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Short Communication

Determination of L-methionine-*dl*-sulphoxide in tissue extracts

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ABSTRACT

The amino acid fraction from rat liver, heart and skeletal muscle was prepared by the separation of sulphosalicylic acid extract on Dowex 50 H^+ form. The presence of L-methionine-*dl*-sulphoxide in these extracts was identified and compared by three independent chromatographic methiods: ion-exchange, Pico-Tag and reversed-phase high-performance liquid chromatography after precolumn derivatisation with diethylethoxymethylenemalonate. Quantitative data indicate that L-methionine-*dl*-sulphoxide is present in the intracellular pool at the levels of free methionine.

INTRODUCTION

Methionine is an amino acid susceptible to oxidation. The products of oxidation of the thioether group of methionine are methionine sulphone and methionine sulphoxide. The formation of methionine sulphone is irreversible and occurs only *in vitro* in the presence of strong oxidative agents, *e.g.* performic acid [1]. The formation of L-methionine-*dl*-sulphoxide, however, is reversible and occurs both *in vitro* in the presence of mild oxidative agents [2] and *in vivo* by action of oxygen-free radicals [3,4]. The oxidation of protein-bound methionine in the living organism has been classified as one of the processes of posttranslational modification [5]. The finding of methionine sulphoxide in feed proteins after food processing has triggered several studies to compare the nutritional value of methionine sulphoxide with that of methionine [6,7]. It was found that L-methionine sulphoxide can either totally or nearly substitute for L-methionine when tested in growing rats. This implies that L-methionine sulphoxide can be metabolised *in vivo* but satisfactory progress in characterising methionine sulphoxide metabolism in mammals has been hampered by difficulties in the determination of methionine sulphoxide in biological samples. We measured the amount of L-methionine sulphoxide in tissue extracts of laboratory rats in order to assess the contribution of methionine sulphoxide to the cellular pool of free amino acids and its ability to serve as a "spare" pool of intracellular methionine. In this report we present the results of analyses for methionine sulphoxide and methio-

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nine using (a) a "classic" ion-exchange method but with a modified elution program [8], (b) a commercially established Pico-Tag system [9] and (c) reversed-phase high-performance chromatography after precolumn derivatization with diethylethoxymethylenemalonate [10]. The pitfalls and advantages of these methods used for determining L-methionine-*dl*-sulphoxide were assessed.

EXPERIMENTAL

Reagents

Boric acid, Dowex 50 W (100–200 mesh), sodium acetate, sodium citrate, and amino acid standards were from Sigma (Dorset, UK). Acetonitrile, methanol and HPLC-grade acetic acid were from Fisons (Loughborough, UK). Deionized, distilled water was used for the preparation of buffers. All solvents and samples used for HPLC were filtered through 0.22-μm filter (Millipore, Bedford, MA, USA).

Instruments

Ion-exchange separation of amino acids was performed using a Locarte amino acid analyser (London, UK). The system employed a 25 cm × 0.9 cm I.D. resin bed temperature controlled at 45°C, and with a buffer flow-rate of 30 ml/h.

A Pico-Tag system from Waters (Millipore) consisted of two pumps Type 510, a 717 autosampler, a 486 UV detector and a Pico-Tag column 300 mm × 3.9 mm I.D. Data acquisition and processing were effected with Baseline software (Waters).

Reversed-phase chromatography using precolumn derivatization with diethylethoxymethylenemalonate was carried out using a Waters 625 LC system with a quaternary low-pressure mixing pump, a 486 UV detector, a Rheodyne 9125 manual injector and a 4-μm Nova-Pak C_{18} column 300 mm × 3.9 mm I.D. with Nova-Pak C_{18} precolumn inserts. Data were acquired using Millennium version 1.1 (Waters).

Sample preparation

Extracts from liver, heart and skeletal muscle from fed adult male rats (Hooded Lister, Rowett strain), 200–250 g body weight, were prepared as follows: animals were killed by cervical dislocation, and tissue samples (200–800 mg) were quickly rinsed in a cold 0.9% NaCl solution, blotted on a filter paper and freeze-clamped in liquid nitrogen with aluminium tongs. Samples were stored at −70°C until used for the extraction. To avoid any oxidation of amino acids, particularly of methionine, the tissues were homogenised with ice-cold 10% (w/v) sulphosalicylic acid thoroughly gassed with nitrogen. The tissue was quickly weighed and homogenised within 10–15 s after its removal from liquid nitrogen. A 20% tissue homogenate was prepared with an Ultra Turrax (Janke & Kunkel) IKA Labortechnik. The homogenate was centrifuged twice at 5000 *g* for 10 min and the pellet was rehomogenised in half of the original volume of sulphosalicylic acid before the final centrifugation. Supernatants were combined, and 20–50 nmol of norleucine (internal standard) were added to each extract. Further separation of the amino acid fraction from the extracts was performed using minicolumns (3 cm × 0.7 cm I.D.) with Dowex 50 W H^+ form [11]. The extracts were applied on the columns and after washing with 6 ml of water the amino acid fraction was collected in the eluate with 6 ml of 4 *M* ammonia. The amino acid fraction was freeze-dried and stored at 4°C until used for analysis.

Under the applied conditions of the sample preparation we have not observed any sulphooxidation of methionine. This was tested by a parallel preparation of tissue extracts with or without 100 nmol of methionine added. These amino acid analyses showed the same amount of methionine sulphoxide in prepared extracts.

The separation of the dextrorotatory stereoisomer (L-methionine-*d*-sulphoxide) from levorotatory isomer (L-methionine-*l*-sulphoxide) from its racemic mixture (L-methionine-*dl*-sulphoxide) was achieved by the formation of picric acid derivatives: the dextrorotatory isomer was water-insoluble contrary to the levorotatory isomer; a detailed procedure of the separation is described in ref. 12.

Chromatography

Ion-exchange chromatography conditions were essentially as described previously [8]. The separation of acidic and neutral amino acids with added methionine sulphoxide was achieved by lowering the pH of the first buffer from 3.25 to 2.97 and that of the second buffer from 4.25 to 4.08.

Conditions for the separation of amino acids using the Pico-Tag system were essentially as described in ref. 9. The temperature of the column and derivatization conditions for the separation of physiological amino acids were controlled as described in *Operation Manual of Amino Acids Analysis Pico-Tag* by Waters Division of Millipore.

The derivatization of samples with diethylethoxymethylenemalonate was carried out essentially as described in ref. 10 with the slight modification that we used 1 μl of derivatization reagent per 1 ml of borate buffer. We also modified the elution conditions to achieve better resolution of methionine sulphoxide from other amino acids: solvent A was 25 m*M* sodium acetate with 5% (v/v) acetonitrile plus 0.02% (w/v) sodium azide pH 6.0; solvent B was 100% acetonitrile. The flow-rate was 0.9 ml/min, and the gradient program was as follows: 0.0–5 min, 100% solvent A; 5.0–45 min, a linear gradient from 0% B to 5% B; 45–50 min, a linear gradient to 15% B followed by isocratic condition up to 70 min; 70–75 min, the gradient up to 30% B was linear with isocratic conditions for the next 10 min; 85–90 min, the column returned to the initial conditions with 100% A.

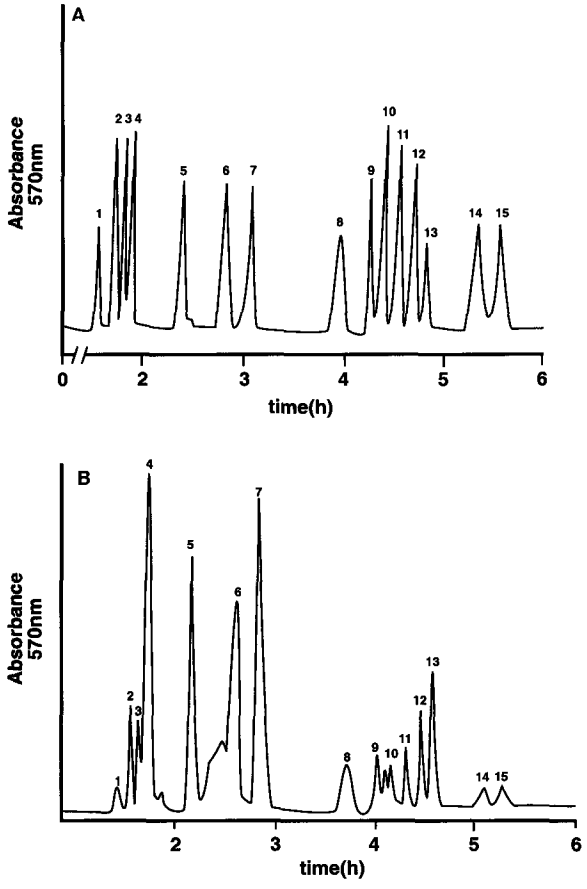

Fig. 1. Elution profiles of (A) standard amino acids and (B) extract from rat liver chromatographed on an ion-exchange column. Each peak of standard mixture represents 25 nmol of amino acid except for L-methionine-*dl*-sulphoxide: 10 nmol. Peaks: 1 = methionine sulphoxide; 2 = Asp; 3 = Thr; 4 = Ser/Gln; 5 = Glu; 6 = Gly; 7 = Ala; 8 = Val; 9 = Cys; 10 = Meth; 11 = Ile; 12 = Leu; 13 = Nleu; 14 = Tyr; 15 = Phe.

RESULTS AND DISCUSSION

The separation of L-methionine-*dl*-sulphoxide from acidic and neutral amino acids by the ion-exchange method is presented in Fig. 1A. The *d*- and *l*-isomers of L-methionine sulphoxide eluted as one peak (peak 1) in front of aspartic acid. Lowering the pH of the first buffer in the ion-exchange analysis resulted in the elution of cysteine at the front of the second buffer but this was well resolved from methionine (see Fig. 1A); under our conditions glutamine was co-eluted with serine. In spite of this, the majority of amino acids could be quantified without interference, especially methionine sulphoxide and methionine as well as leucine and norleucine so the recovery of the amino acids from tissue extracts could be calculated. The profile of amino acid separation in rat liver extract is presented in Fig. 1B.

The elution and detection of the standards of L-methionine-*dl*-sulphoxide by the HPLC pre-column derivatization with phenylisothiocyanate (Pico-Tag method) and with diethylethoxymethylenemalonate are presented in Figs. 2 and 3,

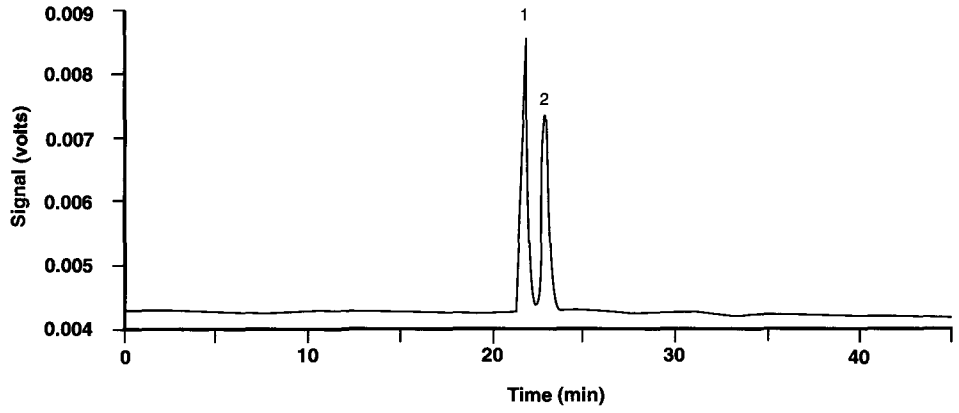

Fig. 2. Separation of a standard solution of L-methionine-*dl*-sulphoxide on a Pico-Tag column. Each peak represents 175 pmol of L-methionine-*d*-sulphoxide (peak 1) and L-methionine-*l*-sulphoxide (peak 2).

respectively. Both methods separate the two isomers of L-methionine-*dl*-sulphoxide. The amount of *l*- and *d*-sulphoxide in the standard solution was equal as calculated from the area of the two

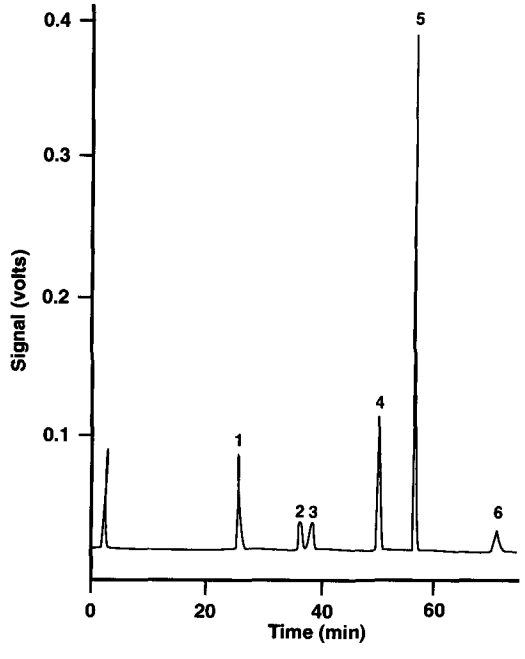

Fig. 3. Elution pattern of a standard mixture of some amino acid and L-methionine-*dl*-sulphoxide chromatographed on a C_{18} column with precolumn derivatization with ethylethoxymethylenemalonate. Peaks: 1 = Ser (50 pmol); 2 = L-methionine-*d*-sulphoxide (75 pmol); 3 = L-methionine-*l*-sulphoxide (75 pmol); 4 = Ala (50 pmol); 5 = ammonia; 6 = internal standard, Norleu (40 pmol).

peaks. However, the resolution of the two stereoisomers of L-methionine sulphoxide from other amino acids was not complete. On the Pico-Tag column alanine co-elutes with L-methionine-*d*-sulphoxide; this is illustrated on skeletal muscle extract analysis in Fig. 4. The separation of stereoisomers of L-methionine-*dl*-sulphoxide with the second HPLC method did not give complete separation; in this case the quantitation of L-methionine-*l*-sulphoxide is hampered due to co-elution with threonine. The resolution of L-methionine-*d*-sulphoxide from histidine was possible in most cases but there was some difficulty when the amount of histidine present was high (data not shown).

At this stage we considered the use of a specific electrochemical detector that has been successfully used for determining plasma methionine [13]. However, within optimal reducing potential for methionine sulphoxide (0.72–0.85 V) the interference of oxygen in the solvent is too high, and selective detection of methionine sulphoxide could not be achieved (data not shown). The conditions of chromatography used with the Pico-Tag system are those routinely used in many laboratories, and the manufacturer claims the satisfactory resolution of most of the physiological amino acids. Therefore we decided to determine only the L-methionine-*l*-sulphoxide isomer using the Pico-Tag system without any further modifications. The HPLC system with the derivatization by di-

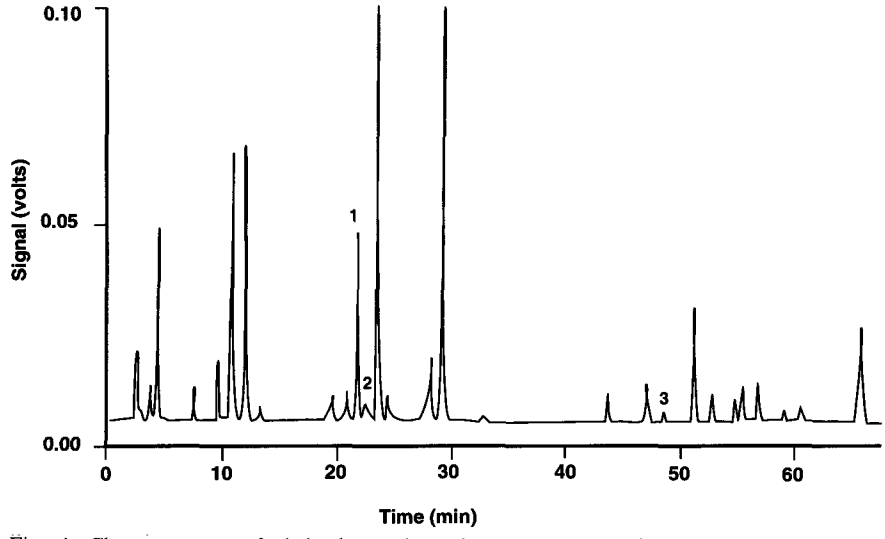

Fig. 4. Chromatogram of skeletal muscle amino acids on a Pico-Tag column. Peaks: 1 = Ala + L-methionine-*d*-sulphoxide; 2 = L-methionine-*l*-sulphoxide; 3 = L-methionine.

ethylethoxymethylenemalonate has the advantage that the derivatized samples are stable and can be stored several months at room temperature without affecting the reproducibility of the analysis. The draw-back of the method is that under the optimum conditions for separation of methionine sulphoxide and methionine, one cannot obtain complete resolution of all the physiological amino acids. The much lower sensitivity of this method and difficulties in quantifying separately the two stereoisomers of L-methionine-*dl*-sulphoxide in the presence of other physiological amino acids mitigated against the use of this method for the routine determination of L-methionine-*dl*-sulphoxide in the biological samples tested. However, the method can be used specifically for determining the dextrorotatory isomer of L-mehionine-*dl*-sulphoxide since this is better resolved than on the Pico-Tag system.

The calculated concentration of L-methionine, L-methionine-*dl*-sulphoxide and some other selected amino acids in extracts of rat tissue are presented in Table I. The results permit the comparison of the intracellular amino acid content in the rat liver, heart and skeletal muscle using Pico-Tag and ion-exchange methods. Quantitation of the selected amino acids (except aspartic acid and methionine) show a high degree of agreement between the Pico-Tag method and ion-exchange chromatography. This confirms the extensive and detailed comparison of these two methods of amino acid analysis reported recently [14]. Under our conditions, however, the reproducibility of the methionine and aspartic acid concentrations within each method is relatively high contrary to that presented in the study of Feste [14]. This may reflect our use of laboratory rats kept under the same feeding regime while in the studies presented by Feste [14] human plasma was taken from volunteers of unknown nutritional status. The amounts of the amino acid (particularly methionine) in our analysis are also in good agreement with data previously published by others [15,16]. This therefore eliminates the possibility that the methionine sulphoxide detected and quantified here is formed from methionine oxidised during the sample preparation.

The amounts of L-methionine-*dl*-sulphoxide detected in the amino acid pool in rat tissues exceeds the concentration of L-methionine. This finding supports the concept that methionine sulphoxide could be a "spare" pool of methionine in the body. It is difficult to know yet how much methionine sulphoxide comes from the rats' diet.

TABLE I

CONCENTRATION OF SELECTED FREE AMINO ACIDS IN THE LIVER, HEART AND MUSCLE OF LABORATORY RATS

Results are the mean ± S.D. of three to five different samples. Values are expressed as nmol/mg tissue.

Amino acid	Liver		Heart		Skeletal muscle	
	Ion-exchange	Pico-Tag	Ion-exchange	Pico-Tag	Ion-exchange	Pico-Tag
Methionine sulphoxide	0.157 ± 0.030	0.062 ± 0.020	0.152 ± 0.006	0.059 ± 0.012	0.098 ± 0.003	0.104 ± 0.025
Methionine	0.038 ± 0.005	0.081 ± 0.010	0.075 ± 0.008	0.115 ± 0.014	0.068 ± 0.002	0.108 ± 0.024
Asp	0.204 ± 0.040	0.097 ± 0.060	0.496 ± 0.093	0.300 ± 0.023	0.086 ± 0.034	0.047 ± 0.008
Gly	1.150 ± 0.055	0.950 ± 0.025	0.352 ± 0.053	0.285 ± 0.015	1.490 ± 0.420	1.346 ± 0.280
Thr	0.175 ± 0.030	0.117 ± 0.020	0.152 ± 0.005	0.107 ± 0.004	0.245 ± 0.034	0.197 ± 0.041
Ala	2.020 ± 0.040	1.590 ± 0.069	1.267 ± 0.190	1.097 ± 0.059	1.635 ± 0.305	1.428 ± 0.132
Val	0.210 ± 0.050	0.155 ± 0.020	0.119 ± 0.020	0.105 ± 0.012	0.165 ± 0.016	0.146 ± 0.017
Ile	0.120 ± 0.030	0.104 ± 0.011	0.055 ± 0.011	0.057 ± 0.010	0.076 ± 0.004	0.088 ± 0.019
Leu	0.240 ± 0.060	0.219 ± 0.031	0.123 ± 0.020	0.129 ± 0.021	0.128 ± 0.008	0.132 ± 0.022

The animals were fed a high-protein diet (18%), with a purported methionine concentration of 3.1 g per kg of diet. Because the ingredients in the diet (soya, 15%; fish meal, 5%) are known to contain appreciable amounts of oxidised methionine (see Table I in ref. 17), the L-methionine sulphoxide found in the rat tissues may be of dietary origin.

After the administration of L-methionine sulphoxide to humam volunteers, its changes in plasma and urine indicates its utilisation *in vivo* [18]. Furthermore, the incorporation of sulphur from L-methionine sulphoxide into hepatic glutathione in laboratory rats has been reported [19]. Our results showing for the first time the quantitative analyses of L-methionine-*dl*-sulphoxide in animal tissues validate the idea of its metabolism in mammals.

Assuming equal amounts of the two stereoisomers of L-methionine-*dl*-sulphoxide in both rat liver and heart, the determination by the Pico-Tag method of the levorotatory form of L-methionine sulphoxide is in good agreement with the half of the amount of the racemic mixture of L-methionine-*dl*-sulphoxide quantified by the ion-exchange method. In skeletal muscle, the data indicate the selective accumulation of only the levorotatory form of L-methionine sulphoxide. At present it is difficult to explain such a finding but recently published observations suggest there may be a difference in the metabolic utilisation of the two epimers of L-methionine sulphoxide in young rats [20]. Thus, Iwami *et al.* [20] suggest that the dextrorotatory isomer of L-methionine sulphoxide is more readily acetylated and reduced than the levorotatory stereoisomer. The full elucidation of the metabolic differences between the two stereoisomers of L-methionine-*dl*-sulphoxide clearly awaits further study, but it is nevertheless clear that the source of methionine and its metabolism needs to take into account the presence of methionine sulphoxide in the diet and the selective transport or use of the specific stereoisomers in different tissues.

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