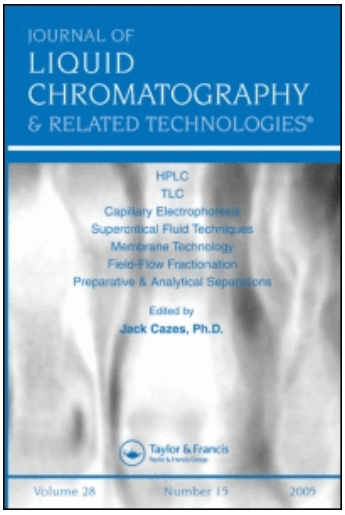

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A RAPID HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINATION OF HOMOCYSTEINE IN PORCINE TISSUE

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ABSTRACT

A simple yet sensitive method for determination of total L-homocysteine (HC) in tissues is described. The assay involves incubation of a tissue homogenate in the presence of a reducing agent to liberate homocysteine from protein followed by derivatization of thiols with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F). Homocysteine is then quantified using fluoresence detection following high performance liquid chromatography on a 5 μM reverse phase column.

The method was applied to heart tissue taken from normal pigs and the results compared to values obtained from pigs intermittently exposed to nitrous oxide (N_2O). It was found that N_2O treatment resulted in a reduction in methionine synthase activity (EC 2.1.1.13) and concomitantly in a massive accumulation of homocysteine in both tissue and plasma.

INTRODUCTION

In recent years there has been an increase in interest in the sulphur containing amino acid homocysteine (HC) which has been implicated in several pathological conditions, most notably coronary artery disease (1,2).

Homocysteine, formed from S-adenosylhomocysteine, can be metabolized to cystathionine, by condensation with serine, or can be remethylated to methionine. This latter reaction is catalyzed by two enzymes the most widely distributed of which is methionine synthase. The anaesthetic gas N_2O has been demonstrated to be a potent inhibitor of methionine synthase giving rise to an accumulation of HC in plasma (3,4).

It has been reported that, in the presence of copper, homocysteine can exhibit prooxidative activity and that this may result in oxidative modification of low density lipoprotein (LDL) (5,6). It is possible that this may contribute to the onset of coronary artery disease. There is also some evidence suggesting that LDL modification may occur not in plasma but in the arterial intima (7).

A method has previously been described for the measurement of HC in mouse and rat tissues (8) based on the measurement of S-adenosylhomocysteine formed by condensing endogenous HC with adenosine, the reaction being catalysed by S-adenosyl-homocysteine hydrolase (AdoHc hydrolase). However, this method is laborious, time consuming and commercially available AdoHc hydrolase is expensive.

Recent advances in the production of fluorogenic thiol derivatives have been utilised in the measurement of HC in plasma (9,10). In this article we detail a rapid method for determining the HC content of tissues after derivatizing with the thiol specific compound ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F), described by Toyo`oka and Imai (9), followed by HPLC with fluoresence detection. This method is sensitive and robust, yet given its simplicity it is suitable

for the analysis of large numbers of samples. The method was used in this study to determine the HC content of heart tissue taken from normal pigs and from pigs intermittently exposed to N_2O.

MATERIALS AND METHODS

Reagents

Methanol (HPLC grade) was obtained from Rhone-Poulenc Ltd (Manchester, UK). D,L-Homocysteine and tri-n-butylphosphine were obtained from Aldrich Chemical Co. Ltd (Gillingham, UK). SBD-F was obtained from Wako (Dusseldorf, Germany). All other reagents were obtained from BDH Ltd (Poole, UK).

Standard solutions

A stock solution of 10 mmol/L D,L-homocysteine was prepared in 0.1 mol/L hydrochloric acid and stored at 4°C for 1 week. This was diluted to give a final concentration of 5 μmol/L in water prior to use.

HPLC instrumentation

The liquid chromatography system consisted of Hewlett Packard HP 1090 liquid chromatograph, a Merck Hitachi F1050 fluoresence detector (EX 385, EM 515) and a Hewlett Packard HP 3392A integrator. The analysis was performed using a LiChrospher 100 RP 18, 25 x 0.4 cm I.D., 5 μM particle size (E. Merck, Darmstadt, Germany) at ambient temperature. A chart speed of 0.3 cm/min was used.

Chromatographic conditions

The mobile phase gradient used was as described by Araki and Sako (10). Briefly, solvent A consisted of 0.1 mol/L acetate buffer (pH 4.0) containing 2 % methanol (v/v) and solvent B of 0.1 mol/L phosphate buffer (pH 6.0) containing 5 % methanol (v/v).

The buffers were filtered through a type HV filter 0.45 μM (Millipore, Bedford, MA, U.S.A.), mixed with methanol and degassed prior to use. A linear gradient from solvent A to solvent B over 20 minutes (0-100%) at a flow rate of 1 ml/min was used.

Animal study

Sixteen 5 week old Landrace pigs were randomly divided into 2 equal groups. Both groups received the same commercial diet. Group 1 was maintained under a polythene canopy in an atmosphere of 15 % (v/v) N_2O for 4 days. The canopy was then raised and the pigs maintained in air for 3 days. This procedure was then repeated. Group 2 was maintained in air for the entire duration of the experiment. All animals were bled and killed after 4 weeks and tissues immediately frozen in, and stored under, liquid nitrogen.

Homocysteine analysis

Two grams of chopped heart were homogenized with 8 ml of 50 mmol/L phosphate buffer (pH 7.4) containing 10 mmol/L EDTA. This homogenate was then derivatized with SBD-F using a similar procedure to that employed for plasma by Araki and Sako (10). In short, 50 μL of 10 % (v/v) tri-n-butylphosphine in dimethylformamide was added to 250 μL of the homogenate and the mixture incubated at 4°C for 30 minutes to release protein-bound homocysteine. This was followed by the addition of 250 μL of chilled 10 % (w/v) trichloroacetic acid

containing 1 mmol/L EDTA under vigorous vortexing, followed by centrifugation at 1500g for 10 minutes at 4°C. An aliquot (100 μL) of the supernatent was then mixed with 200 μL of 2.5 mol/L borate buffer (pH 9.5) containing 4 mmol/L EDTA and 100 μL of SBD-F (1.0 mg/ml) in 2.5 mol/L borate buffer (pH 9.5). The mixture was then incubated for 60 minutes at 60°C. A 5 μL aliquot was then used for HPLC analysis. Each specimen was homogenized and analysed in duplicate and results expressed as nmol homocysteine /gram tissue (wet wt.).

Plasma was analysed by the same method by substituting 250 μL of plasma for 250 μL of tissue homogenate.

Methionine synthase analysis

Total methionine synthase activity was measured radio-enzymatically as described by Keating *et al.* (11).

Statistical analyses

Statistical analyses were carried out using Student's two-tailed t test with equal or unequal variance as appropriate. Differences between means were considered significant at $p < 0.05$. All values cited in the text are means ± SEM.

RESULTS

HPLC analysis

Figure 1 shows the HPLC chromatograms of: (a) homocysteine standard (5 μmol/L), (b) control pig heart extract and (c) the same control pig heart tissue fortified with 20 nmol homocysteine /gram tissue.

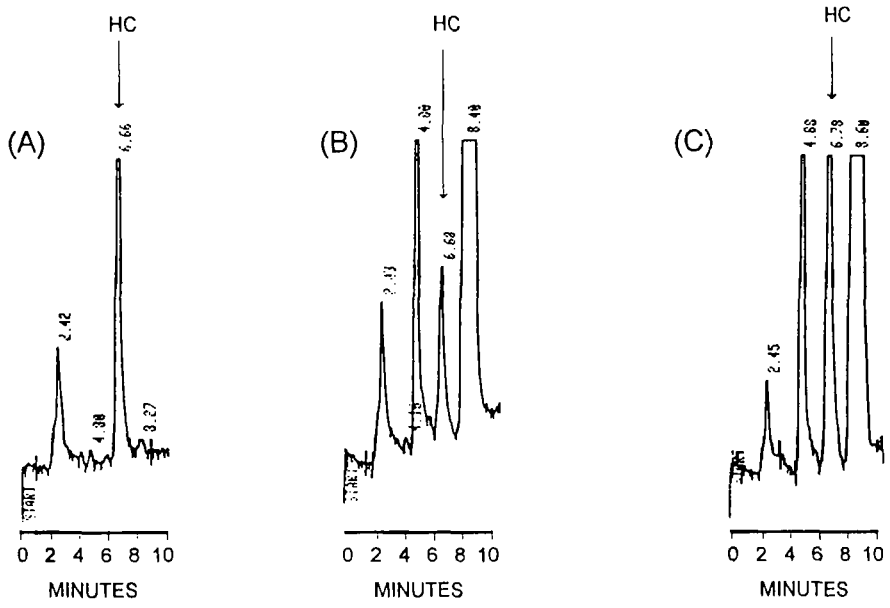

FIGURE 1. Chromatograms of: (A) homocysteine standard (5 μmol/L), (B) a normal pig heart homogenate and (C) the same pig heart homogenate fortified with 20 nmol homocysteine / gram tissue.

The reproducibility of the assay was determined by analysing one sample 10 times within one day and by analysing the same sample over 6 different days. The values obtained within day were 8.9 ± 0.2 nmol/g (n=10, CV 8.5 %) and between day were 9.1 ± 0.4 nmol/g (n=6, CV 8.9 %).

Six different heart samples were fortified with HC at each of two levels (10 nmol/g and 20 nmol/g). The mean recovery of HC from samples fortified at 10 nmol/g was 104.1 ± 3.3 %, and from samples fortified at 20 nmol/g was 106.3 ± 3.8 %.

The linearity of the assay was assessed over a concentration range equivalent to 0-250 nmol/g. The equation of the line of best fit was y =

1.063x - 0.259 (correlation coefficient = 0.9993). The limit of detection, defined as 3 times the signal to noise ratio, was less than 1.25 nmol/g tissue.

Results obtained by analysis of heart tissue from control and N_2O treated pigs for methionine synthase activity and tissue homocysteine content and for plasma homocysteine concentration are summarised in Table 1. All results are the mean of duplicate analysis.

Total methionine synthase activity was significantly lower in the heart of N_2O treated animals than in heart tissue from air controls (38 % of control value). This was reflected in a significantly higher homocysteine content in both tissue and plasma in the N_20 treated group.

DISCUSSION

The present report describes a method for the determination of HC in tissue. The method is a modification of the assay of Araki and Sako (10) who described the determination of HC in plasma by HPLC separation of SBD adducts. The present method proved to be reproducible and reliable and gave good recovery of HC from pig heart homogenate when added at both 10 nmol/g and 20 nmol/g (104.1 ± 3.3 % and 106.3 ± 3.8 %, respectively). A previously reported method (8) produced similar figures in terms of reproducibility and recovery. However detection in that method was based on determining the radioactivity in HPLC fractions, making it tedious and restricting the batch size. With the current method up to 20 tissue specimens can be homogenized and derivatized in duplicate in one working day and with the use of an autosampler the HPLC can be carried out overnight.

Ueland *et al.* (8) demonstrated that the HC content of murine tissue isolated and frozen *post mortem* did not differ significantly from the HC

Table 1. Tissue methionine synthase activity and homocysteine content of plasma and heart tissue from pigs treated with N_2O and corresponding controls.

TREATMENT GROUP	METHIONINE SYNTHASE nmol/g/hr	PLASMA HOMOCYSTEINE µmol/L	TISSUE HOMOCYSTEINE nmol/g
GROUP 1 N2O	49.8 * ± 5.2	496.0 * ± 3.3	88.0 * ± 6.7
GROUP 2 AIR	132.9 ± 12.5	26.3 ± 3.3	9.6 ± 0.7

Values are means ± SEM of eight N_2O-treated pigs and eight air controls. Asterisks indicate significant differences from air control group (* $p < 0.0005$) as assessed by Student's t test.

content of tissue frozen *in vivo*. However, in this study it was found that the HC content of heart tissue increased significantly if the tissue was allowed to stand at room temperature for as little as 10 minutes *post mortem* before being frozen in liquid nitrogen (data not shown).

In this study we have also shown that treatment of pigs with N_2O brings about a significant reduction in methionine synthase activity in heart tissue (activity fell to 38 % of control animal values). This was accompanied by an accumulation of HC in plasma (496 ± 28.9 µmol/L), a concentration which is very much higher than the 58 µmol/L observed by Van der Westhuyzen *et al.* in N_2O treated fruit bats (4). We have also demonstrated that HC is present in measurable amounts in normal porcine heart tissue (9.6 ± 0.7 nmol/g) and that exposure of the animal to N_2O brings about an accumulation of HC in tissue (88.0 ± 6.7 nmol/g). This assay is currently being applied in a study examining the effects of hyperhomocysteinaemia on the pathogenesis of coronary artery disease in pigs.

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