Journal of Chromatography, 382 (1986) 247—252
Biomedical Applications
Elsevier Science Publishers B V., Amsterdam — Printed in The Netherlands

CHROMBIO 3242

Note

Analysis of homocysteine in human urine using high-performance liquid chromatography with electrochemical detection

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(First received February 28th, 1986; revised manuscript received April 29th, 1986)

Homocysteine is a thiol-containing amino acid produced as an intermediate in the metabolism of methionine [1]. The possibility that it may be a precipitating agent in the development of vascular damage is supported by experimental evidence which indicates that increasing the homocysteine level in the circulation leads to rapid cardiovascular injury [2, 3], as well as by observations of patients suffering from the genetic disorder homocystinuria where abnormally high levels of homocysteine accumulate in the blood and urine. If the condition is untreated, cardiovascular disease invariably proves fatal at any time following their early teen years [4].

It has been shown that women using oral contraceptives suffer a higher incidence of thrombosis than non-users [5] and, as outlined in a previous publication [6], a proposal is that oral contraceptive use may in some way interfere with the metabolism of the sulfur-containing amino acids [7], leading to elevated levels of vascular-damaging homocysteine in the circulation and perhaps precipitating the observed thrombotic episodes.

Until recently little, if any, data existed on the amount of homocysteine in tissues under physiological conditions [8] since previous analytical measurements of homocysteine in body fluids relied on methods that lacked sensitivity and selectivity. However, the development of a very sensitive technique for measuring homocysteine which combines amperometric detection at a mercury drop detector with ion-exchange high-performance liquid chromatography (HPLC) has allowed the measurement of physiological levels of homocysteine in the urine of experimental rats [6, 9].

This report describes the modifications made to the analytical system prior to determination of homocysteine in human urine. It also contains data which

0378-4347/86/$03.50

compare the homocysteine concentrations in the urine of males and females, as well as the results of a study in which the effect of oral contraceptive use by a group of women upon their urinary homocysteine concentrations is compared to a control group. Further to this work, the effect of cigarette smoking on the urinary homocysteine concentrations of oral contraceptive users as compared to controls has been assessed, and finally the report contains the urinary homocysteine concentrations over a 28-day collection period for both an oral contraceptive-using female and a male volunteer.

EXPERIMENTAL

Chemicals

All chemicals were as described previously [6]; 2 *M* perchloric acid (AR grade, Ajax Chemicals) was used to prevent thiol group oxidation which can occur on contact with air.

Analytical system

This is identical to the system used previously [6, 9], where a static mercury drop electrochemical detector (Model 310, EG & G Princeton Applied Research) is used following ion-exchange HPLC, except that we have replaced the HPLC column described with a Z-Module radial compression separation system (Waters Assoc.) filled with the same strong cation-exchange material (Partisil-10 SCX, 10 μm, Whatman).

Urine collection

Approximately 50 ml of urine from volunteers participating in this study were collected into vessels containing 10 ml of 2 *M* perchloric acid. In all cases the samples were obtained in the morning immediately upon waking, and for the comparison of oral contraceptive users with controls, the urine samples were obtained on the 21st day of their cycle. Samples were analysed immediately after collection or frozen at −20° C until analysis. Prior to chromatographic analysis the samples were centrifuged (EBA 35, Hettich) at 500 *g* for 5 min, and the supernatant was diluted 1:1 with Nanopure water (Waters Assoc.) to give more manageable HPLC injection volumes.

Homocysteine was quantified by the standard addition technique using peak-height measurement, the determination being carried out in triplicate. The homocysteine concentration was then expressed per mg creatinine (colorimetric method, Boehringer Mannheim). Creatinine is used as the comparative value when quoting homocysteine excretions because the amount excreted per individual is relatively constant, depending mostly on muscle mass, since creatinine arises solely from the spontaneous cyclization of muscle creatine [1].

RESULTS AND DISCUSSION

The analytical system used in previous work [6, 9] was modified to include the Z-Module radial compression separation system instead of the more commonly encountered stainless-steel HPLC column because it achieved a

slightly superior separation of homocysteine from possible interferents than the latter column [6].

Using this analytical system, a comparison of urinary homocysteine excretion by a group of twenty males and twenty females was undertaken. The mean excretion rates and the standard deviations (both expressed as ng homocysteine per mg creatinine) were 394 ± 322 for the males (range 138–1484) and 211 ± 83 for the females (range 60—371). The female group was made up of ca. 50% oral contraceptive users and ca. 50% non-oral contraceptive users, with the urine samples being collected on the 21st day of their cycle. All the participants in the study were apparently healthy, with an average age in the mid-twenties for the females and the late twenties for the males. The significantly ($\alpha < 0.05$) higher excretion rate of homocysteine for the males compared with the females using the Student t distribution test is of interest since the risk of myocardial infarction for males is up to six times greater than for females in the under forty age bracket [10]. The causative nature of infarcts is invariably atherosclerosis of the coronary arteries, with thromboembolic disease being a complicating factor in up to 40% of the cases, so increased homocysteine in the circulation as indicated in our study, may be of some significance, especially since previous research points to elevated levels of homocysteine being involved in the development of cardiovascular injury [2, 3].

The effect of oral contraceptive use on urinary homocysteine excretion has been assessed by comparing an experimental set of apparently healthy women all aged in their early twenties. The set consisted of three groups, the first of which was a control group of 36 women. The second was a group of 32 women using either a triphasic or a constant formulation oral contraceptive where both types of pill contained a low dose of estrogen ($\leqslant$ 50 g) and a low dose of progestogen ($\leqslant$ 150 g). The third group of 12 women were taking an oral contraceptive which combines a low dose of estrogen ($\leqslant$ 50 g) with a high dose of progestogen ($\geqslant$ 500 μg).

The results obtained for the three groups, namely the mean ± S.D. and the range (which are all expressed as ng of homocysteine excreted per mg of creatinine), were 205 ± 111 (range 59—649), 187 ± 96 (range 60—487) and 251 ± 170 (range 78—704), respectively. Application of Duncan's Multiple Range Test to the results indicates that there is no statistical difference in the amount of homocysteine excreted in the urine of women in each of the three test groups. The indication therefore is that neither oral contraceptive formulation causes an increase in the excreted level of homocysteine in the urine of users as compared to the control group. Previous research has shown that rats given ethynyl estradiol in doses designed to resemble the concentration per unit body weight taken by women using oral contraceptives exhibit a significantly increased amount of excreted homocysteine in their urine [6, 9]. The results of those studies, combined with the observations made here, along with evidence showing that homocysteine causes cardiovascular damage in experimental animals which could predispose towards thromboembolic episodes [2, 3], suggest that the components of the oral contraceptive formulation act differently in the human as compared to the rat, and do not lead to elevated homocysteine levels in human urine. Hence it seems that the increased incidence of thrombotic episodes observed among women using oral contra-

ceptives as compared to controls [5] is due to a cause(s) other than an elevated homocysteine level caused by the synthetic steroid components of the oral contraceptive formulation.

Twelve of the urinary homocysteine excretion values were obtained from women who use oral contraceptives and who also smoke between ten and twenty cigarettes per day. The range and mean value ± S.D. for urinary homocysteine excretion from these women were 94—704 and 202 ± 153, respectively, expressed as ng homocysteine per mg creatinine in the urine samples. Because these values do not differ significantly from those obtained from 36 non-smoking subjects, which were 60–487 and 200 ± 99 for the range and mean value ± S.D., respectively, it would appear that smoking has not influenced the urinary excretion levels of homocysteine in women participating in this study. The similarity of the homocysteine excretion levels in the urine of smoking and non-smoking oral contraceptive users is interesting also in that it has been recognised for some time that smoking probably increases the risks associated with oral contraception, including that of developing thrombosis [11]. This observation tends to suggest that as is seen with oral contraceptives, the mechanism by which smoking leads to increased thrombotic incidences seems to be something other than the causation of an elevated circulating level of homocysteine which could lead to vascular damage predisposing to thrombosis.

The amounts of estrogen and progestogen ingested vary during the period in which a woman is taking an oral contraceptive [12]. Fig. 1 shows the daily morning urinary homocysteine excretions of both a healthy male and also a healthy female volunteer, the latter taking a combination triphasic ethynyl estradiol/levonorgestrel oral contraceptive over 28 days which constitutes one complete oral contraceptive cycle for a female. Previously, it has been shown that administration of ethynyl estradiol to female rats in a dose per body

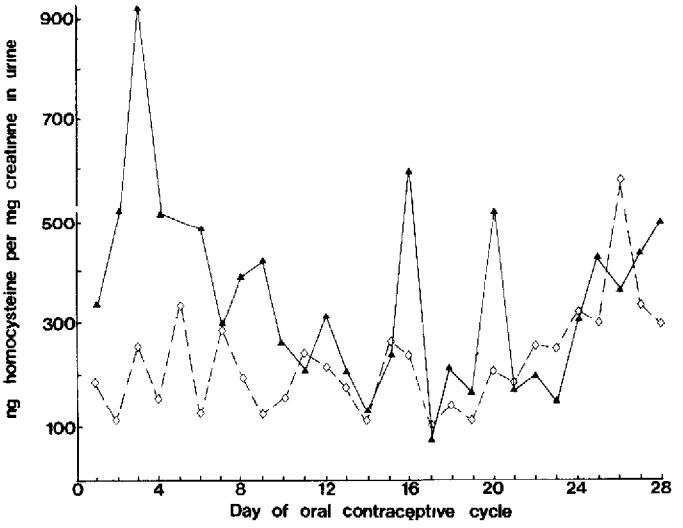

Fig. 1. Homocysteine excretion in the first urine sample of the morning for both a female (▲) and a male (◇). The study was conducted over 28 days, which is the period of one complete female oral contraceptive cycle.

weight similar to that encountered by oral contraceptive users caused significant increases in the levels of urinary homocysteine excreted [9]. The figure shows that homocysteine excretion of the male is relatively constant over the test period. This would be expected if estrogen levels influenced urinary homocysteine levels since males do not have any cyclical patterns of their sex hormones. In the case of the female oral contraceptive user, however, the fact that the estrogen pattern contained in the oral contraceptive formulation over-rides natural estrogen levels in the body [13] means that there is a virtually constant circulating estrogen level for a predominant part of the oral contraceptive cycle, except that it would be expected to be slightly different on days 7 through to 11 when the ethynyl estradiol content of the formulation is 25% higher than at other times of the cycle, and also from days 21 to 28 when no synthetic estrogen is being taken by the oral contraceptive user. The female homocysteine excretion pattern in the urine tends to go from a high level of excretion at the start and end of the oral contraceptive cycle to a low level in the middle of the cycle (although it is certainly not a dramatic change) in a manner which in no way follows the estrogen pattern contained in the oral contraceptive formulation, nor the progestogenic component of the oral contraceptive which increases step-wise during days 1 through to 21 [12]. Therefore it appears that there is no observable link between the synthetic steroid levels and urinary homocysteine levels, and it would seem that if there were any changes in homocysteine excretion which could be correlated with either estrogen or progestogen level changes in the body, they would be masked by other influences such as diet. This hypothesis is based on the results of a dietary history taken coincidently with the collection of the urine samples which indicated that each of the three peaks observed in the female study followed days where the diet included amounts of red meat and alcohol in excess of the average diet of the subject. Since methionine, which is the amino acid whose catabolism produces homocysteine, is mainly derived from foods of animal origin [14], it would appear that these particular incidences of elevated urinary homocysteine excretion are probably due to the dietary factor, rather than any hormonal changes.

In summary, a method for the reliable quantification of homocysteine in human urine has been described. Using this procedure a variety of experiments were undertaken, one result indicating that the mechanism(s) by which both oral contraceptive use and cigarette smoking can lead to elevated incidences of thrombosis in women must be something other than the causation of an elevated level of homocysteine which could lead to pre-thrombotic vascular damage. Other results show that any homocysteine urinary excretion changes which might follow the estrogen pattern of a triphasic oral contraceptive formulation during a cycle would appear to be impossible to detect. This is because dietary effects, among other things, may cause sufficiently large changes in the level of homocysteine excreted in the urine to mask any effects upon the excretion rate that hormonal changes may produce.

The finding of most interest, however, was that males in this study excreted significantly larger amounts of homocysteine in their urine than did females of approximately the same age, which may be of some significance in terms of the increased incidence of myocardial infarction exhibited by males in the population, and certainly warrants further study.

ACKNOWLEDGEMENTS

This work was made possible by a research grant from Wyeth International and Schering AG. The assistance of Mrs. E. McLennan, Miss A. Billett and Mr. B. Stewart in the collection of urine samples is gratefully acknowledged.

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