Journal of Chromatography, 338 (1985) 201—208
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2398

Note

Determination of homocysteine in urine*

S.B. THOMSON and D.J. TUCKER*

Division of Chemical and Physical Sciences, Deakin University, Deakin, Victoria 3217 (Australia)

and

M.H. BRIGGS

Division of Biological and Health Sciences, Deakin University, Deakin, Victoria 3217 (Australia)

(First received July 4th, 1984; revised manuscript received October 16th, 1984)

Homocysteine is a product of methionine metabolism rarely detectable in normal human tissue fluids [1]. This thiol-containing amino acid is present in excessive amounts in the blood and urine of persons suffering from homocystinuria (an enzyme deficiency blocking the metabolic pathway between homocysteine and cystathionine) [2, 3]. Homocystinurics exhibit a wide range of physical manifestations including skeletal defects and mental retardation; if allowed to continue unchecked, cardiovascular disease invariably kills the patient at any time after their early teen years [4, 5].

The prematurity and pronounced nature of this vascular damage, coupled with research showing that an increased level of homocysteine in the circulation is a major causative factor in accelerated cardiovascular injury [6, 7], has led us to develop a more sensitive, routine analytical technique for measuring homocysteine in biological fluids and to look at the possibility that hormone treatment may lead to increased levels of homocysteine which may predispose to cardiovascular damage.

Surveys suggest that women taking oral contraceptives have an increased risk of suffering thrombosis as compared to non-users [8, 9]. It has been proposed

*The paper was presented at the Melbourne International Symposium on High-Performance Liquid Chromatography in the Biological Sciences, Melbourne, Australia, February 20—22, 1984. The majority of papers has been published in J. Chromatogr., Vol. 336, No. 1.

0378-4347/85/$03.30

that oral contraceptives may interfere with sulfur amino acid metabolism [10, 11], leading to altered homocysteine production and/or breakdown in the methionine pathway, and elevated homocysteine levels in tissue fluids. This would increase cardiovascular damage, perhaps leading to thrombotic episodes.

This report describes a technique for determining the homocysteine concentration in urine. The procedure involves separation on an ion-exchange column followed by electrochemical detection (ED) at a hanging mercury drop. Preliminary studies involving urine from rats treated with estrogen and progestogen used in oral contraceptives are also reported.

METHOD

Chemicals

Buffers were made up with sodium dihydrogen phosphate (A.R. grade, BDH) in deionized, distilled water. The pH was adjusted with A.R.-grade orthophosphoric acid (BDH) and prior to chromatographic work, buffer filtration was carried out using a 0.45-μm filter (Millipore). Mercury was triply distilled (Englehard). Amino acid standards were: L-cysteine and L-methionine (Puriss grade, Fluka) and D,L-homocysteine (Sigma). Urine specimens were treated with acid (either orthophosphoric or hydrochloric acid, both A.R. grade, BDH) to prevent oxidation of the thiol groups prior to analysis.

Analytical system

An anodic reaction of cysteine at mercury electrodes leads to the formation of an adsorbed inorganic–metallic species:

$$2R{-}SH + Hg \rightarrow (R{-}S)_2Hg + 2H^+ + 2e$$

This has been the basis of several methods for the electrochemical detection of cysteine, as well as other biologically important thiols such as penicillamine and glutathione [12–17]. The reaction has been used to detect homocysteine in biological samples [12, 13, 17], but only recently has it been shown that the formation of an adsorbed homocysteine–mercury complex following oxidation is the basis for homocysteine detection [18]. Most electrodes for flowing systems utilize mercury-coated gold surfaces, operated amperometrically at a potential sufficiently positive to allow the anodic reaction to occur when the thiol group passes the electrode surface.

Adsorption products build up on the electrode with each subsequent detection, leading to a reduced electrochemical response over time, and rather than clean and renew the solid electrode surface each time, we have incorporated a static mercury drop electrochemical detector (Model 310, EG and G Princeton Applied Research), so a new mercury drop of specified size can be formed whenever significant surface contamination occurs. The thiol detection limit is about 0.5 ng, making it similar to freshly prepared mercury-coated gold electrodes. This is the first time this method has been used to detect biologically important thiols, although it has been used with success for other compounds [19].

Homocysteine is polarographically inseparable from cysteine [18], and strong cation-exchange high-performance liquid chromatography (HPLC)

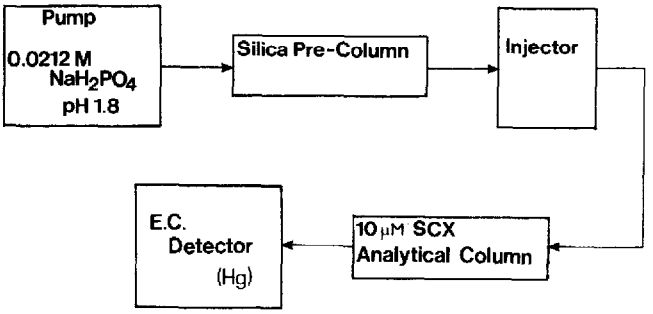

Fig. 1. Schematic representation of the HPLC—ED system for homocysteine determination.

(25 cm × 4.6 mm I.D. stainless-steel column containing Partisil-10 SCX 10 μm, Whatman) has been incorporated into the system to allow separation of the thiols prior to detection. The choice of ion exchange as the chromatographic medium is based upon previously developed methods to separate thiol-containing compounds [12, 13, 16, 17]. A unique feature of our method (Fig. 1) is the use of a silica pre-column (Silica Pre-Column Kit, Whatman) prior to the injector in the HPLC system, for a very acidic eluent is needed to allow satisfactory separation of homocysteine from other electrochemically active species. Without this silica pre-column, the silica backbone of the ion-exchange column can degenerate within 24 h, rendering the column useless. As the buffer passes through the silica pre-column first, silica dissolves and saturates the buffer, thus greatly reducing dissolution of silica from the backbone of the analytical column, resulting in extended column life [20—22]. Incorporation of the pre-column kit has enabled use of an analytical column on a daily basis for two months (about 800 injections) with no appreciable drop in column efficiency. Inclusion of the silica pre-column before the injection does not affect the separation of the substances of interest, since they only pass through the analytical column.

Urine collection

Rats used were female Sprague—Dawley. They were fed rat pellet food and water ad libitum during the experiments, except for the one fed a methionine (0.35%, w/w) loaded diet, where the amino acid was pre-mixed into the food. Urine was collected over 24-h periods from rats isolated in metabolic cages into vials containing known volumes of acid (either orthophosphoric or hydrochloric acid) to act as antioxidant, preventing thiols forming disulfides from atmospheric contact. Prior to chromatographic analysis, the samples were centrifuged (EBA 35, Hettich) at 500 *g* for 5 min to separate any solid material, and then the supernatant was diluted 1:1 with Nanopure water (Waters Assoc.) to give a more manageable injection volume.

Ethynyl estradiol (Sigma) was dissolved in absolute alcohol (0.83 mg/ml) as was levonorgestrel (Wyeth International) (0.83 mg/ml). Estradiol valerate (Sigma) was dissolved in 1:1 benzyl benzoate—castor oil (0.03 mg/ml).

RESULTS AND DISCUSSION

An acidic eluent (pH 2.2, 0.02 *M* sodium dihydrogen phosphate) was

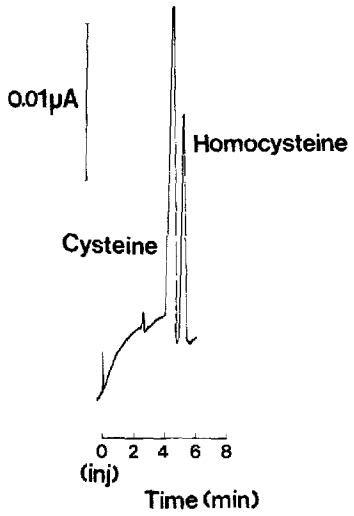

Fig. 2. HPLC profile obtained using a Partisil SCX-10 column followed by electrochemical detection at a hanging mercury drop electrode (at −0.15 V versus Ag/AgCl) of an injected sample containing $2.5 \cdot 10^{-5}$ *M* concentrations of homocysteine and cysteine in 5 μl of eluting buffer. Eluting buffer is 0.02 *M* sodium dihydrogen phosphate, pH 2.2 at a flow-rate of 1.0 ml/min.

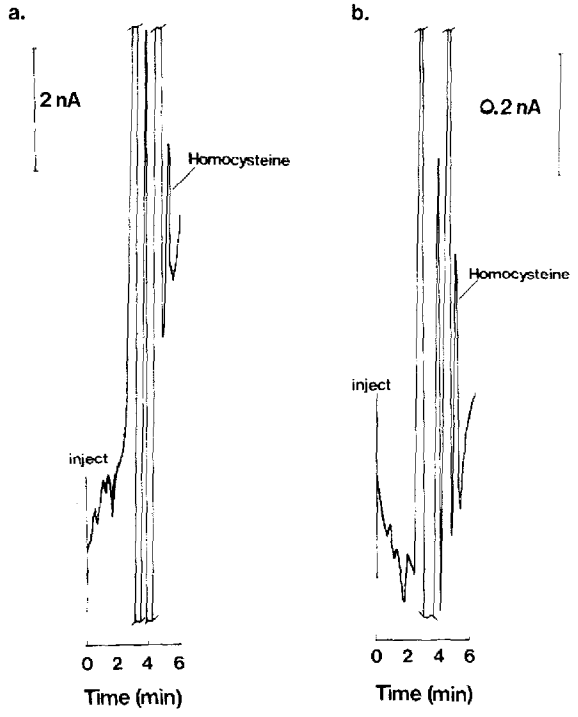

Fig. 3. HPLC profile obtained using a Partisil SCX-10 column followed by electrochemical detection at a hanging mercury drop electrode (at −0.15 V versus Ag/AgCl) of (a) 10 μl of a rat urine sample (diluted 1:1 with Nanopure water) and (b) 10 μl of a rat urine sample (diluted 1:1 with Nanopure water) which has been spiked with homocysteine to give an effective amount of 6.75 μg in the injected sample. Eluting buffer is 0.0212 *M* sodium dihydrogen phosphate, pH 1.8 at a flow-rate of 1.0 ml/min.

required to separate homocysteine from other thiols such as cysteine (Fig. 2), penicillamine and glutathione (the latter two compounds are retained on the column longer than homocysteine). Only a very minor adjustment of pH and molarity of the eluting buffer was required to obtain adequate separation of the homocysteine peak from other components in a urine sample which are electrochemically responsive at the applied potential (Fig. 3a). Presumably the buffer adjustments compensate for effects of urinary material on the cation-exchange column (e.g., such as occupying exchange sites) which alter the separating capability of the column. Co-elution occurred when a known amount of homocysteine was added to a urine sample which contained homocysteine (Fig. 3b).

The reliability and reproducibility of the quantitative HPLC—ED system for homocysteine was assessed by injecting various amounts of homocysteine in 10 μl of buffer and measuring the resulting peak heights. Good linearity was obtained over the range 0—20 ng of homocysteine, with the graph having a slope of $y = 0.4x$. Application of the system for quantitatively determining homocysteine concentrations in urine was also demonstrated by spiking urine samples with known amounts of homocysteine and injecting 10 μl of the resultant solution (after acidification and centrifugation as described previously) into the system. The peak height responses, measured by fitting a tangent across the base of the peak, and measuring to the apex, fell very close to the calibration line, indicating that other urine components did not alter the homocysteine peak height or shape. These responses also showed that other substances contained in urine do not interact with the mercury electrode surface to reduce its sensitivity to homocysteine.

Having demonstrated the reliability of the analytical system, a preliminary study was conducted into effects of synthetic steroids on urinary homocysteine in rats. We initially looked at the effect of a synthetic estrogen on homocysteine excretion. Three mature female rats were kept in separate metabolic cages for seven days. One received a single 45-μg injection of estradiol valerate in oil intramuscularly, the second a methionine-loaded diet (described in the method section), while the third was an untreated control. Table I gives the average value of triplicate determinations performed on each sample collected, and shows obvious differences in the amounts of homocysteine excreted by these rats over the 24 h test periods.

TABLE I

EFFECT OF A SYNTHETIC ESTROGEN ON HOMOCYSTEINE EXCRETION IN 24-h URINE SAMPLES COLLECTED FROM RATS

Excretion rates expressed as ng homocysteine per 24 h.

Periods of collection (h)	Control rat	Rat fed 0.35% methionine in diet	Rat injected with 45 μg estradiol valerate
24—48	5755	1637	2928
96—120	1584	8478	31210
120—144	2450	10041	20859
144—172	6098	14000	15627

The results show that the estrogenic hormone increased the homocysteine excretion rate. Five days after the administration of a single large dose of estradiol valerate (45 μg) the homocysteine content of the urine of the treated rat was five times greater than that of the urine of a control rat. During the following three days the level of homocysteine in the urine of the treated rat dropped. These results may indicate that the mild anabolic effect of estrogen [23] is responsible for elevating the activity of the methionine pathway, resulting in turn in an increase in the excretion of homocysteine which is an intermediate metabolite. The results also suggest that the effect only lasts as long as the elevated estrogen level exists, and as the active form of estrogen is metabolically inactivated, the effect on the methionine metabolic pathway becomes less pronounced, causing the homocysteine content in 24-h urine samples to tend toward control levels.

The treatment of a rat with methionine added to the diet at a level of 0.35% was undertaken because methionine is metabolised partly to homocysteine and we expected homocysteine excretion rates to rise as the methionine level in the diet was raised, increasing the pressure on that metabolic cycle. This hypothesis was supported by the increasing excretion of homocysteine during the seven days of the trial. Malloy et al. [24] have shown that homocysteine may be bound more strongly to plasma proteins than cysteine, and this may be part of the reason that the amount of homocysteine in the urine of the methionine-diet-treated rat is still within the daily range of homocysteine excreted by the control rat up to 48 h after the trial began. As the feeding continues, a new equilibrium between homocysteine, cysteine and the protein binding sites will be established, and the homocysteine excretion rate will rise. The results obtained for this rat also assist in confirming that the chromatographic peak we are observing is homocysteine since it increases during the trial as expected, and is not therefore some other component that has the same retention time as homocysteine when standard solutions of the latter are chromatographed.

The second animal study looked at the effects of a synthetic estrogen, a synthetic progestogen and a combination of the two on homocysteine excretion in 24-h urine samples collected from weanling rats after 21-days treatment as described. All the experimental rats were treated exactly as the controls, except that two received daily subcutaneous injections of 0.2 μg ethynyl estradiol, two were injected with 2 μg levonorgestrel, and another pair had daily injections of 0.2 μg ethynyl estradiol plus 2 μg levonorgestrel. The

TABLE II

EFFECTS OF SYNTHETIC ESTROGEN AND PROGESTOGEN ON HOMOCYSTEINE EXCRETION IN URINE SAMPLES COLLECTED FROM RATS AFTER THE DESCRIBED TREATMENTS FOR 21 DAYS

Excretion rates expressed as ng homocysteine per 24 h.

Control	Daily injection of 0.2 μg/day ethynyl estradiol	Daily injection of 2 μg/day levonorgestrel	Daily injection of both 0.2 μg/day ethynyl estradiol and 2 μg/day levonorgestrel
11,600	33,600	11,500	28,100

dosages of these experiments were designed to resemble the amount per body weight of the synthetic steroids that oral contraceptive users take on a daily basis [25]. Table II shows the differences between the groups, each value being the average homocysteine excretion exhibited by the pair of rats in that group after all collected samples had been analysed in triplicate.

These results confirm that estrogenic substances elevate homocysteine excretion rates, since treatment with daily doses of ethynyl estradiol caused a marked increase in the excretion of homocysteine in the urine of these rats compared to the control rats. Daily administration of levonorgestrel (a synthetic progestogen) caused no apparent change in the level of homocysteine excreted in the 24-h urine samples when compared with the results obtained from the control rats. The combination of estrogen and progestogen administered daily also caused a marked increase in the levels of homocysteine excreted when compared to the control rats, and the similarity in the size of the increase by this group and the estrogen-treated group leads to the conclusion that the increased amount of homocysteine excreted is due to the estrogen in the combination.

We are currently undertaking a study involving a large number of rats to statistically test these observations. We also plan to ascertain whether women who use oral contraceptives have increased urinary levels of homocysteine.

ACKNOWLEDGEMENTS

This study was made possible by a research grant from Wyeth International and Schering A.G. The help of Mr. D. Geraghty, Mr. B. Biviano and Ms. S. Lee in setting up and maintaining the rat metabolic studies is gratefully acknowledged.

REFERENCES

1 S.-S. Kang, P.W.K. Wong and N. Becker, Pediatr. Res., 13 (1979) 1141.
2 D. Valle, G.S. Pai, G.H. Thomas and R.E. Pyeritz, Johns Hopkins Med. J., 146 (1980) 110.
3 R. Griffiths, in J.M. Rattenburg (Editor), Amino Acid Analysis, Ellis Horwood, Chichester, 1981, p. 337.
4 V.J. Gupta and D.E.L. Wilcken, Eur. J. Clin. Invest., 8 (1978) 205.
5 R.W. Hund, E.J. Hammond and D.J. Wilder, Brain Res., 209 (1981) 250.
6 K.S. McCully and R.B. Wilson, Atherosclerosis, 22 (1975) 215.
7 L.A. Harker, R. Ross, S.J. Slichter and C.R. Scott, J. Clin. Invest., 58 (1976) 731.
8 P.E. Sartwell, A.T. Masi, F.G. Arthes, G.R. Green and H.E. Smith. Amer. J. Epidemiol., 90 (1969) 365.
9 M.P. Vessey and R. Doll, Brit. Med. J., 2 (1969) 651.
10 K.S. McCully, Amer. J. Clin. Nutr., 28 (1975) 542.
11 L.T. Miller, M.J. Dow and S.C. Kokkerer, Amer. J. Clin. Nutr., 31 (1978) 619.
12 D.L. Rabenstein and R. Saetre, Anal. Chem., 49 (1977) 1036.
13 R. Saetre and D.L. Rabenstein, Anal. Biochem., 96 (1978) 684.
14 R. Eggli and R. Asper, Anal. Chim. Acta, 101 (1978) 253.
15 L.A. Allison, Curr. Sep., 4 (1982) 38.
16 L.A. Allison and R.E. Shoup, Anal. Chem., 55 (1983) 8.
17 L.A. Smolin and N.J. Benevenga, J. Nutr., 112 (1982) 1264.
18 A.M. Bond, S.B. Thomson, D.J. Tucker and M.H. Briggs, Anal. Chim. Acta, 156 (1984) 33.

19 S.K. Vohra and G.W. Harrington, J. Chromatogr. Sci., 18 (1980) 379.
20 J.G. Atwood, G.J. Schmidt and W. Slavin, J. Chromatogr., 171 (1979) 109.
21 F.M. Rabel, J. Chromatogr. Sci., 18 (1980) 394.
22 F.M. Rabel, Amer. Lab., 12 (1980) 81.
23 E.B. Astwood, in L.S. Goodman and A. Gilman (Editors), The Pharmacological Basis of Therapeutics, Macmillan, New York, 4th ed., 1970, p. 615.
24 M.P. Malloy, D.K. Rassin and G.E. Gaull, Amer. J. Clin. Nutr., 34 (1981) 2619.
25 F.H. Meyers, E. Jawetz and A. Goldfien (Editors), Review of Medical Pharmacology, Lange Medical Publications, CA, 6th ed. (c), 1978, p. 396.