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Short communication

Quantitation of total homocysteine in human plasma by derivatization to its N(O,S)-propoxycarbonyl propyl ester and gas chromatography–mass spectrometry analysis

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Abstract

Much evidence supports the hypothesis that mild or moderate hyperhomocysteinaemia represents an important and independent risk factor for occlusive vascular diseases. Therefore, the accurate and reliable determination of total plasma homocysteine has gained major importance for risk assessment. Furthermore, it can help in the detection of folate and vitamin B_{12} deficiency. This has prompted us to develop a sensitive gas chromatography–mass spectrometry (GC–MS) method in order to quantify total homocysteine in human plasma. Prior to chromatography, reduced homocysteine was released from disulfide bonds by incubation with excess dithiothreitol and converted into its N(O,S)-propoxycarbonyl propyl ester by derivatization with *n*-propyl chloroformate. Aminoethylcysteine served as internal standard. The method proved to be highly linear over the entire concentration range examined (corresponding to 0–266 μM homocysteine) and showed intra-assay and inter-assay variation (relative standard deviations) of approximately 5 and 5–10%, respectively. External quality control by comparison with duplicate analyses performed on a HPLC-based system revealed satisfactory correlation. The newly developed GC–MS based method provides simple, reliable and fast quantitation of total homocysteine and requires only inexpensive chemicals, which are easy to obtain. © 1997 Elsevier Science B.V.

Keywords: Homocysteine; N(O,S)-Propoxycarbonyl propyl ester

1. Introduction

The sulfur-containing amino acid homocysteine is an intermediate formed in the metabolism of the essential amino acid methionine. Homocystinuria, i.e. the occurrence of elevated homocyst(e)ine concentrations in urine, is known to be caused by rare inherited enzyme defects, predominantly by a defect of cystathionine synthase. Among its typical clinical features are vascular diseases and thrombosis, which often lead to early death [1].

Much evidence supports the hypothesis, that not only marked hyperhomocysteinaemia (as observed in homocystinuria), but also mildly to moderately elevated total plasma homocysteine concentration, is associated with occlusive vascular diseases and represents an important and independent risk factor for arteriosclerosis in the general population [1,2]. This has resulted in growing interest in the detection and quantitation of only slightly increased total

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homocysteine concentrations in human plasma. Elevated plasma concentrations of total homocysteine can also indicate folate and vitamin B_{12} deficiency [3]. Except for pronounced hyperhomocysteinaemia, reduced homocysteine accounts only for trace amounts of total plasma homocysteine. 75–90% of the homocysteine in normal plasma is covalently bound to plasma proteins by disulfide bonds, another part forms low-molecular-mass disulfides, including homocystine and homocysteine–cysteine mixed disulfide [4]. Therefore, it is necessary to reduce disulfides in order to recover total homocysteine.

Most previously reported methods for the determination of total homocysteine in plasma (reviewed in Refs. [3] and [5]) used HPLC with precolumn derivatization or an amino acid analyzer (postcolumn derivatization), although a GC–MS method has also been described, which includes derivatization of homocysteine to its *tert.*-butyldimethylsilyl derivative and the use of expensive deuterated internal standards [3,6].

We used a different GC–MS approach and achieved reliable and highly sensitive quantitation of total homocysteine in plasma after converting homocysteine and the internal standard aminoethylcysteine to the corresponding N(O,S)-propoxycarbonyl propyl esters.

2. Experimental

2.1. Chemicals

DL-homocystine and internal standard S-2-aminoethyl-L-cysteine hydrochloride (both of >99% purity) were obtained from Fluka (Buchs, Switzerland) and Calbiochem (La Jolla, CA, USA), respectively. The internal standard compound is now sold by ICN Biomedicals (Aurora, OH, USA) instead of Calbiochem. Dithiothreitol, *n*-propyl chloroformate (PCF), as well as trichloroacetic acid and pyridine, were Aldrich products purchased from Sigma–Aldrich (Vienna, Austria), who also provided the hydrocarbons (Supelco product) used in this study. Chloroform and *n*-propanol were obtained from Merck (Darmstadt, Germany). Deionized water was prepared with a Milli-Q water purification system (Millipore, Milford, MA, USA).

2.2. Collection of plasma

Venous human blood samples were collected into tubes containing heparin or EDTA and immediately centrifuged at 2000 *g* and 4°C for 10 min. The plasma layer was removed and stored at −20°C until further sample preparation.

The choice of EDTA or heparin as the anticoagulant did not affect the results significantly.

2.3. Sample preparation

Sample preparation consisted of reduction of disulfides, protein precipitation, derivatization with PCF and of the extraction of the formed derivatives. The derivatization technique for homocysteine with PCF was based on the method described by Husek [7,8], who converted other amino acids with methyl and ethyl chloroformate prior to GC analysis with flame-ionization detection. We used PCF for derivatization because it yields greater fragments in mass spectrometry than its lower homologues.

To release homocysteine from disulfide bonds, 500-μl plasma aliquots were mixed with 500 μl deionized water, 100 μl internal standard solution (1 m*M* S-2-aminoethyl-L-cysteine hydrochloride), as well as 25 μl of an aqueous solution of dithiothreitol (12.5%, w/v) and incubated for 30 min at 40°C.

After the incubation, proteins were precipitated by addition of 100 μl 72% (w/v) trichloroacetic acid in water and thorough mixing. Precipitates were sedimented by short centrifugation at room temperature. A 600-μl volume of the resulting supernatant was diluted with 400 μl of a 4:1 (v/v) mixture of *n*-propanol and pyridine. Subsequently, derivatization was started by addition of 50 μl PCF to each sample and brief vortex. After 2 min at room temperature, the formed N(O,S)-propoxycarbonyl propyl esters were extracted with 1000 μl chloroform containing 1% (v/v) PCF. A 1200-μl volume of the lower layer, which separated from the upper one in a few minutes, was evaporated to dryness under a nitrogen stream at room temperature. Residues were then solved in 100 μl chloroform containing 1% (v/v) PCF.

2.4. Chromatographic conditions

A 2-μl aliquot of the solved residues was injected into the injection port of a Hewlett–Packard (HP) 5890 series II "plus" gas-chromatograph, using a HP 7673 auto injector. The injector port temperature was set to 260°C, splitless injection was performed. Separation was achieved with a HP 5 M.S. capillary column (30 m lengths, 0.25 mm inner diameter, 0.25 μm film thickness) at a helium flow-rate of 1.0 ml/min. The run time of a single analysis was 25 min. An initial oven temperature of 100°C was held for 2 min and subsequently raised at a rate of 10°C/min. Finally, 300°C were reached and held for 3 min. We did not observe any problems attributable to coextracted lipids, however, as a precaution, the liner was occasionally replaced. Coinjection of lipids might be minimized by hexane extraction following the TCA induced precipitation.

2.5. Mass-selective detection

A HP 5972 series mass-selective detector was used for selective ion monitoring of the homocysteine derivative (target ion m/z 349.3 and qualifier m/z 289) as well as of the derivative of the internal standard compound (target ion m/z 172.3; qualifier m/z 188). Ions were selected from spectra of both derivatives, which had been obtained in the scan mode (m/z 50 to m/z 500) after an aqueous solution of both homocystine and internal standard compound

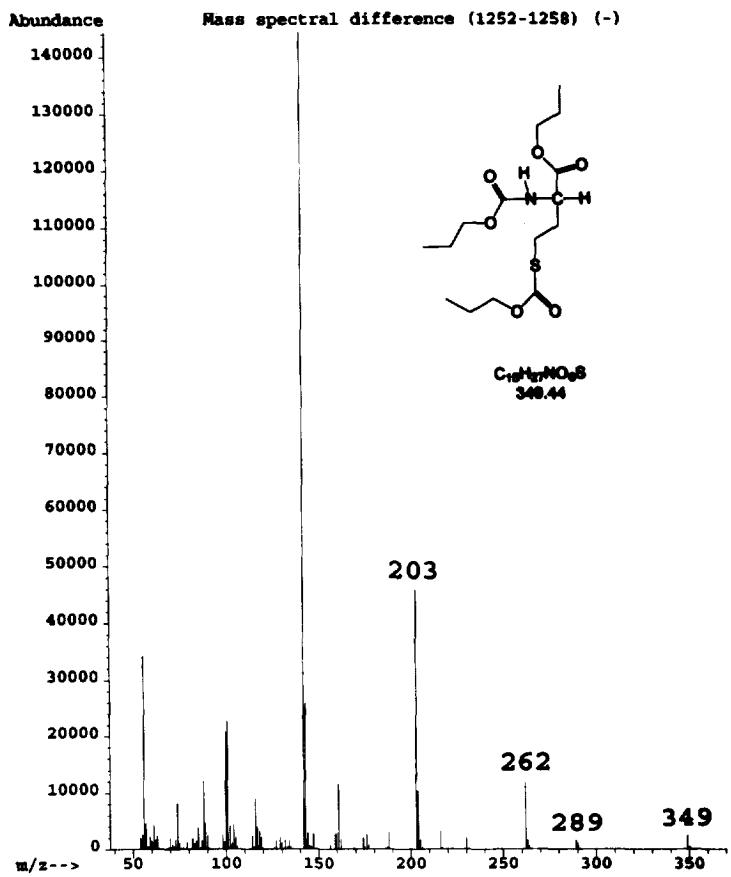

Fig. 1. Mass spectrum (after subtraction of a background spectrum obtained from a scan close to the peak) and structural formula of the N(O,S)-propoxycarbonyl propyl ester of homocysteine. m/z values are indicated for characteristic ions.

had been submitted to the sample preparation procedure outlined above (Section 2.3).

2.6. Calibration and validation

Standards for external calibration were prepared by adding known amounts of homocystine to the 500 μl water component of the incubation mixtures of normal human plasma. Final concentrations of added homocystine were 0, 5, 10, 20, 30, 50, 75, 100 and 133 μ*M* (calculated as twice these concentrations to obtain homocysteine values). The data obtained were corrected for the endogenous total homocysteine in the plasma.

Linear regression was performed by least squares analysis of the response ratios and the amount ratios of the derivatives of homocysteine and internal standard compound. Retention index values (methylene units×100) were obtained by comparison of the retention times of the two PCF derivatives in question with those of 9 *n*-alkanes from $C_{12}H_{26}$ to $C_{32}H_{66}$.

Intra- as well as inter-assay reproducibility was tested on three days with non-spiked normal human plasma (8.4 μ*M* total homocysteine), as well as with aliquots of the same plasma spiked with 133 μ*M* homocystine (corresponding to 266 μ*M* homocysteine).

External quality control was performed by blind analysis of 18 plasma samples (covering a range of values), which were generously provided by Mr. C. Angst and Dr. B. Fowler (Kinderspital Basel, Switzerland), who had determined total homocysteine by

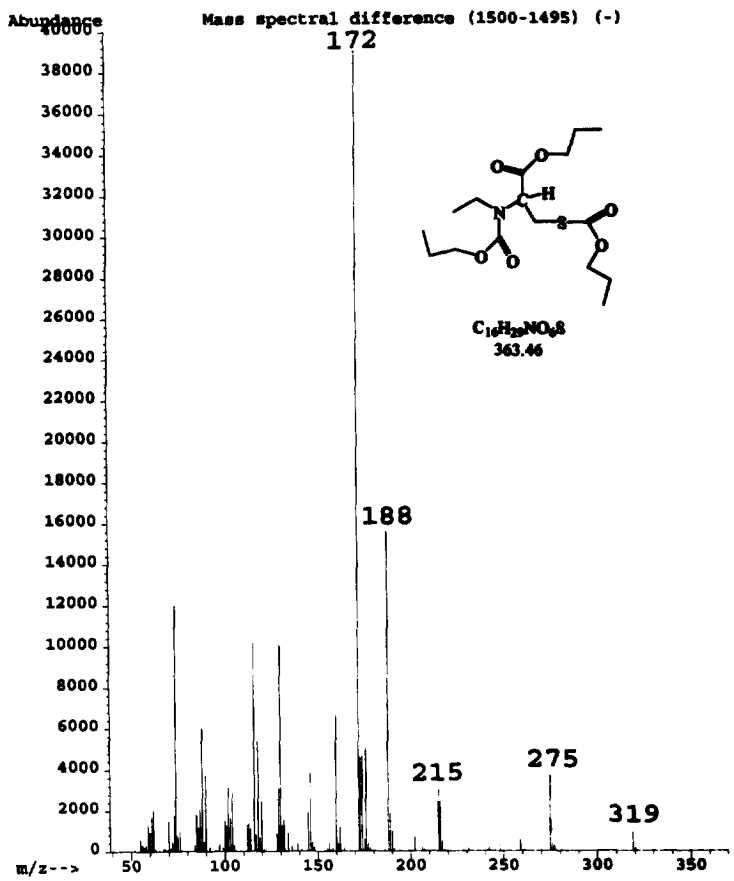

Fig. 2. Mass spectrum (after subtraction of a background spectrum obtained from a scan close to the peak) and structural formula of the N(O,S)-propoxycarbonyl propyl ester of aminoethylcysteine (internal standard compound). *m/z* values are given for characteristic ions.

their modification [9] of the HPLC-based method described by Vester and Rasmussen [10].

3. Results and discussion

Fig. 1 and Fig. 2 display the mass spectra of the products resulting from derivatization of homocysteine and aminoethylcysteine with PCF.

Fragmentation of the homocysteine derivative (Fig. 1) is suggested to be the following: $[M]^+=349$, $[M-\text{propanol}]^+=289$, $[M-\text{propoxycarbonyl}]^+=262$ and $[262-\text{propoxy radical}]^+=203$. For the aminoethylcysteine derivative (Fig. 2) this fragmentation is proposed: $[M]^+=363$, $[M-\text{propyl}-H]^+=319$, $[M-\text{propoxycarbonyl}-H]=275$, $[275-\text{propanol}]^+=215$, $[275-\text{propoxycarbonyl}]^+=188$, $[215-C_2H_5N]^+=172$.

From these spectra the ions for selective ion monitoring were chosen: m/z 349.3 and m/z 172.3 became target ions for the derivatives of homocysteine and internal standard compound, respectively. The ions m/z 289 and 188 served as qualifiers.

Fig. 3 shows a typical homocysteine signal obtained from a human plasma sample of a healthy male volunteer. The retention index of this signal was 2360, the corresponding value for the internal standard derivative 2650.

The assay was linear over the entire concentration range examined (0–133 μM homocystine, corresponding to 0–266 μM total homocysteine). The linear correlation coefficient was $r=0.998$.

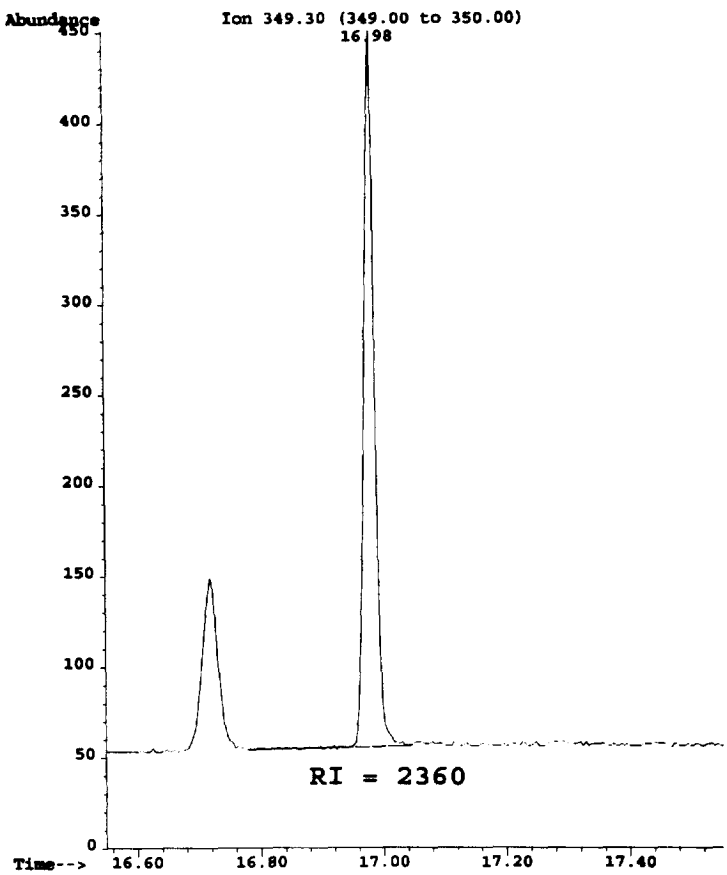

Fig. 3. Mass-selective detection (selective ion monitoring at m/z 349.3) of the homocysteine derivative signal (retention index [RI]=2360) obtained from a normal human plasma sample with 8.2 μM total homocysteine.

Table 1
Reproducibility of the assay expressed as relative standard deviation (R.S.D.)

	Intra-assay R.S.D. (%) (n=3–8)			Inter-assay R.S.D. (%) (n=3)
	Day 1	Day 2	Day 3	Day-to-day
Normal plasma sample	5.27	4.78	5.72	11.07
Normal plasma sample spiked with 133 μ*M* homocystine	2.82	5.12	4.12	5.63

As demonstrated in Table 1, the new method was highly reproducible, both at a low, normal level and at a high level of total homocysteine, correponding to total plasma homocysteine concentrations found in patients with homocystinuria.

External quality control revealed a satisfactory degree of linear correlation between values determined by the newly described GC–MS method and the compared HPLC-based technique. Linear regression over all 18 data points resulted in a correlation coefficient r=0.8, significantly following a linear model of regression. The P value of the runs test was 0.6, indicating that there is not a significant departure from linearity.

4. Conclusion

This highly sensitive GC–MS method allows the reliable determination of total homocysteine in plasma even at low levels. The described technique includes fast and simple sample preparation and only requires nonexpensive chemicals, which are easy to obtain. Determination of total homocysteine is performed on a GC–MS system which is also well suited to the determination of urinary organic acids (following a modification of the method developed by Bachmann et al. [11]) without the need for any hardware modifications.

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