Estimation and Application of Biological Variation of Urinary δ-Aminolevulinic Acid and Porphobilinogen in Healthy Individuals and in Patients with Acute Intermittent Porphyria

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Background: Diagnosis of an attack of acute intermittent porphyria (AIP) is based on the demonstration of increased concentrations of porphobilinogen (PBG) and δ-aminolevulinic acid (ALA) in urine, but many AIP patients also have high baseline concentrations in remission. The aim of this study was to estimate the biological variations of ALA, PBG, and porphyrins in healthy individuals and AIP patients to improve interpretation of test results.

Methods: Fifteen healthy individuals and 15 AIP patients were included, and biological variations were calculated based on urine samples collected weekly for 10 consecutive weeks. For the AIP patients, long-term variations were also estimated based on 7 samples collected through a 2-year period.

Results: The porphyrin variances were inhomogeneously distributed; biological variations of porphyrins were therefore not calculated. The within-subject biological variations of ALA and PBG were 16%–20% in the short-term settings and for PBG, 25% in the long-term setting, giving reference change values of ~50% and 70%, respectively. The probability of detecting a 100% real change in PBG was 97% in the short-term setting and 80% in the long-term setting.

Conclusions: In an AIP patient, a 2-fold increase in PBG, independent of the baseline concentration, will be detected with a probability >80% and is most likely related to the patient's disease and not caused only by analytical and biological variation. When PBG is used in the assessment of AIP-related symptoms, both the PBG concentration in remission and the length of time since the previous sample must be considered.
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Acute intermittent porphyria (AIP)[3] is characterized by acute attacks of abdominal pain and neuropsychiatric symptoms that are precipitated by various factors such as drugs, hormonal changes, and physical/mental stress. It is assumed that all attacks are accompanied by increased excretion of primarily urinary porphobilinogen (PBG), δ-aminolevulinic acid (ALA), and to some extent, porphyrins. The increases in PBG and ALA are used diagnostically when assessing patients with symptoms *(1)* and when monitoring the use of potentially porphyrinogenic drugs. However, both patients with manifest disease and patients who have never experienced symptoms (latent AIP) can have baseline PBG concentrations up to 50-fold higher than the upper reference limit *(2)*. The precise correlation of the PBG concentration with AIP symptoms is also difficult to discern *(1)*. Urinary PBG is nevertheless expected to increase in relation to an attack *(1–5)*. To be able to distinguish an increase in porphyrin metabolites caused by the disease from their natural variations, it is necessary to know the analytical and within-subject biological variations in asymptomatic AIP patients in short- and long-term steady-state conditions to calculate reference change values (RCVs). Similarly, to improve the initial biochemical diagnosis of AIP, it is necessary to know the between- and within-subject variations for healthy individuals. Information about biological varia-

tions can also be used to set analytical quality specifications and to calculate the index of individuality *(6)*. The aims of the present study were thus to estimate the analytical and biological variations of urinary porphyrin precursors and porphyrins in both healthy individuals and in AIP patients in short- and long-term steady-state conditions to improve the diagnosis and monitoring of AIP.

Materials and Methods

PARTICIPANTS

Fifteen adult healthy individuals (6 males, 9 females; hereafter denoted healthy individuals) were recruited from the staff of our laboratory. Fifteen patients (5 men, 10 women; age range, 10–68 years; hereafter denoted AIP patients) with AIP diagnosed at the Norwegian Porphyria Centre based on criteria given by Sandberg and Elder *(7)* were also included. Nine had been in remission for at least 2 years, and 6 were latent. Ten of the patients belonged to 3 different families. Patient 1 was prepubertal. One patient used regular medication classified as "probably porphyrinogenic" *(8)*. The study was designed in accordance with guidelines from the Regional Committee for Medical Research Ethics, and all patients and healthy controls gave informed consent to participate in the study.

SPECIMENS AND ANALYSIS

Material for estimation of biological variations in the healthy individuals consisted of urine samples obtained on the same weekday for 10 consecutive weeks. Material for the assessment of short-term biological variation in the AIP patients was collected similarly (10 weeks; short-term study period). Samples for assessment of long-term biological variation in the AIP patients consisted of the samples collected during weeks 1 and 10 in addition to 5 consecutive samples collected at 3-month intervals for a total of 2 years (long-term study period). All were first morning urine samples, protected from light. For the healthy individuals, urine samples were delivered within 1 h after collection, and for the AIP patients, urine samples were sent by overnight mail to the Norwegian Porphyria Centre. For all participants, a questionnaire recording any occurrence of disease, medications, and for females, the first day of the last menstruation accompanied all samples. None of the AIP patients reported any porphyria-related symptoms. Two AIP patients used "probably porphyrinogenic" drugs *(8)* for a period of 1 to 2 weeks.

To study the effect of transportation by post on porphyrin concentrations and variations, 2 subsamples were taken from all specimens for the 30 participants (10-week study period) on arrival in the laboratory. To imitate shipment by mail, the subsamples were then transported by 2 different people (~2 h) and kept (~22 h) under various conditions.

All samples were immediately stored at −80 °C after arrival. The samples from each participant were analyzed in the same run except for the long-term samples, which were analyzed consecutively within 1–2 weeks after arrival. All samples were analyzed in duplicate for ALA, PBG, porphyrins, and creatinine. ALA/PBG and porphyrins were analyzed by the Bio-Rad Column Test® (Bio-Rad Laboratories Hemel Hempstead). Internal quality-control material was also analyzed in every run. There was no systematic change in the concentrations of controls during the study period. The total analytical CVs (CV_{AT}; within- and between-run variation) were as follows: for ALA, 1.2% at 38.1 μmol/mmol creatinine; for PBG, 3.5% at 5.8 μmol/mmol creatinine; for porphyrins, 3.1% at 21.0 nmol/mmol creatinine; and for creatinine, 1.4% at 5.4 and 9.5 mmol/L.

STATISTICS

There were no systematic increases or decreases in the concentrations of ALA, PBG, or porphyrins in either the healthy individuals or the AIP patients throughout the study periods. The homogeneity of duplicates, the distribution of the mean of duplicates for each individual, and the extreme means for participants were examined according to Fraser and Harris *(9)*. One extreme mean was found among the AIP patients for all constituents. However, the within-subject biological variations for this patient were comparable to those for the other AIP patients for all analytes; this patient was therefore included in the analyses. Testing for homogeneity of variances was performed according to Neuilly *(10)*, and no outliers were detected for ALA and PBG with Cochrane values well below the critical values ($P = 0.05$). Outliers were detected for porphyrins in all the different settings, however. Variance homogeneity was also illustrated in relation to the χ^2 distribution as cumulated fraction frequency on a rankit scale of CVs and $\sqrt{(\chi^2/df)}$ where *df* is the degrees of freedom. Plots showed homogeneity for ALA and PBG, but not for porphyrins, as exemplified in Fig. 1.

All data analyses were done assuming both gaussian and ln-normal distribution of data. The results were comparable, and only results assuming a gaussian distribution are presented. General statistical analysis (median, range, paired *t*-tests) was carried out with SPSS® 13.0 for Windows, whereas the biological and within-series analytical CVs (CV_{AW}) were estimated from the statistical model for repeated balanced subsampling (nested design) *(9)*. Biological variation is divided into between-subject (CV_{BS}) and within-subject (CV_{WS}) biological variations. For the short-term study, the variances were calculated for both healthy individuals and AIP patients based on analyses of 10 samples each from 15 persons, and for the long-term study, the variances were calculated from 7 samples from each of the 15 AIP patients. Confidence intervals and significance testing for variances were calculated according to Kringle and Bogovich *(11)*. Transportation-induced variation was estimated by the difference (d_i) between the 2 transportation methods, with CV calculated as:
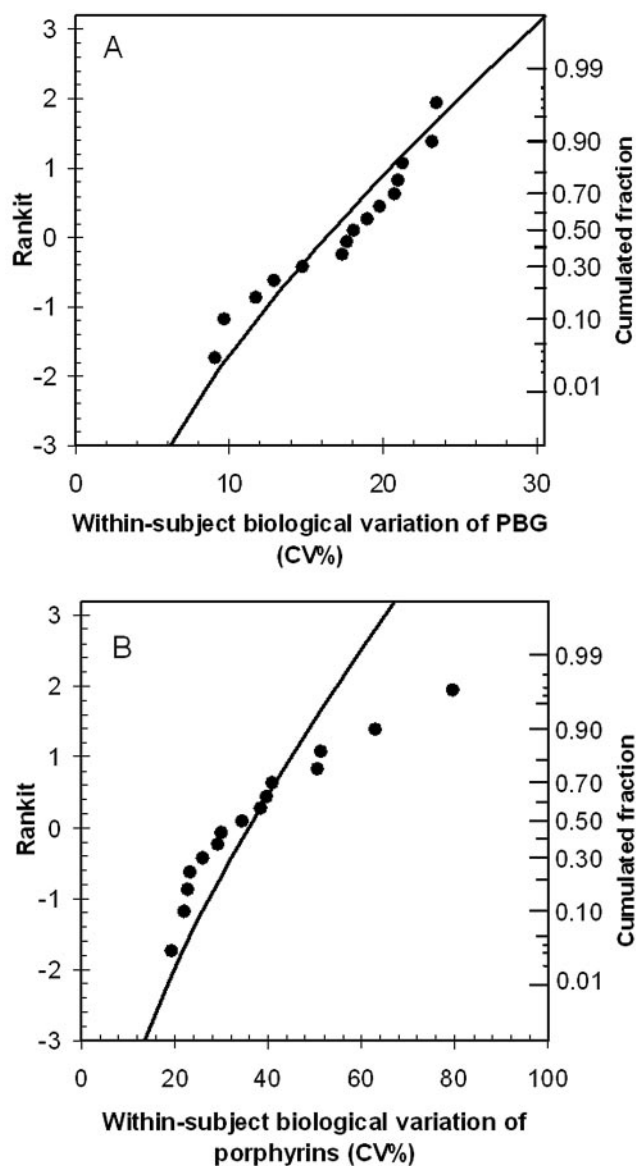
Fig. 1. Variance homogeneity plots.

Rankit plots show the accumulated fractions as function of within-subject biological CVs. The ● represent individual CVs for healthy individuals; the *line* indicates the expected distribution of measured CV values ($\sqrt{(\chi^2/df)}$) with $df = 9$) for "true" CV values of 17.3% for PBG (*A*) and 38.1% for porphyrins (*B*). The Cochrane test *(10)* gives values of 0.11 for PBG and 0.25 for porphyrins, and a value indicating lack of homogeneity should be >0.17. For the AIP patients, the plots for ALA and PBG were similar to *panel A*, and for porphyrins the plot was similar to *panel B*.

$$CV = \sqrt{\frac{\sum (d_i / \text{mean}_i)^2}{2n}}$$

For all analyses, significance was set to $P < 0.05$.

Results

Complete sets of urine samples and questionnaires with clinical data were received for all study participants. The median ALA, PBG, and porphyrin concentrations for the healthy individuals and for the AIP patients in the short-

and long-term settings are given in Table 1. There were no significant differences between the short- and long-term concentrations of the metabolites or between the male and female participants in either the AIP patients or in the healthy individuals (see Fig. 2 and Table 1). There was no impact of reported symptoms, use/change of medications, or menstrual cycle in fertile women on the concentrations of urinary ALA, PBG, or porphyrins.

EFFECT OF TRANSPORT

Calculating the variation caused by shipment by mail for 1 day gave transportation CVs (95% confidence intervals) of 2.8 (2.5–3.2)% for ALA, 5.9 (5.3–6.7)% for PBG, and 9.3 (8.3–9.6)% for porphyrins, all of which were significantly different from zero. For the healthy individuals, transportation (day 0 to day 1) caused mean (SE) decreases of 0.9 (0.5)% and 1.1 (1.6)% for ALA and PBG, respectively, and an increase of 50 (4.3)% for porphyrins. For the AIP patients, transportation (day 1 to day 2), caused slightly larger decreases for ALA and PBG, whereas the increase for porphyrins was smaller.

ANALYTICAL VARIATION

CV_{AW} values for the short- and long-term AIP patient study period were ~2%–4% for ALA, PBG, and the porphyrins (Table 1). The CV_{AW} values for PBG and porphyrins were smaller in AIP patients than in healthy individuals, probably because of the low concentrations of these metabolites in the healthy individuals. The CV_{AT} values for the control materials were similar to the CV_{AW} values.

BIOLOGICAL VARIATION

For biological variation to be of any real value, the variance of a constituent must be homogeneous. This was the case for ALA and PBG, but not for the porphyrins (Fig. 1), and biological variations were therefore not calculated for porphyrins. Calculating ALA and PBG per millimole of creatinine significantly reduced the within-subject biological variation (Table 2). The within-subject biological variation of urinary ALA was ~16%–20% in both healthy individuals and AIP patients. The within-subject biological variation of PBG was 18% in both healthy individuals and AIP patients in the short-term setting; however, the long-term within-subject biological variation of PBG (25%) was significantly higher (Table 2). The individual within-subject biological variations of ALA and PBG in the form of CV values were not related to the concentrations of the analytes in the urine, the sex of the individuals, or whether they had ever experienced attacks. Fertile women did not have higher within-subject biological variations than the nonfertile women or the men. The 1 prepubertal AIP patient did not exhibit lower biological variation than the other patients. Exclusion of 1 patient who regularly used medication assessed as probably porphyrinogenic did not significantly change the results. We found no significant differences in the within-subject

Table 1. Median measured concentrations and CV$_{AW}$ values for urinary ALA, PBG, porphyrins, and creatinine in healthy individuals over a 10-week period and in AIP patients during the short-term (10 weeks) and long-term (2 years) study periods.

		ALA[a]	PBG[a]	Porphyrins[b]	Creatinine[c]
Healthy individuals	Median (range)	1.9 (0.9–4.3)	0.3 (0.1–0.6)	3.6 (0.6–11.9)	13.8 (2.7–38.9)
	CV$_{AW}$,[d] %	2.5 (2.2–2.8)	8.2 (7.3–9.3)	14 (13–16)	<1.0
AIP patients					
10-week study period	Median (range)	6.9 (1.8–31.2)	5.0 (0.2–49.8)	56.7 (1.5–283.5)	7.45 (2.2–21.7)
	CV$_{AW}$,[d] %	3.4 (3.0–3.9)	3.0 (2.7–3.4)	3.1 (2.8–3.5)	<1.0
2-year study period	Median (range)	6.7 (1.4–35.2)	5.0 (0.2–49.0)	49.0 (2.5–201.8)	8.3 (1.9–22.3)
	CV$_{AW}$,[d] %	2.2 (1.9–2.6)	2.2 (1.9–2.6)	3.8 (3.3–4.4)	<1.0

[a] Concentration in μmol/mmol creatinine.
[b] Concentration in nmol/mmol creatinine.
[c] Concentration in mmol/L.
[d] Values in parentheses are the 95% confidence intervals.

biological variations for the different families that participated.

CLINICAL APPLICATION OF THE DATA

The RCV, i.e., the change in a result that makes it significantly different from a previous result with $P < 5\%$ (bidirectional changes between 2 measurements in the same patient), is calculated as:

$$RCV = \sqrt{2} \times 1.96 \times \sqrt{(CV_{WS}^2 + CV_{AT}^2)}$$

The RCVs for ALA were ~50% in all settings. For PBG, the RCV was ~50% for both healthy individuals and AIP patients in the short-term setting, whereas for AIP patients in the long-term setting it was 70% (Table 3). The analytical goals for imprecision, defined as one half of the biological within-subject variation, and the index of individuality, defined as the ratio of the biological within-subject variation and the between-subject variation (CV_{WS}/CV_{BS}), for ALA and PBG are shown in Table 3.

Discussion

The within- and between-subject biological variations of porphyrins and porphyrin precursors in urine have not been assessed previously. In the present study, the variances of porphyrins in urine were inhomogeneously distributed, and biological variations were therefore not calculated. A substantial part of the porphyrins found in urine is formed by polymerization from PBG *(1)*, and it is likely that this is the cause of the inhomogeneous distribution. This also explains the increase in porphyrins during storage and transportation.

Knowing the within-subject variations of ALA and PBG is of great value both when considering whether a patient's symptoms are caused by porphyria and similarly when evaluating a patient with a possible diagnosis of AIP. In this study, the within-subject variation was lower for PBG in a short-term setting than when measured over a 2-year period (Table 2). PBG is the metabolite most closely linked to AIP and disease activity, and it is possible that in a longer time span this reflects a higher

within-subject biological variation of PBG attributable to the disease itself. The within-subject variations of some analytes are higher in patients with specific diseases than in healthy individuals *(12–14)*, whereas in other diseases they are similar to or smaller than in healthy individuals *(15–17)*. The within-subject biological variations of ALA and PBG compared fairly well to those seen for other types of metabolites in urine *(18)*. Our data also show the importance of relating the concentrations of porphyrin metabolites in urine to the excretion of creatinine, as this considerably decreases the within-subject biological variation (Table 2). Many porphyria centers are national reference laboratories and therefore receive patient samples by overnight mail, and this leads to samples being subjected to at least 1 day of shipment by post before reaching the laboratory. It is, however, not necessary to consider test results from samples sent by overnight mail any differently than test results from samples that are analyzed directly because the variation introduced by transportation is small.

If analytical quality specifications are defined as being equal to or less than one half of the within-subject biological variation, both ALA and PBG meet this goal (Table 3).

The concentration to which PBG must change for it to confirm or exclude an acute attack or porphyria-related symptoms is not known. In a relevant clinical setting, some use the finding of increased concentrations of PBG per se to confirm the diagnosis of an acute attack *(19, 20)*. The European Porphyria Initiative (EPI) *(3)* and Sandberg and Elder *(7)* state that in an attack, the PBG concentration in urine is usually at least 10-fold higher than the upper reference limit, whereas other suggestions are a 2- to 4-fold increase in an individual *(4)* or a 20- to 200-fold increase compared with reference values *(4, 21)* or the finding of increased concentrations of 88–884 μmol/L *(22)*. Our data show that for, e.g., a patient with a urinary PBG baseline concentration of 5.0 μmol/mmol creatinine, a change to a value between 2.5 and 7.5 μmol/mmol creatinine (50%) in a short-term setting can be explained
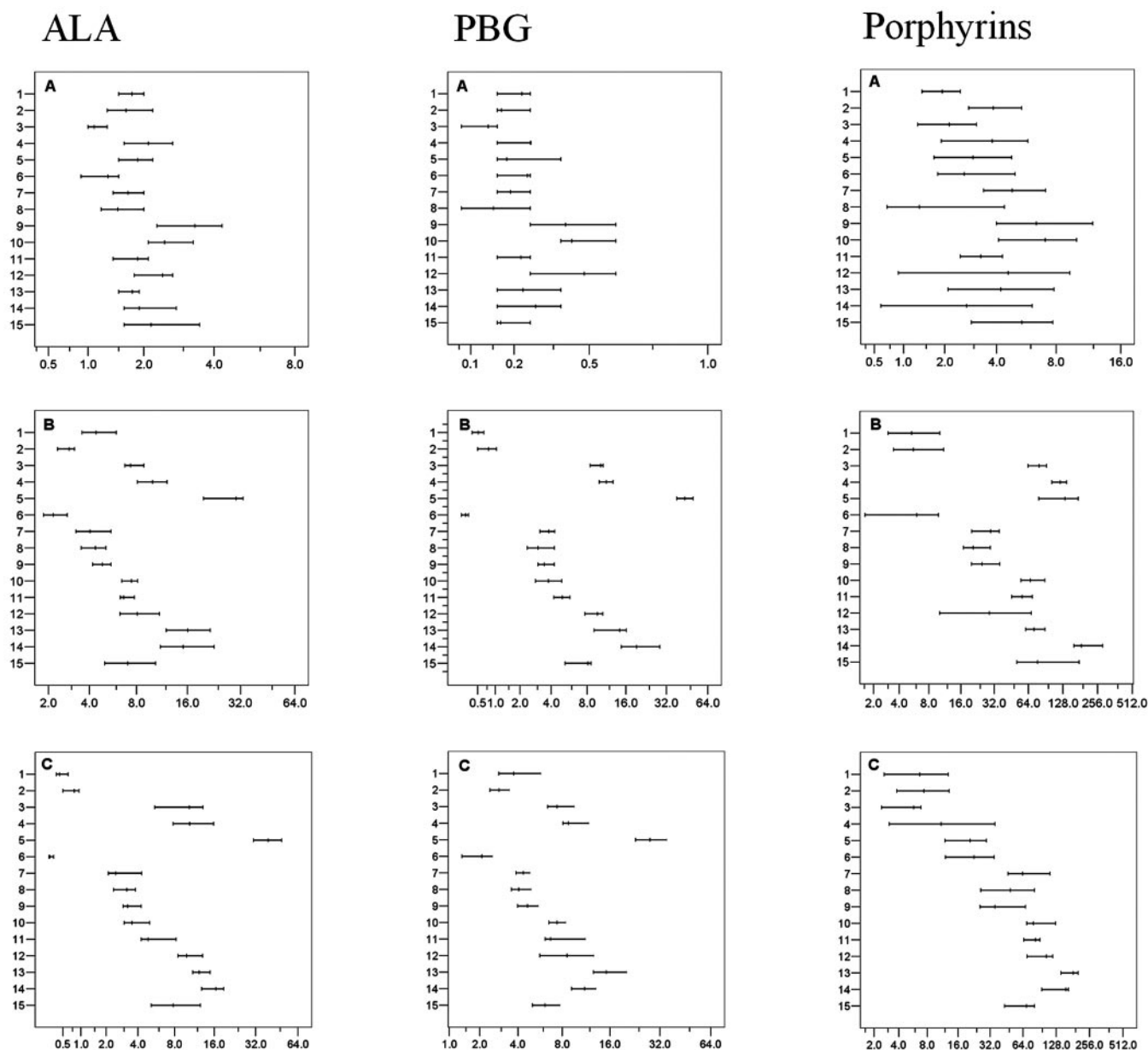
Fig. 2. Medians and ranges (*error bars*) for ALA, PBG, and porphyrin concentrations in healthy individuals (*A*) and in AIP patients in the short-term (10 weeks; *B*) and long-term (2 years; *C*) studies.

For the healthy individuals (*y axes* in *panels A*), individuals 1–6 are male and individuals 7–15 are female, and for the AIP patients (*y axes* in *panels B* and *C*), patients 1–5 are male and patients 6–15 are female. The upper reference limits (99th percentiles) are <5.5 μmol/mmol creatinine for ALA, <0.8 μmol/mmol creatinine for PBG, and <35.0 nmol/mmol creatinine for porphyrins.

by biological and analytical variation within the 95% interval of distributions during the steady state, whereas the corresponding values are 1.5–8.5 μmol/mmol creatinine (70%) in the long-term setting. When dealing with AIP patients, the clinical questions are often whether the symptoms patients are experiencing are caused by their disease or whether an increase in PBG seen while monitoring potentially porphyrinogenic drugs is of relevance to a patient's condition. In this case the difference must be unidirectional; the RCV in Table 3 therefore reflects a 2.5% ($2\alpha = 0.05$) probability for detecting an increase in the

concentration under steady-state conditions. However, the probability of detecting a true increase of the same size as the RCV is only 50% ($\beta = 0.5$), and the power to detect a true change of this size is therefore ($1 - \beta = 0.5$) (*23*). The probability (power) of detecting a true change can be estimated by the probability corresponding to a z-value, obtained with the formula (*23, 24*):

$$z = \frac{\text{true change} - \text{RCV}}{\sqrt{2} \times \sqrt{(\text{CV}_{\text{AT}}^2 + \text{CV}_{\text{WS}}^2)}}$$

Table 2. CV$_{BS}$ and CV$_{WS}$ values for ALA and PBG in healthy individuals over a 10-week period and in AIP patients in the short-term (10 weeks) and long-term (2 years) study periods.

Group		Calculated per	CV, %		
			ALA	PBG	Creatinine
Healthy individuals	CV$_{BS}$	mmol creatinine	27 (24–31)	31 (28–35)	36 (32–41)
	CV$_{WS}$	mmol creatinine liter	16 (15–19)	18 (16–21)	
			35 (31–39)	37 (33–42)	33 (30–38)
AIP patients					
10-week study period	CV$_{BS}$	mmol creatinine	80 (72–91)	121 (108–137)	37 (33–42)
	CV$_{WS}$	mmol creatinine liter	20 (18–22)	18 (17–21)	
			48 (43–53)	53 (48–59)	31 (28–34)
2-year study period	CV$_{BS}$	mmol creatinine	80 (70–94)	117 (102–137)	40 (35–47)
	CV$_{WS}$	mmol creatinine liter	20 (18–23)	25 (23–29)	
			45 (40–51)	59 (52–67)	32 (28–36)

This is shown in Fig. 3 for PBG for the short- and long-term situations, respectively. Thus, in a patient with a baseline PBG concentration of 40 μmol/mmol creatinine, a result of 60 μmol/mmol creatinine obtained with the RCV for the short-term period or of 68 μmol/mmol creatinine obtained with the RCV for the long-term period can be explained by biological and analytical variations within the expected 97.5% limit, if indeed no attack is present and the patient is in steady state. However, if there is a true change in PBG equal to the RCV, there will be only a 50% chance of detecting this. A true change, e.g., from 40 to 80 μmol/mmol creatinine, a 2-fold increase, will be detected with probabilities of 97% and 80% when the RCVs for the short- and long-term situations, respectively, are used (Fig. 3). Similar calculations can be performed for ALA. When using PBG in the assessment of AIP-related symptoms or when monitoring a patient on porphyrinogenic drugs, it is therefore important to take into account both the patient's PBG concentration when in remission as well as the length of time since the previous sample.

Ninety-nine percent of healthy individuals have PBG values <0.8 μmol/mmol creatinine. To be 95% certain that a healthy individual does not have a PBG value above

0.8 μmol/mmol creatinine (false positive), the value should be <0.6 μmol/mmol creatinine when only 1 sample is obtained and <0.65 μmol/mmol creatinine for the mean of 2 replicates. Calculating the false-negative rate is more difficult because approximately one third of patients with AIP have "normal" values between attacks (4).

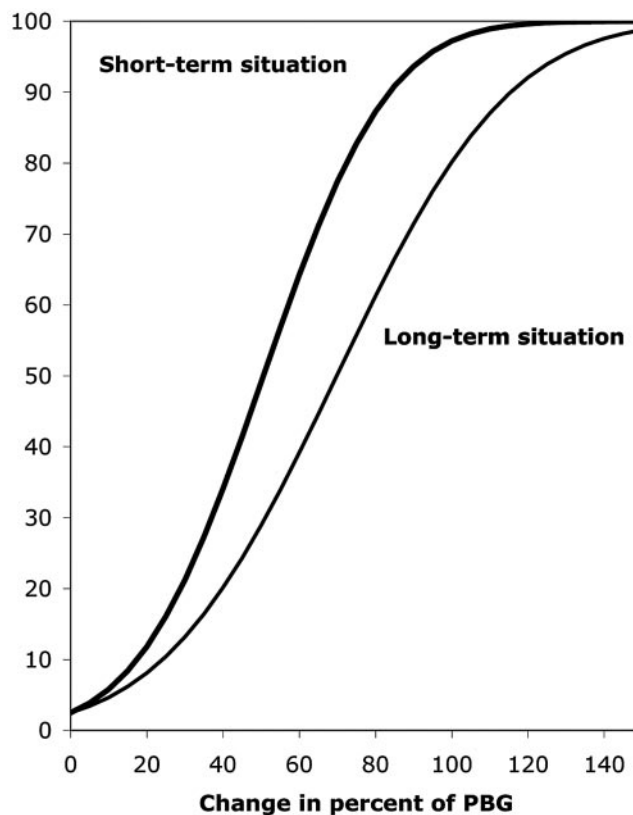In our study, the between-subject biological variation

Fig. 3. Power functions for PBG in the short-term situation (CV$_{WS}$ = 18% and RCV = 51%) and for the long-term situation (CV$_{WS}$ = 25% and RCV = 70%).

The *y axis* shows the probability of detecting a change larger than the RCV as the function of a true change (as %) in PBG concentration.

Table 3. Index of individuality, RCVs, and analytical goals.

Group		ALA	PBG
Healthy individuals	Index of individuality[a]	0.6	0.6
	RCV,[b] %	46	55
	Analytical goal (CV),[c] %	8.1	9.1
AIP patients			
10-week study period	Index of individuality	0.3	0.1
	RCV, %	56	51
	Analytical goal (CV), %	10	9.1
2-year study period	Index of individuality	0.2	0.2
	RCV, %	56	70
	Analytical goal (CV), %	9.9	13

[a] Index of individuality = CV$_{WS}$/CV$_{BS}$.
[b] RCV = $\sqrt{2} \times 1.96 \times \sqrt{(CV_{WS}^2 + CV_{AT}^2)}$.
[c] Analytical goal for imprecision = 0.5 × CV$_{WS}$.

was moderately larger than the within-subject variation in the healthy individuals, whereas in the AIP patients the between-subject biological variation was very large (Table 2), as is expected with the differing concentrations of ALA and PBG in the urine of patients *(2, 4)*. The index of individuality is by definition based on the ratio CV_{WS}/CV_{BS} in healthy individuals *(6)*. Sometimes the nominator also includes the analytical variation. It is suggested that if the index of individuality is between 0.6 and 1.4, then reference limits may be more useful than RCVs *(23, 25)*. Calculating the index of individuality in the healthy volunteers gave indices of 0.6 for both ALA and PBG. If, however, we apply the concept of index of individuality to patients with stable disease, then the indices for ALA and PBG would be 0.3 and 0.1, respectively (Table 3), suggesting that repeated sampling with comparison of the measured differences with the RCV may be superior to reference intervals for clinical interpretation *(24–26)*.

In conclusion, the RCVs for ALA and PBG in a short-term setting are ~50%, whereas for AIP patients in a long-term setting the RCV of PBG increases to 70%. Thus, when using urinary PBG in the assessment of potentially AIP-related symptoms, both the concentration in steady state as well as the length of time since the last analyzed sample must be taken into account. In an AIP patient, a greater than 2-fold increase in PBG, independent of the baseline concentration, will be detected with a probability >80% and is most likely related to the patient's disease and not the result of only analytical and biological variation. Our data also emphasize the need for prospective well-defined trials to establish the changes in urinary porphyrin metabolite concentrations in acute attacks.

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