 5.

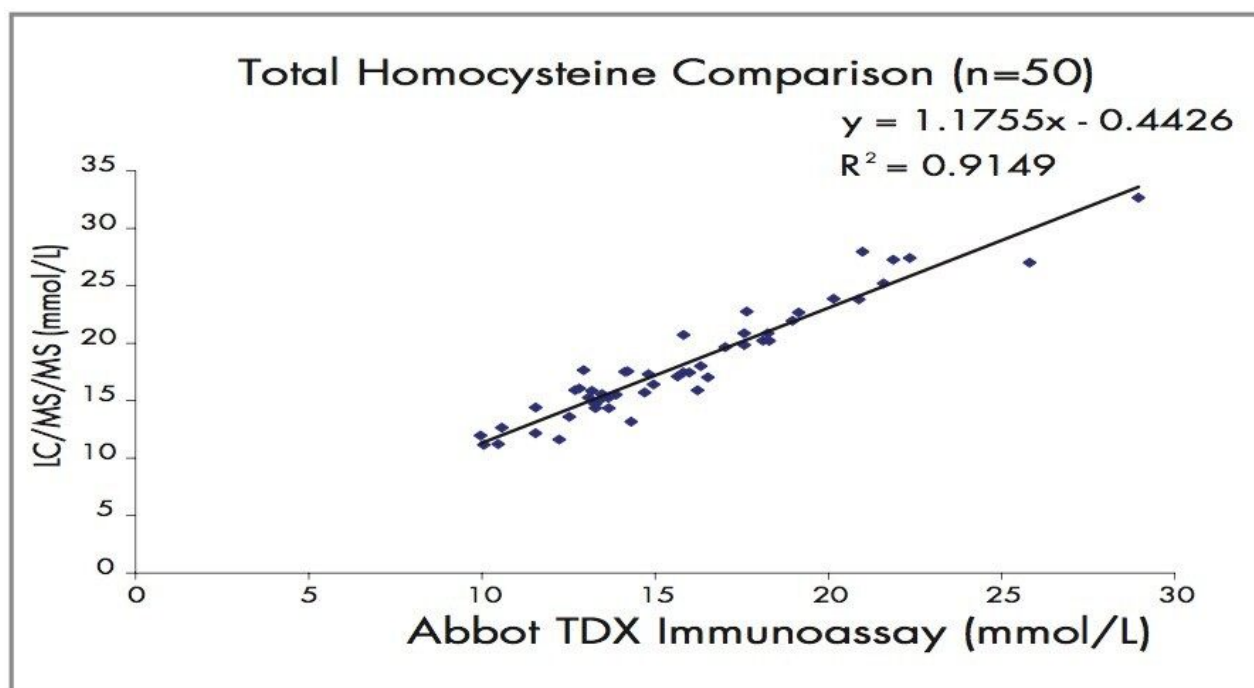


Figure 5. tHcy comparison using LC-MS/MS and an Abbot TDx kit.

Conclusion

This method has been shown to be rapid, precise, and accurate for the measurement of total homocysteine.

The simplicity of the assay makes it ideal both for non-specialized staff and routine high throughput.

References

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