Quantitative urinary biomarkers of epilepsy as a pyridoxine-dependent condition

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Abstract

Introduction

The study testifies the hypothesis on epilepsy as an inborn error of pyridoxine (vitamin B6) metabolism and suggests non-invasive quantitative biomarkers for evaluation of clinical status and monitoring an individual treatment by antiepileptic drugs.

The hypothesis

In children with different forms of epilepsy and matched healthy controls urinary parameters of pyridoxal-phosphate (PLP)-dependent tryptophan degradation were measured by HPLC method with simultaneous ultraviolet and fluorimetric detection. Concentrations of compounds formed or metabolized in the course of tryptophan degradation (kynurenines, indoxyl-sulfate) and correlations between them turned out to be quantitative biomarkers useful for evaluation of patient’s clinical state and monitoring individualized antiepileptic treatment.

In particular, the value of the ratio 4-pyridoxic acid to kynurenine appears to be the index of experienced seizures, while the ratio of 3-hydroxyanthranilic acid to 3-hydroxykynurenine reflects activity of kynureninase, the enzyme of critical sensitivity to PLP supply. Growing progressively worse, epilepsy is accompanied by aggravation of PLP-dependent disturbances of tryptophan metabolism and expanding inhibition of kynureninase.

Conclusion

The affected pyridoxine metabolism is discussed as an inborn genetic sign of epilepsy open of many years of pyridoxine replacement therapy. Non-invasive quantified urinary biomarkers based on indices of B6-dependent TRP metabolism are suggested for more precise evaluation of patients’ state and tailoring individualised AED treatment.

Introduction

Described more than 50 years ago\textsuperscript{1}, pyridoxine-dependent epilepsy (PDE) has been considered as a rare (1:100.000) autosomal recessive genetic disorder, occurring in the uterus, or subsequently in infancy or early childhood. Familial cases were repeatedly described. The resistance to conventional antiepileptic drugs (AED) and response to pyridoxine administration are accepted as the main characteristics of PDE\textsuperscript{2,3,4}. First, an inborn abnormality of pyridoxal phosphate (PLP)-dependent GABA synthesis by glutamate decarboxylase (GAD) had been postulated as a cause of the disease and life-long pyridoxine administration had been recommended\textsuperscript{5,6,7}. Later on in the search for the gene responsible for PDE, the primary involvement of the GAD 1 gene on chromosome 2q31 and the GAD 2 on 10p23 were excluded\textsuperscript{8}.

At present PDE is considered as a result of mutations in the ALDH7A1 gene, encoding antiquitin. Antiquitin deficiency in the cerebral lysine degradation pathway leads to accumulation of piperidein-6-carboxylic acid – the compound, which inactivates PLP\textsuperscript{9,10,11}.

Recently, several cases of other inborn errors of vitamin B6 metabolism, i.e., pyridox(am)ine 5-phosphate oxidase deficiency and type 2 hyperprolinemia, have also been described\textsuperscript{12,13,14}.

So, the data accumulated have shown that autosomal recessive pyridoxine-dependent seizures are genetically heterogeneous.

However, neither data accumulation, nor recommendations for pyridoxine administration in early started intractable cases of epilepsy\textsuperscript{3,16,17}, or pyridoxine applicability as the first line drug for infantile spasms\textsuperscript{18,19,20,21} have changed conventional perception of the strictly limited role of pyridoxine in the pathogenesis of epilepsy in the whole.

Meanwhile, disturbances in the metabolism of glutamate, GABA, tryptophan, serotonin, tauroine, dopamine and norepinephrine – i.e., aminoacids and neurotransmitters, which synthesized and/or metabolized by PLP-dependent enzymes\textsuperscript{22,23} – have been repeatedly found in epileptic patients. The increased levels of excitatory amino acids – glutamate, aspartate and glycine\textsuperscript{24,25,26,27,28,29,30} - along with the reduced levels of inhibitory amino acids and amines – GABA, serotonin and tauroine\textsuperscript{31,32,33,34,35,36} - were detected in the plasma, CSF and epileptogenic foci of patients with different forms of epilepsy.

Moreover, a moderate increase in the activity of glutamic acid dehydrogenase, the glutamate synthesizing enzyme, which is specifically inhibited by PLP, has been found in epileptic fog\textsuperscript{23}.

These clinical data along with experimental results obtained in genetically epilepsy-prone seizure-naive animals\textsuperscript{40,41,42,43} enable us to hypothesize that an inborn error of pyridoxine metabolism (accentuated by high pyridoxine requirement during early development) is inherent in epilepsy. Being a starting point for neurotransmitter disorders, such an error may be a key determinant of epileptic diathesis.

An impairment of GABA (as well as serotonin and tauroine) – mediated inhibition along with an enhancement of glutamate (and aspartate) – mediated excitatory transmission evidently facilitate spreading of ictal...
activity throughout the brain and generation of seizures.

Disturbances of PLP dependent tryptophan (TRP) degradation, in particular, over-excess of neurotoxic 3-HOKYN, have been repeatedly shown in epilepsy starting from 50-54,45,46,47. Summarizing the data obtained48,49, we suggested that quantitative correlations between metabolites formed in the course of TRP degradation (Figure 1), might be indicative of clinical status in epileptic patients. Some of these correlations are accepted in literature as reflecting activity of enzymes of TRP degradation50. Specifically, the ratio of KYN to TRP serves as an index of activity of indoleamine 2, 3-dioxygenase (IDO), the rate-limiting enzyme of TRP degradation. As a heme-containing enzyme, IDO is apparently PLP-dependent, in as much as heme synthesis is PLP-dependent.

The ratio between the levels of 3-HOAA and 3-HOKYN is considered an index of activity of kynureninase, the enzyme of critical sensitivity to PLP supply51,52,53. The ratio between 4-PA and KYN turned out to be an indicator of recently experienced seizure attack. We have used these and some other quantitative urinary biomarkers for clarification of patient’s status at different stages of epilepsy and for tailoring of individualized AED treatment.

The Hypothesis

This article suggests to consider epilepsy as an inborn error of pyridoxine (vitamin B6) metabolism and to use non-invasive quantitative biomarkers for evaluation of clinical status and individual monitoring antiepileptic treatment. The protocols of this study have been approved by the ethics committees of Kaplan hospital, in which patients were under treatment.

Subjects

Urine samples were analysed in children of 4–17 years of age with different clinical forms and stages of epilepsy, but healthy in all other respects. Patients with absence and atonic seizures were not included into the study. Altogether, 109 subjects divided into the following groups were comparatively studied:

1. Newly diagnosed epileptic patients experienced their first epileptic attack on the previous day and had not been treated yet with AED (n = 11);
2. Epileptic patients successfully treated with AED and seizure free for at least three months, regardless of the type of epilepsy (n = 19);
3. Epileptic patients partially responsive to AED treatment, i.e., those having repeated seizures in spite of antiepileptic treatment (n = 19);
4. Control group of healthy children matched by sex and age (n = 37).

About 250 urine samples were analysed. Patients’ samples were provided by the Paediatric Department of Kaplan’s Hospital (Rehovot). Control samples were collected from healthy children in local kindergartens and elementary schools.

Determination of tryptophan and its metabolites in urine by HPLC with simultaneous ultraviolet and fluorimetric detection

Urinary TRP and its metabolites were determined by the modified Herve et al.54 HPLC method. In addition to KYN, 3-HOKYN and 3-HOAA detected by these authors, some other TRP metabolites, i.e., anthranilic acid (AA), kynurenic acid (KA), Indoxyl sulfate (IND) and 4-PA were measured.
All standards were purchased from Sigma. All solvents were HPLC graded.

Sample preparation
Mixed standard solutions (1 mM of each compound) were stored at -80° C for up to 3 months. Urine samples were collected into 20 mL glass scintillation vials and stored in aliquots at -80° C.

Samples were acidified by addition of 100 µL of 2.4 M perchloric acid to 900 µL of urine. After centrifugation (5000 g, 15 min, 4° C) supernatants were filtered (0.22 µL Millipore filter) into HPLC vials and analysed at the same day.

Chromatography
Reverse phase HPLC analysis was performed with an Inertsl (C-18, 5 µm) column (250 mm × 4.6 mm) and Merck Hitachi system equipped with a Quaternary Pump L-7100 and interface D-7000.

Peaks detection and quantification were carried out with a scanning fluorescence detector L-7485 connected to the programmable photodiode array detector L-7450A.

Samples were analysed using the following gradient: 28 min isocratic elution of 100% solvent A, 6 min linear gradient from 100% to 75% of solvent A, 61 min isocratic elution 75% of solvent A, 2 min linear gradient from 75% to 100% of solvent A, and 8 min isocratic elution of 100% solvent A. Solvent A was 1 M ammonium acetate buffer, pH 5.2. Solvent B was 6% acetonitrile in 1 M ammonium acetate buffer pH 5.2. The mobile phase was prepared at the day of analysis.

Acquisition and processing of chromatograms were performed using HSM software (Merck-Hitachi). Standard compounds showed linearity range from 0.03 μM to 10 μM. Concentrations were calculated based on peak areas of external standards. TRP, its metabolites, and 4-PA were determined with UV and fluorescence detection at two different excitation and emission wavelengths: 3-HOAR and 4-PA, AA, TRP, IND and KA were detected by UV absorption at 365 nm and eluted at 14.3, 41.4 and 87.0 min, respectively; while 3-HOAA, 4-PA, AA, TRP, IND and KA were detected by fluorescence and eluted at 31.6, 58.9, 72.4, 84.5, 92.8, 99.9 min, respectively.

Statistical analysis
The data were expressed as mean ± SEM. Paired Student's t-test was used to assess the difference between groups; p < 0.05 were considered as statistically significant difference between values of parameters.

Figure 3: The value of 4-PA/KYN as a marker for diagnosing of recently experienced seizure attacks. Designations: A - healthy controls (n = 37); B - patients experienced their first epileptic attack previous day (n = 9); C - seizure -free patients successfully treated by AEDs (n = 18); D - patients partially controlled by AEDs (n = 5).

Figure 4: Histograms of distribution of the KYN/TRP ratio in epileptic patients in comparison with healthy controls. Designations: A - healthy controls (n = 42); B - patients experienced the first epileptic attack (n = 9); C - seizure -free patients successfully treated by AEDs (n = 11); D - patients partially controlled by AEDs (n = 12).
Urinary biomarkers for detection of the first seizure attack. In patients, who had seizures for the first time in life the day or two before admission to the ward and had not yet been treated by AEDs, the mean level of TRP, and especially of KYN, were sharply increased, in comparison with healthy control group, thus providing the elevated mean value of KYN/TRP ratio and decreased value of 3-HOKYN/KYN ratio. The concentration of KA was twofold elevated and the IND level was almost twice reduced.

Taken together, these changes in “first seizure attack” patients formed a pattern strongly distinguishable from that of healthy controls (Table 1). The level of urinary 4-PA was statistically indistinguishable from healthy controls, though in four out of eleven patients it was reduced to 1μM or even lower. The mean value of 4-PA/KYN ratio in children of this group turned out to be almost sevenfold less than in healthy controls (Table 1, Figure 3) and appeared to be the marker of an occurred seizure attack, regardless of its type.

The alterations in the urinary levels of TRP and KYN lead to some rightward shift in the histogram of distribution of the KYN/TRP ratio and strongly leftward shift in the histogram of distribution of the ratio 3-HOKYN/KYN (Figures 4 and 5).

At the same time, the histogram of distribution of 3-HOA/3-HOKYN ratios leans distinctly to the left (Figure 6), reflecting some reduction of kynureninase activity, though in this group the mean value of the ratio is decreased insignificantly (Table 1).

The correlations between both TRP and KYN and the ratio indicative for kynureninase activity, i.e., TRP: (3-HOAA/3-HOKYN) and KYN: (3-HOAA/3-HOKYN), in "first attack" children are strongly higher, than in the control group.

Combination of increased concentrations of TRP and KYN with decreased concentration of IND provides a drastic diminution in both IND/TRP and IND/KYN ratios in these patients in comparison with healthy controls. In approximately 70% of these patients the IND/KYN ratio is lower than 100, while in healthy children it is always higher than 100 and even higher than 300 in 60% of them (Table 1, Figure 7).

**Urinary markers in seizure-free patients treated by AEDs**

In patients well controlled by AEDs (in our study those, who were seizure-free for at least 3 months) most of studied parameters are practically indistinguishable from those in healthy controls. The mean values of KYN/TRP, 4-PA/KYN, 3-HOKYN/KYN and 3-HOAA/3-HOKYN ratios are coincided with those of the control group (Table 1). Though the urinary concentrations of TRP and KYN in seizure-free patients are significantly higher than in the healthy group, correlations between

### Table 1: Urinary Tryptophan Metabolites and Correlations between them in Epileptic Patients and Healthy Controls.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1: Healthy controls (n = 37)</th>
<th>Group 2: Experienced the first seizure attack (n = 11)</th>
<th>AED treated Group 3: Seizure-free (n = 19)</th>
<th>Group 4: Partially AED controlled (n = 19)</th>
<th>P values between compared groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
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<tr>
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<td>20</td>
<td>36</td>
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<td>KYN, µM</td>
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<td>5.7</td>
<td>1.4</td>
<td>2.4</td>
</tr>
<tr>
<td>3-HOKYN, µM</td>
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<td>0.1</td>
<td>1.8</td>
<td>0.5</td>
<td>1.1</td>
</tr>
</tbody>
</table>

**Figure 5:** Histograms of distribution of the 3-HOKYN/KYN ratio in epileptic patients in comparison with healthy controls. Designations as at Fig. 4.
concentrations of these compounds and the ratio indicative for kynureninase activity, i.e., TRP (3HOAA/3-HOKYN) and KYN (3HOAA/3-HOKYN) practically coincide with corresponding correlations in healthy controls.

Histograms of distribution of the most of studied parameters in seizure-free children are similar to those in healthy controls, with the exception of the histogram of distribution of 3-HOKYN/KYN (Figure 4, Figure 5 and Figure 6). Similarity between the histogram of distribution of 3-HOKYN/KYN ratios in children of this group and those, who experienced their first attacks (Figure 5) is signifying that kynureninase activity is not yet completely restored in seizure-free AEDs treated patients (though statistical differences between values of the pointed ratios are absent in two compared groups).

The mean concentration of IND in seizure-free patients remains as low as in “first attack” children, but values of IND/TRP and IND/KYN ratios (due to reduction of TRP and KYN levels) are higher than in those patients. The histogram of distribution of IND/KYN ratios remains still shifted to the left (Figure 7 and Table 1).

**Urinary markers in patients partially responsive to AED treatment**

Repeated convulsive episodes, which occur in epileptic patients in spite of AED treatment, result in decreased values of 4-PA/KYN ratio- the marker of recently experienced seizure attacks (Figure 3). In three out of five patients, who had seizures shortly before, or on admission to the hospital, this ratio was less than one, decreasing thereby the mean value of the group (Table 1). The fourfold elevated concentration of toxic 3-HOKYN and the twofold elevation of the ratio 3-HOKYN/KYN along with dramatically reduced value of 3-HOAA/3-HOKYN ratio are the most remarkable signs of this group. The mean value of 3-HOAA/3-HOKYN ratio in these patients is reduced to 0.9 and represents only 15% of the corresponding value in seizure-free patients (Table 1, Figure 5 and Figure 6). The strongly right skewed histogram of distribution of 3-HOKYN/KYN ratios and strongly left skewed histogram of distribution of 3-HOAA/3-HOKYN ratios correlate with severe disturbances of kynureninase activity in partially AED controlled patients. The reduction in kynureninase activity results also in the accumulation of TRP, KYN and KA. Accordingly, the correlation of each of these compounds to the 3-HOAA/3-HOKYN ratio, i.e., KYN: (3-HOAA/3-HOKYN); KA: (3-HOAA/3-HOKYN) and TRP: (3-HOAA/3-HOKYN) reach extremely high values in patients partly sensitive to AEDs. The intensive inpatient AED treatment distinctly changes the examined parameters. First of all, the value of 4-PA/KYN ratio is increased.

The value of 3-HOAA/3-HOKYN ratio is also increased, reflecting an increase of kynureninase activity. Accordingly, values of KYN: (3-HOAA/3-HOKYN);
KA: (3-HOAA/3-HOKYN) and TRP: (3-HOAA/3-HOKYN) are significantly diminished.

In successful cases favourable changes, once attained, remain stable (Figure 8; patients E and K). In unsuccessful cases the initial increase in the 3-HOAA/3-HOKYN ratio suddenly reverts back, and the related parameters are changed accordingly (Figure 8, patient D).

In spite of the increased level of IND in patients of this group, the ratios IND/TRP and IND/KYN remain quite low because of increased levels of both TRP and KYN (Table 1).

**Discussion**

Disturbances of vitamin B6 metabolism, revealed in epileptic children through the example of disorders of PLP-dependent degradation of TRP, confirm the suggestion that epilepsy is the PLP-dependent disorder. The severity of these disorders varies at the different stages of the disease. The parameters reflecting kynureninase activity seems to be the most sensitive link of PLP-dependent disorders. The same biochemical disturbances have been found in asymptomatic first degree relatives of epileptic patients (in press), confirming thereby a hypothesis on an inborn error of pyridoxine metabolism inherent in epileptic families.

The data obtained testify that the concentrations of compounds formed or metabolized in the course of PLP-dependent TRP degradation and some correlations between them may serve the quantitative urinary biomarkers for determination of clinical status and monitoring individualized AED treatment in epileptic patients.

Once the initial seizure attack has occurred, the drastically increased levels of TRP, KYN, and toxic 3-HOKYN, as well as reduced level of IND, indicate that PLP-dependent connections of TRP degradation process are disordered. Low values of the IND/TRP and IND/KYN ratios, as well as 4-PKA/KYN and 4PA/3HOKYN ratios, completely change the pattern of TRP metabolites in children, who have endured convulsions, in comparison with healthy controls (Figure 1 and Table 1). Specifically, the low value of 4-PA/KYN ratio may distinguish an epileptic episode from paroxysmal loss of consciousness of non-epileptic origin. It is important to trace, how long this index remains at such low a level after a single seizure episode.

The effective AED treatment normalizes most of discussed parameters (Table 1). Kynureninase activity is also restored by effective anticonvulsants. The ratio 4-PA/KYN is increased almost up to the control values. We believe that maintaining of this ratio within the range between two and four (Figure 3) would help to provide adequate seizure control and reduce a risk of pharmacological overtreatment.

However, the increased levels of TRP, KA and KYN, reduced concentrations of IND and IND/TRP and IND/KYN ratios (Table 1 and Figure 7) still distinguish effectively AED treated seizure-free patients from healthy controls. The IND/KYN and IND/TRP ratios as parameters indicative of patients’ condition require separate consideration.

Intestinal PLP-dependent tryptophanase of bacterial origin is the key enzyme of alternative indole pathway of TRP degradation, inhibited by KYN. A drastic drop in the level of IND (and accordingly – the reduction of pointed ratios) is presumably caused by the increased concentrations of KYN in all studied groups.

Parameters reflecting activity of kynureninase at the different stages of disease indicate that aggravation of epilepsy is accompanied by expanding inhibition of kynureninase, with its critical sensitivity to PLP supply.

The accumulation of 3-HOKYN, along with twofold increase in the ratio 3HOAA/KYN and six-fold decrease in the ratio 3-HOAA/3-HOKYN are the most characteristic signs of sharply reduced kynureninase activity in patients partially controlled by AEDs (Table 1). The decrease in the value of 3HOAA/3HOKYN ratio leads to accumulation of TRP, KYN and KA (Table 1, Figure 4, Figure 5 and Figure 6). The similar pattern is reproduced by kynureninase inhibitors, once even considered as possible anticonvulsants. The intensive inpatient AED treatment of partially AED controlled patients decreases the 3-
HOKYN/KYN ratio and increases the 3-HOAA/3-HOKYN ratio. Stability of attained parameters signifies successful treatment (Figure 8). The data obtained indicate that indices of kynurenine activity are the reliable markers for evaluation of clinical status and effectiveness of individual antiepileptic therapy.

Normalization of PLP-dependent reactions by effective AED therapy is a separate point for discussion. It is possible to suggest that decreased activity of alkaline phosphatase (ALP), the enzyme, which provides pyridoxal transport trough brain membranes by PLP dephosphorylation, is one of the key factors of the disease. Seizures in mice that lack tissue non-specific ALP and rescue of lethal convulsions by administration of pyridoxal39,60 substantiate such a suggestion. In our experiments, the activity of ALP in the cortex and hippocampal of genetically seizure-naive epilepsy-prone BALB/c mice was significantly lower than in genetically epilepsy-resistant controls (Bresler, Dolina, unpublished). Presumably, increase in ALP activity under effective AED treatment51,61,62,63,64 intensifies pyridoxine transport, normalizing thereby PLP-dependent systems.

In summary, the suggested quantifiable urinary biomarkers, based on dynamic alterations of TRP metabolites in the course of the disease and antiepileptic treatment, are potentially helpful for:
1. Identifying of patients, who recently experienced seizure episode regardless of seizure type;
2. Detecting minimal effective doses of AED and gradual improvement of clinical status in the course of AED treatment;
3. Evaluating seizure-free status with the greater precision;
4. Identifying of inadequate seizure control, and rapid evaluation of the effectiveness of the novel treatment regimen;
5. Tracing the stability of results attained in the course of individualized AED treatment.

Taking into account the overall misdiagnosis rate of epilepsy (26%) and the rate of seizure recurrence after discontinuation of AED treatment (ranging through 12%– 66%65,66), it seems expedient to put the suggested biomarkers into practice.

**Conclusion**

The pilot clinical trial carried out in children with different forms of epilepsy (excluding absence and atonic forms) has confirmed our previous assumption of affected pyridoxine metabolism as an inborn genetic sign in epilepsy. We believe that inborn errors of pyridoxine metabolism in PDE and other forms of epilepsy are a kind of clinical continuum, ranging from extremely severe states resistant to AEDs to those more common, which are corrected by different AEDs.

This clinical continuum is open to high dose pyridoxine replacement therapy.

According to our experience67, prolonged (up to ten years) pyridoxine treatment in pharmacological doses (10 mg/kg, without exceeding 200 mg/daily) is valuable for different types of epilepsy (excluding – at least at present – absence and atonic forms). Being started at early stages of the disease and targeted at the stable correction of PLP-dependent metabolic disturbances, pyridoxine treatment will be effective by itself, or as a background for AED management.

Non-invasive quantified urinary biomarkers based on indices of PLP-dependent TRP metabolism are suggested for more precise evaluation of patients’ state and tailoring the individualized AED treatment.

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