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THE ENANTIOMER AS INTERNAL STANDARD FOR THE QUANTITATION OF THE ALKYLATED AMINO ACID S-METHYL-L-CYSTEINE IN HAEMOGLOBIN BY GAS CHROMATOGRAPHY–CHEMICAL IONISATION MASS SPECTROMETRY WITH SINGLE ION DETECTION

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SUMMARY

A method has been developed using gas chromatography–chemical ionisation mass spectrometry for the determination of S-methyl-L-cysteine in rat and human haemoglobin. The D-enantiomer of S-methylcysteine is added as an internal standard prior to the protein hydrolysis and a partial purification of the protein hydrolysate made by ion-exchange column chromatography. The enantiomers of S-methylcysteine are separated as their N-trifluoroacetyl *n*-butyl ester derivatives on a capillary column coated with the chiral stationary phase Chirasil-Val. The use of single ion detection in the assay is three times more sensitive than the use of multiple ion detection with a deuterium labelled internal standard. The method has been applied to haemoglobin samples from rats and humans exposed to methylating agents.

INTRODUCTION

Humans are frequently exposed to small traces of alkylating agents and as many of these compounds are carcinogens, methods for detecting such exposure are currently being sought. Measurement of the formation of S-alkylcysteine and N-alkylhistidine derivatives in haemoglobin has been proposed as a method of assessing the degree of exposure to these carcinogens[1–6]. For human exposure the amount of alkylating agent–protein reaction products are extremely small. For example, Calleman *et al.*[6] who have developed a gas chromatographic–mass spectrometric (GC–MS) method for quantifying 3-(2-hydroxyethyl)histidine in haemoglobin found levels of this alkylated amino acid in workers exposed to ethylene oxide ranging from 0.4 to 13.5 nmoles/g protein. In order therefore to quantitate accurately these trace levels the assay method must have a very high degree of specificity and sensitivity.

We have recently reported a method using GC–chemical ionisation (CI) MS for the determination of S-methyl-L-cysteine in haemoglobin employing multiple ion detection with a deuterated internal standard[7]. We have now attempted to increase the sensitivity of this analytical method by operating the mass spectrometer in the

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single ion rather than the multiple ion monitor mode. This has been made possible by using as an internal standard the unnatural D-enantiomer of S-methylcysteine which can be resolved from the corresponding L-enantiomer using a capillary column coated with the chiral phase Chirasil-Val[8,9].

MATERIALS AND METHODS

S-Methyl-L-cysteine was obtained from Sigma London (Poole, Great Britain). S-Methyl-D-cysteine was prepared by the methylation of D-cysteine with dimethyl sulphate following the procedure of Du Vigneaud *et al.*[10]. S-Trideuteromethyl-L-cysteine was obtained similarly. *n*-Propanol, isopropanol, *n*-butanol and isobutanol (BDH, Poole, Great Britain) of analytical-reagent grade were redistilled before use. Trifluoroacetic anhydride, pentafluoropropionic anhydride and heptafluorobutyric anhydride were purchased from Pierce (Rockford, IL, U.S.A.) and were redistilled over P_2O_5 and stored at 0°C. Dowex 50W-X4 ion-exchange resin was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.).

Isolation of S-methyl-L-cysteine from haemoglobin

The analytical procedure used which was similar to that previously reported[7] is summarised in the schematic diagram shown in Fig. 1. Following isolation of globin from blood the internal standard S-methyl-D-cysteine (250 ng) was added to samples of protein (1–10 mg) which were then hydrolysed in 6 *N* HCl containing mercaptoethanol (1%) and *n*-octanol (1%) at 110°C for 24 h *in vacuo*. The S-methyl-cysteine in the resulting amino acid mixture was partially purified by chromatography on a column (23 × 1 cm) of Dowex 50W-X4 using 1 *N* HCl as eluting solvent. The

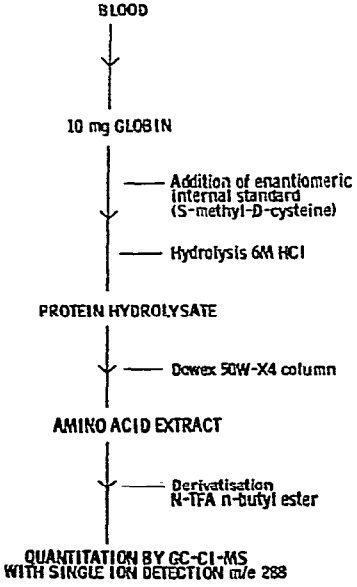

Fig. 1. A schematic diagram of the method for the determination of S-methyl-L-cysteine in haemoglobin.

fraction containing the L- and D-S-methylcysteine (72–108 ml) was concentrated to dryness and the residue derivatised for GC–MS analysis.

Derivatisation of samples

The extracts were dried following addition of methylene chloride by evaporation under nitrogen at 70°C and were then treated with 3 *N* HCl in *n*-butanol at 120°C for 1 h in tubes with PTFE-lined caps. The resulting *n*-butyl esters were dried under nitrogen at 50°C and were acylated with a solution of trifluoroacetic anhydride (30 μl) in ethyl acetate (100 μl) at 140°C for 10 min. The reaction mixtures were taken to dryness under nitrogen while the tubes were immersed in ice–water and the products redissolved in ethyl acetate (100 μl) prior to GC–CI-MS analysis.

Gas chromatography–mass spectrometry

Capillary columns were coated with Chirasil-Val using the static coating procedure to give a film thickness of *ca.* 0.2 μm. The wall surface of capillaries drawn from borosilicate glass was modified prior to coating with stationary phase by depositing a microparticular layer of NaCl on the walls of the column according to the procedure of De Nijs *et al.*[11]. More recently capillaries of fused silica have been used which we have found can be coated with Chirasil-Val without any surface modification.

Capillary columns were installed in a Packard-Becker Model 419 gas chromatograph equipped with flame ionization detectors and in a Pye-Unicam Series 204 gas chromatograph interfaced with glass-lined stainless steel tubing to a 70-70 VG Micromass double focusing mass spectrometer. Helium was used as carrier gas at a flow-rate of 2 ml/min measured at 180°C. Samples were introduced into the GC column with a falling needle solid injector[12] made of quartz glass using an injector port temperature of 200°C. The mass spectrometer operating conditions were as follows: source temperature 200°C, accelerating voltage 4 kV; chemical ionisation was carried out using isobutane as reagent gas with an emission current of 500 μA and electron energy of 50 eV.

For the determination of S-methyl-L-cysteine in haemoglobin samples the mass spectrometer was operated in the single ion monitor mode recording mass *m/e* 288. Quantitation was made by reference to a standard calibration curve determined for each batch of samples analysed. Standard mixtures containing varying amounts of the L-enantiomer (0–400 ng) in the presence of 250 ng D-enantiomer were subjected to the hydrolysis procedure and chromatographed on Dowex ion-exchange columns prior to derivatisation and GC–CI-MS analysis. Since partial racemisation occurs during the work up procedure the observed peak heights of the L-enantiomer (H_L) and D-enantiomer (H_D) are related to the original quantities of the L-enantiomer (Q_L) and D-enantiomer (Q_D) as follows:

$$H_L = (1-\alpha)Q_L f_L + \alpha Q_D f_L \quad (1)$$

$$H_D = \alpha Q_L f_D + (1-\alpha)Q_D f_D \quad (2)$$

where α is the degree of racemisation (assuming this to be equal for both enantiomers) and f_L and f_D are the corresponding factors relating peak height to quantity of each

enantiomer. Based on the above equations a calibration curve relating the peak height ratio of S-methyl-L-cysteine:S-methyl-D-cysteine measured at *m/e* 288 to the amount of S-methyl-L-cysteine added was obtained using an iterative least means squares procedure.

RESULTS AND DISCUSSION

The quantitation of amino acids by GC or GC–MS using enantiomer labelling has only recently become feasible with the introduction by Frank *et al.*[8] of the thermally stable chiral stationary phase Chirasil-Val. Using this phase it is possible after forming suitable esterified acylated derivatives to resolve almost all the common protein amino acids and their enantiomers in a single programmed high-resolution GC separation[8,9]. We have used a short capillary column coated with this phase in our previous described GC–MS method for S-methyl-L-cysteine[7]. Advantage was taken of the favourable separation characteristics of the phase which allowed the methylated cysteine to be resolved and quantitated without interference from the large amounts of contaminating amino acids present in the partially purified hydrolysate. The N-heptafluorobutyryl *n*-butyl ester derivatives of the deuterated and non-deuterated amino acid were used in this multiple ion monitoring assay. These same derivatives were however unsuitable for use in this present method because adequate resolution of the S-methylcysteine enantiomers could not be obtained. In order therefore to find more suitable derivatives a study was made of the GC elution characteristics of the N-trifluoroacetyl (N-TFA), N-pentafluoropropionyl (N-PFP) and N-heptafluorobutyryl (N-HFB) derivatives of the *n*-propyl, isopropyl, *n*-butyl and isobutyl esters of D- and L-S-methylcysteine. Baseline resolution of the enantiomers could be obtained with all these derivatives with the exception of the N-HFB *n*-butyl esters. The enantiomeric separation factor increased with decrease in molecular weight of the acyl group and was affected by the structure of the alkyl ester group in the order isopropyl > *n*-propyl > isobutyl > *n*-butyl. The highest selectivity of enantiomeric separation was therefore obtained with the N-TFA isopropyl esters. The acylated *n*-propyl and isopropyl esters were considered unsuitable for use in the assay since these derivatives were too volatile for quantitative transfer onto the GC column using the solid injection device. Some uncontrollable losses also occurred when these more volatile derivatives were concentrated prior to GC–MS analysis. The N-TFA *n*-butyl esters were found to be the most suitable having a lower volatility and favourable MS properties. The GC separation on the Chirasil-Val coated capillary of a standard mixture of amino acid enantiomers using these derivatives is illustrated in Fig. 2. L-Valine and L-proline are the two major contaminating amino acids present in the protein hydrolysate after chromatography on the Dowex ion-exchange column.

The electron impact (EI) mass spectrum of the S-methylcysteine derivative showed extensive fragmentation and in order to increase the *m/e* value of the ion used for quantitation and to decrease background contamination CI mass spectrometry was employed. The isobutane CI mass spectrum of the *n*-butyl ester of N-TFA S-methyl-L-cysteine (Fig. 3) showed MH^+ (*m/e* 288) as the base peak. The mass spectral fragmentation and the percentage ion current carried by the molecular ion were similar to those observed with the N-HFB *n*-butyl ester of S-methyl-L-cysteine[7].

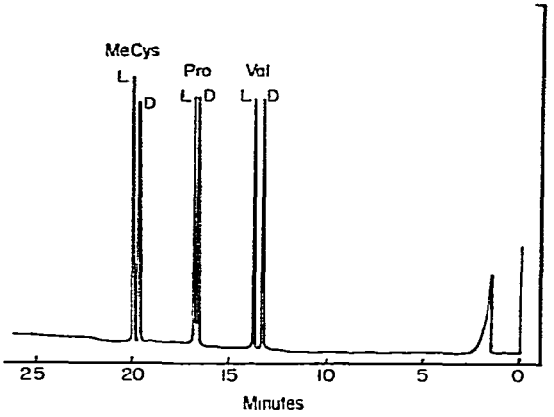

Fig. 2. Gas chromatographic separation of the D-and L-enantiomers of valine (Val), proline (Pro) and S-methylcysteine (MeCys) on a 20 m Chirasil-Val coated capillary. The derivatives were the N-TFA *n*-butyl esters. Initial column temperature 80°C for 3 min followed by a 5°/min programme to 180°C.

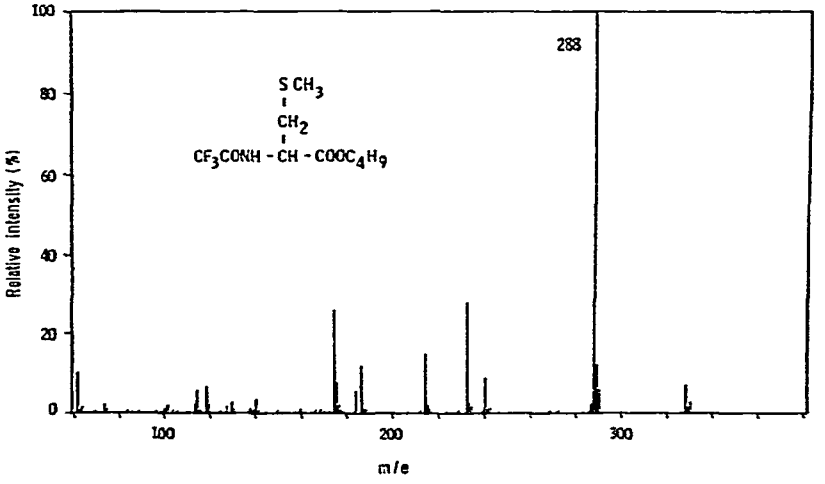

Fig. 3. Isobutane CI mass spectrum of the N-TFA *n*-butyl ester derivative of S-methyl-L-cysteine.

A CI single ion monitoring of the protonated molecular ion *m/e* 288 of an equimolar mixture of the D- and L-enantiomers of derivatised S-methylcysteine is shown in Fig. 4. Each peak on the tracing corresponds to 200 pg of compound injected. A typical calibration curve used for the quantitation of S-methyl-L-cysteine in haemoglobin is shown in Fig. 5. Since racemisation of both the D- and L-enantiomers of S-methylcysteine occurs during the work up procedure the curve is non-linear and does not pass through the origin. Eqns. 1 and 2 which were used to fit the calibration curve are equivalent to those developed by Frank *et al.*[9]. The degree of racemisation for S-methylcysteine, which occurs predominately during the hydrolysis step, was estimated to be 6.4%.

The use of single ion monitoring employing the enantiomeric internal standard was compared with the use of multiple ion monitoring in conjunction with a stable

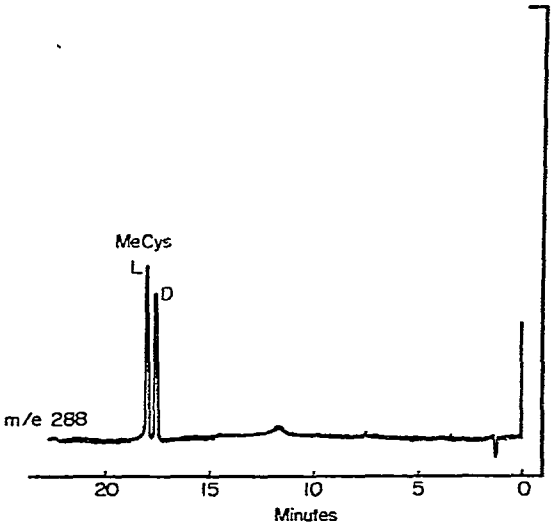

Fig. 4. Single ion monitor trace (*m/e* 288) of a standard of 200 pg L- and D-S-methylcysteine N-TFA *n*-butyl ester. The GC conditions are similar to those given in Fig. 2.

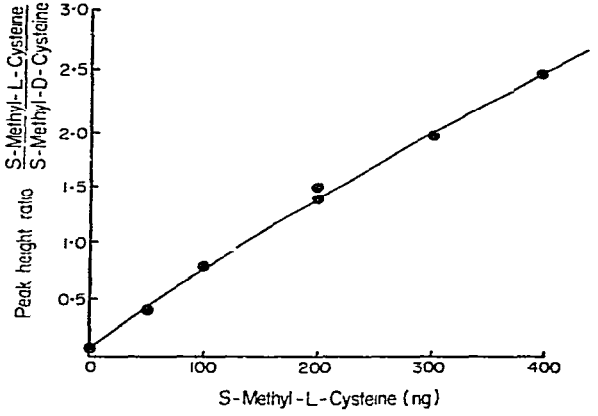

Fig. 5. Calibration curve for the determination of S-methyl-L-cysteine in haemoglobin. The peak height ratio at *m/e* 288 (S-methyl-L-cysteine: S-methyl-D-cysteine) was plotted against the amount of S-methyl-L-cysteine added.

isotope labelled internal standard. Theoretically, at low levels of compound, as the mass spectrometer scans more ions the signal-to-noise ratio for each *m/e* value should decrease[13]. An equimolar mixture of [$^2H_0$] and [$^2H_3$]S-methyl-L-cysteine was derivatised and samples containing 1 ng of each compound were monitored either on single ion (*m/e* 288) or on two ions (*m/e* 288 and 291) corresponding to the MH^+ ions of the $^2H_0$ and $^2H_3$ species. An approximate three-fold increase in signal:noise ratio for [$^2H_0$]S-methyl-L-cysteine was obtained when the mass spectrometer was operated only in the single ion monitor mode.

GC–CI-MS method described in this paper is currently being used to assay the degree of alkylation of cysteine in proteins of animals treated *in vivo* and *in vitro*

with carcinogenic methylating agents and in the proteins of humans exposed to cancer chemotherapeutic alkylating agents. In the course of these studies low levels of naturally occurring S-methyl-L-cysteine were found in the haemoglobin of various species[14]. Fig. 6 shows the application of the assay to the analysis of the background level of S-methyl-L-cysteine in the haemoglobin of the guinea pig.

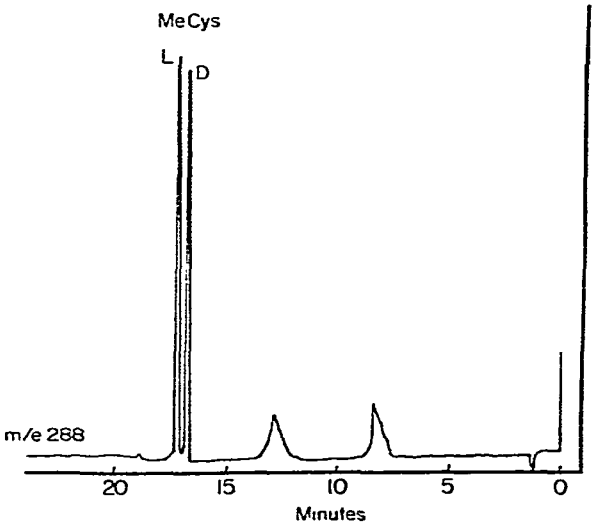

Fig. 6. Single ion monitor trace (*m/e* 288) of an extract from guinea pig haemoglobin. The background level of S-methyl-D-cysteine was found in this animal to be the 16.1 ng/mg globin. The GC conditions are similar to those given in Fig. 2.

CONCLUSIONS

The GC–MS method described in this paper allows the determination of S-methyl-L-cysteine down to levels of 0.1 nmole/g protein with a high degree of precision and accuracy. The assay uses an unnatural enantiomer as an internal standard which is a recent approach developed by Frank *et al.* for the quantitation of amino acids[9,15] and optically active drugs and metabolites[16]. The advantages of using such an internal standard in a GC or GC–MS analysis are as follows.

(1) The standard has identical chemical properties to the compound being quantified.

(2) For the determination of amino acids, the enantiomers are generally readily available commercially and of lower cost than the equivalent stable isotope labelled compound.

(3) For quantitation involving the use of GC–MS, the mass spectrometer can be operated in its most sensitive single ion monitor mode. As a consequence the mass spectrometer does not have to be equipped with multiple ion detection facilities.

Quantitation by enantiomer labelling does however require a correction to be made for the extent of racemisation which may occur during the work up of the sample. This is only a disadvantage if the level of racemisation is high and/or is variable which is not the case with this assay for S-methyl-L-cysteine.

The Chirasil-Val coated capillary columns used in the analysis can be readily prepared of a high separating efficiency particularly if columns made of fused silica are used. It is likely that with the increasing availability of thermally stable chiral phases enantiomer labelling will, in the future, be extensively used as a method for quantifying optically active compounds.

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