Detection of Heterozygotes for Phenylketonuria. Total Body Phenylalanine Clearance and Concentrations of Phenylalanine and Tyrosine in the Plasma of Fasting Subjects Compared

Rudolf Jagenburg,1 Carl-Gunnar Regårdh,2 and Stig Rödjö3,4

Two tests have been compared for detection of heterozygotes for phenylketonuria, one based on determination of plasma phenylalanine and tyrosine concentrations in fasting individuals and the other on kinetic evaluation of the plasma elimination curve after intravenous loading with L-phenylalanine. The plasma elimination curve was biexponential and the kinetics were evaluated according to the two-compartment model. The constant, $\beta$, expressing the rate of elimination from plasma at pseudo-equilibrium, the rate constant for the elimination from the central compartment, and the total body clearance were determined. Of these three, total body clearance, which on the average was reduced by 32% in the phenylketonuric heterozygotes, showed the best discriminatory ability, but was not better than the information on concentrations of phenylalanine and tyrosine in detecting heterozygotes for phenylketonuria.

Additional Keyphrases: tyrosine/phenylalanine relationships, genetics, diagnostic acids, inherited disorders, sex, and age-related differences

Phenylketonuria is an autosomal recessive disease characterized by a lack of phenylalanine hydroxylase (phenylalanine 4-monooxygenase, EC 1.14.16.1) activity. The enzyme is located almost exclusively in the liver (1). Direct measurement of its activity and characterization of it in the liver would probably be the best method for detecting the heterozygous state, but liver biopsy is not now a routine method than can be performed in healthy subjects. Quite recently phenylalanine hydroxylase activity has been demonstrated in fibroblast cultures, and this activity was less in cultures from patients with phenylketonuria (2). The possibility of detecting the heterozygous state by applying the same technique has still to be tested.

Attempts have been made to detect the heterozygous state in indirect ways, by measuring the plasma concentrations of phenylalanine and tyrosine in the postabsorptive state (3-6) or the concentrations of these amino acids after oral (7-9) or intravenous (3, 10, 11) loading with phenylalanine. However, none of these methods has discriminated completely between phenylketonuric heterozygotes and normal homozygotes.

We have previously reported that after intravenous injection of L-phenylalanine the plasma concentration curve declined biexponentially and that the kinetics could be evaluated according to the two-compartment model (12). In the present study this method has been applied for investigation of heterozygotes for phenylketonuria. Furthermore, the possibility to discriminate phenylketonuric heterozygotes from normal homozygotes by determination of the concentrations of phenylalanine and tyrosine in the plasma of fasting subjects has been tested.

Material

Reference group. This group consisted of 27 volunteers, 16 men 22 to 47 years old (mean age 28 years) and 11 women 22 to 31 years old (mean age 24 years). The weight of the men ranged between 63 and 94 kg (mean 74 kg), that of the women between 42 and 62 kg (mean 52 kg). None of the subjects had any family history of phenylketonuria.

One of the men was considered to be a heterozygote for phenylketonuria according to the results in this (Figures 1, 3, and 6) and a subsequent study that included experiments with constant infusion of L-phenylalanine (13). The results obtained for this individual were therefore excluded from the statistical calculations.

Heterozygotes for phenylketonuria. This group consisted of 14 parents (eight men and six women) of phenylketonuric children. The age of the eight men varied between 29 and 49 years (mean 34 years) and their weight ranged between 61 and 89 kg (mean 76 kg). The age of the six women varied between 31 and 34 years and their weight ranged between 52 and 60 kg (mean 56 kg).

The diagnosis of phenylketonuria in the children was

---

1 Department of Clinical Chemistry, University of Gothenburg, Sahlgren's Hospital, S-413 45 Gothenburg, Sweden.
2 Research Laboratories, AB Hässle, S-431 20 Mölndal, Sweden.
3 Department of Medicine II, University of Gothenburg, Sahlgren's Hospital, S-413 45 Gothenburg, Sweden.
4 To whom correspondence should be addressed.
Received Mar. 14, 1977; accepted May 21, 1977.
based on the following criteria: plasma phenylalanine concentration \( \geq 1.2 \) mmol/liter and normal tyrosine concentration before dietary treatment was started; dietary restriction of the daily phenylalanine intake to 1.5–3.0 mmol/day to reach a plasma phenylalanine concentration between 0.2 and 0.6 mmol/liter; and normal development of the children during the dietary therapy.

All subjects were apparently healthy and in good condition at the time of the study. None had any history of hepatic, renal, or endocrine disorders, and all had normal serum concentrations of creatinine, bilirubin, and activities of alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase. Four of the women in the reference group and two of the heterozygotes were taking oral contraceptives.

Methods

Intravenous L-phenylalanine loading. The loading was done in the morning, 0800 hours, after an overnight fast. L-Phenylalanine, 300 \( \mu \text{mol/kg} \) body weight, was given intravenously during 3–4 min. Venous blood, collected in heparinized tubes, was drawn before the infusion and thereafter at intervals of 5 min during the first hour and of 10 min during the next 2 h.

Analytical assays. Plasma phenylalanine and tyrosine were determined by ion exchange chromatography using a column (55 \( \times 0.9 \) cm) of Aminex A-6 resin in a Beckman Model 120C amino acid analyzer. Phenylalanine and tyrosine were eluted with a sodium citrate buffer (0.2 mol/liter, pH 4.50) at a column temperature of 56 °C. The plasma (1 ml) was deproteinized with sulfosalicylic acid (2 ml, 296 mmol/liter) containing D,L-\( \beta \)-2-thienylalanine as internal standard.

Calculations. The concentration (C) of phenylalanine in plasma minus the fasting concentration after the intravenous infusion declined biexponentially in accordance with the equation:

\[
C = A'e^{-\alpha t'} + B'e^{-\beta t'}
\]

where \( A' \) and \( B' \) are the intercepts of the exponential terms at the end of the infusion, \( \alpha \) (first slope) and \( \beta \) (second slope) are the rate constants, and \( t' \) represents the time after the infusion was stopped. Numerical values of the parameters were determined by least squares analysis using the DECUS No. 8-661 nonlinear regression program in conjunction with a Digital PDB 8 computer. Unweighted data were found to give the best agreement between the theoretical curves and the experimental results. The intercepts (A, B), corrected with regard to the time of the infusion, were calculated by the method of Loo and Riegelman (12) by use of the equations:

\[
A = \left( \frac{\alpha \cdot \tau}{1 - e^{-\alpha\tau}} \right) A', \quad B = \left( \frac{\beta \cdot \tau}{1 - e^{-\beta\tau}} \right) B'
\]

where \( \tau \) is the time of the infusion.

The elimination and distribution of phenylalanine were calculated according to the following two-compartment model:

\[
\begin{align*}
\text{Central compartment} & \quad \frac{k_{12}}{k_{21}} \quad \text{peripheral compartment} \\
\left(V_c\right) & \quad \left(V_p\right) \\
\end{align*}
\]

Assuming that the compound is eliminated from the central compartment, as indicated in the scheme, the rate constants associated with this model were calculated from the following relationships:

\[
\begin{align*}
k_{21} &= A \beta + B \alpha \\
A &= \frac{A}{A + B} \\
k_{el} &= \frac{\alpha \cdot \beta}{k_{21}} \\
k_{12} &= \alpha + \beta - k_{el} - k_{21}
\end{align*}
\]

The volume of the central compartment \( (V_c) \) was determined from \( V_c = \text{dose}/(A + B) \) and \( V_p \), the volume of the peripheral compartment from \( V_p = V_{d,\beta} - V_c \) where \( V_{d,\beta} \) is the apparent volume of distribution in the \( \beta \)-phase and is determined from the relationship:

\[
V_{d,\beta} = \frac{\text{dose}}{\beta \cdot \int_0^{\infty} Cdt}
\]

The integral \( \int_0^{\infty} Cdt \), which is identical to the area under the plasma concentration curve from zero to infinite time, was determined by the equation

\[
\int_0^{\infty} Cdt = \frac{A}{\alpha} + \frac{B}{\beta}
\]

Total body clearance was calculated by the product \( k_{el} V_c \). Body surface area (BSA), was estimated according to the method of Isaksson (14).

Statistical analysis. Significance levels of differences between mean values were calculated by Student's \( t \)-test. Statistical significance has been defined as \( P < 0.05 \).

The possibility of discriminating between normal homozygotes and heterozygotes for phenylketonuria was evaluated with a nonparametric method. A discriminant function was determined visually in such a manner that a minimum number of subjects in both groups were incorrectly classified. Discriminant analysis assuming normal distribution was also used (14).

Results

Fasting concentrations of phenylalanine and tyrosine. The fasting plasma phenylalanine concentration was significantly higher \( (P < 0.001) \) in the heterozygotes for phenylketonuria \( (75 \pm 10 \mu\text{mol/liter}, \text{mean} \pm \text{SD}) \) than in the controls \( (56 \pm 9 \mu\text{mol/liter}) \), but there was no difference in the tyrosine concentration (Figure 1). A lower tyrosine concentration was observed in the women using oral contraceptives than in the other subjects. The fasting concentration of tyrosine in each individual was plotted vs. the fasting concentration of
phenylalanine (Figure 1). The equation of the discriminatory line that gave the best discrimination between heterozygotes for phenylketonuria and normal homozygotes was \[ y = 1.56x - 43.5 \], where \( x \) is the phenylalanine and \( y \) the tyrosine concentration in \( \mu \text{mol/liter} \), calculated by discriminant analysis. The probability of erroneous classification on using this equation was 9%. A line could be drawn visually that completely separated all phenylketonuric heterozygotes, except for one man, from the control subjects — i.e., one subject out of 40 was incorrectly classified.

**Kinetics of phenylalanine elimination.** The time-course of the phenylalanine concentration in plasma after the intravenous administration of \( \text{L-phenylalanine} \), corrected for the fasting value, followed a biexponential curve in all individuals. The average concentrations in the different groups are shown in Figure 2 and the kinetic constants of the two-compartment model in Table 1.

The constant \( \beta \) was about 20% lower in the men than in the women (Tables 1 and 2). No sex-related difference was observed in the elimination rate constant \( k_e \) or in the total body clearance \( (k_{el}V_r) \) after correction to constant body surface area or constant body mass. The former correction gave the least interindividual variation in the clearance.

The mean value of \( \beta \) was significantly lower in the heterozygotes than in the controls (Tables 1 and 2), although the overlapping between the groups was great. The mean half-life \( (t_{1/2} = 0.693/\beta) \) was 1.81 ± 0.36 h (mean ± SD) and 2.25 ± 0.34 h in male controls and heterozygotes, respectively. The corresponding values for women were 1.51 ± 0.24 and 1.79 ± 0.25 h.

The elimination rate constant from the central compartment \( (k_e) \) was lower \( (P < 0.001) \) in the phenylketonuric heterozygotes \( (0.95 ± 0.24 \, \text{h}^{-1}) \) than in the control subjects \( (1.32 ± 0.35 \, \text{h}^{-1}) \) whereas the volume of the central compartment \( (V_r) \) was similar in both groups (controls \( 0.24 ± 0.06 \, \text{liter/kg}, \) corresponding to \( 8.8 ± 2.1 \, \text{liter/m}^2 \) heterozygotes \( 0.24 ± 0.08 \, \text{liter/kg}, \) corresponding to \( 8.6 ± 2.7 \, \text{liter/m}^2 \). This resulted in a reduction of the total body clearance in the heterozygotes. The clearance value for all controls \( (n = 26) \) was \( 11.0 ± 1.3 \, \text{liter-h}^{-1} \, \text{m}^{-2} \) (mean ± SD) and for all heterozygotes \( (n = 14) \) \( 7.5 ± 1.6 \, \text{liter-h}^{-1} \, \text{m}^{-2} \), i.e., the total body clearance was decreased by 32% in the heterozygotes.

Total body clearance was the constant that most efficiently discriminated between heterozygotes for phenylketonuria and homozygotes for the normal gene but no complete separation between the groups was attained (Figure 3). Four subjects (two controls and two heterozygotes) of the 40 examined were incorrectly classified when a clearance of \( 9.0 \, \text{liter-h}^{-1} \, \text{m}^{-2} \) was chosen as the discriminatory value. This value was
chosen to obtain the lowest number of incorrectly classified subjects in both groups.

Women who were taking oral contraceptives had a significantly \((P < 0.05)\) larger volume of the central compartment than the other women \([0.32 \pm 0.02 \text{ liter/kg, corresponding to } 10.6 \pm 1.1 \text{ liter/m}^2 (n = 4), \text{ and } 0.23 \pm 0.07 \text{ liter/kg, corresponding to } 7.7 \pm 2.1 \text{ liter/m}^2 (n = 7), \text{ respectively}\), but a lower value of the constant \(k_{el}\) (1.05 \pm 0.07 and 1.44 \pm 0.12 h\(^{-1}\), respectively). Use of oral contraceptives had no apparent effect on the total body clearance of phenylalanine. The mean values of the different constants presented in Table 1 include all women. The proportion of subjects using oral contraceptives was similar in both groups.

**Tyrosine concentrations after L-phenylalanine loading.** The plasma tyrosine concentration rose significantly less after phenylalanine loading in the male heterozygotes than in the male controls (Figure 4). The area under the curve describing the increase in the plasma tyrosine concentration was on average 48% smaller in the heterozygotes than in the control subjects

| Table 1. Kinetic Constants (Mean Value ± SD) of Phenylalanine Calculated According to the Two-Compartment Model after Intravenous Injection of L-Phenylalanine |
|---------------------------------|---------------------------------|---------------------------------|
|                                 | **Male**                        | **Female**                      | **Heterozygotes for phenylketonuria** |
|                                 | **Reference group**             | **Contraceptives**              | **Male**                        | **Female** |
|                                 | **n = 15**                      |                                 | **n = 8**                       | **n = 6** |
| \(\alpha\) h\(^{-1}\)           | 5.14 ± 1.43                     | 5.19 ± 1.29                     | 4.25 ± 0.66                    | 5.37 ± 1.44 |
| \(\beta\) h\(^{-1}\)           | 0.39 ± 0.08                     | 0.47 ± 0.08                     | 0.31 ± 0.05                    | 0.39 ± 0.05 |
| \(V_C\), 1-kg\(^{-1}\)         | 0.24 ± 0.06                     | 0.27 ± 0.07                     | 0.25 ± 0.08                    | 0.22 ± 0.08 |
| \(V_p\), 1-kg\(^{-1}\)         | 0.53 ± 0.12                     | 0.44 ± 0.10                     | 0.40 ± 0.08                    | 0.32 ± 0.06 |
| \(k_{12}\), h\(^{-1}\)         | 2.62 ± 0.88                     | 2.46 ± 0.88                     | 2.12 ± 0.46                    | 2.67 ± 1.07 |
| \(k_{21}\), h\(^{-1}\)         | 1.59 ± 0.52                     | 1.91 ± 0.48                     | 1.64 ± 0.38                    | 2.06 ± 0.19 |
| \(k_{el}\), h\(^{-1}\)         | 1.33 ± 0.38                     | 1.30 ± 0.32                     | 0.84 ± 0.14                    | 1.03 ± 0.31 |
| Clearance \(1\text{-}h^{-1}\hbox{m}^2\) | 11.22 ± 1.53                    | 10.76 ± 1.04                    | 7.66 ± 1.42                    | 7.38 ± 1.93 |

| Table 2. Significance Levels of Differences between Mean Values of the Kinetic Constants in Table 1 |
|---------------------------------|---------------------------------|---------------------------------|
|                                 | **Controls vs. heterozygotes**  | **Males vs. females**           | **Heterozygotes**              |
|                                 | **Males**                       | **Females**                     | **Controls**                  | **Males vs. females** |
| \(\alpha\)                      | NS*                            | NS                             | NS                            | NS                  |
| \(\beta\)                      | <0.05                          | <0.05                          | <0.05                         | <0.01             |
| \(V_C\)                        | NS                             | NS                             | NS                            | NS                  |
| \(V_p\)                        | <0.05                          | <0.05                          | <0.05                         | NS                  |
| \(k_{12}\)                     | NS                             | NS                             | NS                            | NS                  |
| \(k_{21}\)                     | NS                             | NS                             | NS                            | <0.05             |
| \(k_{el}\)                     | <0.01                          | NS                             | NS                            | NS                  |
| Clearance                       | <0.001                         | <0.001                         | NS                            | NS                  |

\*(NS, not significant \((P > 0.05)\).*
during the first 3 h after the injection. A similar difference (56%) was seen when comparing the female groups when those using oral contraceptives were excluded. There was a less pronounced increase in the tyrosine concentration in the females of the control group using oral contraceptives than in the others (Figure 5). The heterozygote group was too small to permit any conclusions in this respect.

The greatest difference in the increase in the plasma tyrosine concentration between heterozygotes and controls was obtained early after the phenylalanine injection. Figure 6 shows that only two subjects were incorrectly classified if an increase in the tyrosine concentration of 17 μmol/liter at 15 min was chosen as the discriminatory level. One male heterozygote showed a greater increase than 17 μmol/liter and one woman control who was taking oral contraceptives showed a smaller increase. The total area under the increase in the plasma tyrosine concentration did not give a better discrimination between heterozygotes and normal homozygotes.

**Discussion**

The heterozygotes for phenylketonuria have only one gene coded for the enzyme phenylalanine hydroxylase and so have less of this enzyme activity than do normal homozygotes. The enzyme activity has been assessed in indirect ways. Haia et al. (7) were the first to report that higher plasma phenylalanine concentrations were seen in heterozygotes for phenylketonuria than in controls after oral administration of L-phenylalanine. Since then, oral loading tests have been widely used (9). Rampini et al. (8) determined the phenylalanine and tyrosine concentrations 1, 2, 3, and 4 h after the loading. By adding these concentrations, corrected by coefficients obtained by discriminant analysis, a discriminatory score was obtained. Using this score, they found a classification error about 1% if three of the 44 subjects were excluded from the original material. This result is surprisingly good, because variations in stomach emptying and gastrointestinal absorption might affect the results. Other studies have shown a considerably higher classification error (15, 16). To avoid the potential sources of errors associated with oral administration, intravenous loading with L-phenylalanine can be done. This route of administration has previously been used in only a few studies dealing with the problem of detecting heterozygotes for phenylketonuria (3, 10, 11). Only four to five blood samples were taken in these previous studies and the possibilities of performing kinetic analysis were consequently limited.

In the present study we have followed the plasma phenylalanine concentration in the time interval 5–180 min after the intravenous administration of a single dose of L-phenylalanine, with blood sampling every 5 to 10 min. The rate constants α and β were estimated by computer simulation using a nonlinear regression program. It was found that the first component (Ae−αt) did not approach zero until 50 to 70 min after the loading. Therefore, if the β-constant is calculated from the plasma concentrations in the time interval 30–150 min after the injection, as in the study of Bremer and Neumann (10), values were falsely high.

The determination of the rate constants in the present study was based on 24 observations. One would therefore expect a higher degree of precision, and consequently less overlapping, between heterozygotes for phenylketonuria and normal homozygotes than in previous studies (10, 11). Instead, we obtained a more pronounced overlapping between the groups. Previously the rate constant for the second component of the elimination (β) has been used for detection of heterozygotes for phenylketonuria (10, 11). Using this constant, which is a function of both distribution and elimination, Woolf et al. (11) found a classification error of 8% on using the β-constant. In the study of Bremer and Neumann (10) a β-value could be obtained visually, which incorrectly classified only two of 26 subjects studied. In our study about a third of the subjects were incorrectly classified in spite of the fact that we have considered that there appears to be a sex-related difference in this constant. A higher value was observed in women, whether control subjects or heterozygotes, but there was no sex-related difference in the total body clearance corrected to constant body surface area. In previous studies (10–12) no sex-related difference has
been observed. It has to be pointed out that clearance also is equal to β-V_d,β, i.e., an increase in β, a decrease in total distribution volume and a constant clearance, as seen in women as compared with man, are compatible results. The smaller value of the total distribution volume in the women was due to a decrease in the volume of the peripheral compartment, which might reflect differences in muscle mass.

Total body clearance and k_el gave a better discrimination of heterozygotes for phenylketonuria than did the β-constant. The total body clearance of phenylalanine, corrected to constant body surface area, gave an incorrect classification of four (two heterozygotes and two controls) of the 40 subjects examined when 9.0 liter·h⁻¹·m⁻² was used as the discriminatory value.

The mean total body clearance of phenylalanine in the reference group of the present study was significantly greater than in a previously studied group (12). This difference is most likely due to a decrease in clearance with age as the mean age of the previous control subjects was 58 years as compared to the present 27 years. The phenylketonuric heterozygotes and the reference subjects in the present study are fairly well matched as far as age is concerned. The heterozygotes were on average six years older than the reference subjects, and this difference might have resulted in a slight overestimation of the discriminatory ability.

The mean total body clearance in the heterozygotes was 68% of that in the control group. The reduction was about the same as observed in a group of patients with cirrhosis of the liver (12). The decrease, however, seems to be due to different mechanisms. In the cirrhotic patients the reduction of the total body clearance was due to a lower volume of the central compartment, probably due to a reduced liver pool size, whereas the elimination rate from the central compartment was unaffected. In the heterozygotes for phenylketonuria V_c was normal but the elimination rate k_el was less, reflecting the decreased activity of phenylalanine hydroxylase.

In several reports concerning the detection of heterozygotes for phenylketonuria, not only the change in the phenylalanine concentration but also the increase in plasma tyrosine after phenylalanine loading has been used as a measure of the decreased hydroxylation of phenylalanine (9). We also found a lower tyrosine increase in the heterozygotes. The area under the curve for increase in the plasma tyrosine concentration during the observation period was about 50% of the corresponding area in the control subjects. However, the area was not a better discriminatory variable than the plasma tyrosine increase at 15 min after the start of the injection (Figure 6). We also noticed that use of oral contraceptives resulted in a lowering of the concentration of tyrosine in the plasma of the fasting subject and a smaller increase in the tyrosine concentration after phenylalanine loading, in agreement with earlier observations (17–20). Thus, there was a risk of incorrectly classifying normal homozygotes who were taking oral contraceptives as heterozygotes for phenylketonuria.

Although the elimination rate of phenylalanine after intravenous loading was calculated from a considerable number of observation points, the discrimination between heterozygotes for phenylketonuria and control subjects was not total. During the past year Griffin and Elsas (6) have reported good results by the simple method of determining the semifasting concentrations of phenylalanine and tyrosine. A discriminant analysis of our data in a similar way gave a probability of incorrect classification of 9%. However, it was possible to draw a line by which only one of the 40 subjects was incorrectly classified.

We conclude that the more time- and resource-consuming method of kinetic evaluation of the curve for elimination from plasma after intravenous injection of L-phenylalanine offers no advantage for detecting heterozygotes for phenylketonuria over the method with determination of the fasting or semifasting plasma concentrations of phenylalanine and tyrosine. However, neither of these methods will permit a complete separation of heterozygotes for phenylketonuria from normal homozygotes.

We thank Dr. L. Palmer, Department of Medicine, AB Håsle for his valuable assistance in the computer processing of the data. This work was supported by grants from the Swedish Medical Research Council (3X-652) and the Medical Faculty in Gothenburg.

References