

Elevated hydroxylactam of hemopyrrole level in urine in perpetrators of extremely violent acts diagnosed with psychosis

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Summary

Aim. Hydroxylactam of hemopyrrole (HPL) is an abnormal side product of porphyrin biosynthetic pathway, which may have a devastating impact on the behavior. The link between an aggressive behavior and an increased HPL level was postulated in the 1960s. Further researches concerning HPL brought contrary results and did not clarify its function and possible role in the pathogenesis of aggression. In our research we hypothesize that a heightened level of HPL may correspond to an extreme aggressive behavior in subjects diagnosed with psychosis.

Methods. We performed an analysis of HPL level in urine samples, collected from 36 male subjects diagnosed with a mental illness who presented an extreme aggressive behavior. The control group included 22 male subjects, matched with age.

Results. The variable HPL/creatinine quotient differs significantly between the study group and the control group.

Conclusions. We used successfully proprietary method for marking HPL level in urine, developed for the purposes of the project. The results of our study indicate that in a group of subjects with a history of an extreme aggressive behavior a corrected level of HPL may be elevated, compared to subject without history of extreme aggressive behavior. Further

studies are needed to evaluate the reasons of HPL elevation and its clinical implications in this group of patients.

Key words: aggression, violence, hydroxylactam of hemopyrrole

Introduction

An aggressive behavior correlated to a mental illness is a severe clinical and social problem. Among patients with a diagnosed mental illness an aggressive behavior occurs more frequently than in a population without any mental impairment. The published research demonstrates that among individuals with schizophrenia the risk of an aggressive behavior is fourfold in comparison to mentally healthy subject [1, 2]. The causes of an aggressive behavior in psychotic individuals might be associated with the symptomatology of the illness (among others: imperative hallucinations, delusion of control) as well as with antisocial traits of personality and increased impulsiveness, but in numerous cases the origins of an aggressive behavior are unknown and inexplicable. An extremely aggressive or autoaggressive behavior may lead to serious consequences, as a severe body injury, homicide/suicide attempt or homicide/suicide. Therefore, for over half a century, several research groups tried to identify biological factors associated with increased levels of aggression, especially among subjects with a mental illness.

In 1958 Hoffer described a “mauve factor”, metabolite reacting with Ehrlich’s reagent, but different from all the well-known Ehrlich-reactors, associated with psychiatric disorders in general and with schizophrenia in particular. He correctly assigned the structure of the new metabolite to the pyrrole family [3, 4]. Sohler et al. introduced a colorimetric quantitative assay and identified the metabolite as kryptopyrrole (2,4-dimethyl-3-ethylpyrrole) [5]. Hoffer’s group observed that the recovery from acute schizophrenia was associated with the disappearance of the “mauve factor” from the urine and regression of symptoms with reappearance of the “mauve factor” [3, 6]. In 1960s and 1970s several groups reported finding the “mauve factor” in the urine of schizophrenics and subjects with acute intermittent porphyria [7–9]. Clinical data showed that a combined zinc and pyridoxine therapy suppressed the “mauve factor” and improved symptoms of schizophrenia [10]. Reports confirmed the mauve elevation in many mental disturbances, including affective psychosis, cognitive disturbance and alcoholism [11, 12]. The “mauve factor” was identified mistakenly as kryptopyrrole till late 1970s. The incorporation of a more sophisticated methodology and further research clarified that the “mauve factor” term should be referred to its structural analogue – hydroxylactam of hemopyrrole (hydroxyhemopyrrolin-2’one; HPL) [13].

Hydroxylactam of hemopyrrole is an abnormal side product of a porphyrin biosynthetic pathway [14], which bound with pyridoxine and zinc and when present in large amounts produces zinc and pyridoxine deficiency. Pyridoxine is crucial as a coenzyme for several metabolic pathways, including amino acid and neuromediators metabolism. HPL level increases with physical or psychosocial stress, therefore is called “a stress-induced factor”, which was confirmed with an experimental model [13, 15]. Neurotoxicity of HPL have been repeatedly proven in the animal model [16]. A theoretical mechanism of neurotoxicity may be related to heme depression, structural

similarity to pyroglutamate and kainic acid – and its influence on neurotransmission, and intracellular metabolism disturbance (reviewed in details by McGinnis et al., 2008) [15]. Elevation of kryptopyrroles may be associated with an inborn error of pyrrole chemistry, but can also result from porphyria or exposure to heavy metals, toxic chemicals, and other conditions enhancing oxidative stress.

The available literature regarding hydroxylactam of hemopyrrole function, its concentration in urine and correlation with aggression is not extensive and in most of the cases derived from 1960s and 1970s. Some authors contest the link of HPL and psychosis [17]. The implementation of modern analytical techniques may improve sensitivity of measurements and make the identification of HPL in urine reliable. The link between an aggressive behavior and increased hydroxylactam of hemopyrrole level was postulated by Hoffer. McCabe [18] characterized subjects with elevated HPL level as persons displaying attention deficit disorder, memory disorders, hyperactive, irritable, prone to uncontrolled agitation and rage. Walsh et al. [19] in 2004 reported that pyroluria was exhibited by 32.9% of 207 patients presenting a diagnosed various behavior disorder (including attention-deficit disorder, conduct disorder, oppositional-defiant disorder). Elevated levels of HPL have been observed in serial killers. Kraus [20] described highly elevated levels of HPL, next to the identified genetic disorders (XYY karyotype) and neurodegenerative disturbance of CNS, as one of the possible etiological factors of extreme aggressiveness of the serial killer of 11 women. In our research we hypothesize that an increased level of hydroxylactam of hemopyrrole may correspond to an extremely aggressive behavior and aggressiveness in subjects diagnosed with psychosis (schizophrenia, schizoaffective disorder, persistent delusional disorder). Therefore, we perform our research among the group of chronic forensic patients, placed in the forensic institution with the maximal level of security, characterized as subjects with the highest level of aggressiveness. In such an institution, evaluation of mental state and aggressiveness is obligatory every 6 months. To ensure, that the aggressive behavior led to the forensic treatment is the trait of aggressiveness, we incorporated into the trial only chronic patients.

Project description and hypothesis presented for the first time during 18th European Congress of Psychiatry in Munich, 2010 [21]. In order to reduce the dispersion caused by an individual kidney function, a new variable has been introduced: HPL/creatinine. HPL/creatinine level was correlated with the control group, composed of 22 male subjects, matched with age. To the best of our knowledge, it is the first report on urine HPL level in the selected group of psychiatric inpatients exhibiting an extremely aggressive behavior.

Material

Subjects

The study comprised 36 inpatients, male subjects aged 24–63 (mean 39.84; *SD* 11.4), placed in detention in the Regional Center of Forensic Psychiatry in Gostynin. The Regional Center of Forensic Psychiatry in Gostynin is one of the three forensic psychiatric hospitals featured with a maximal level of security in Poland. All subjects have been diagnosed according to ICD-10 criteria with a serious mental illness, including: schizophrenia ($n = 25$), schizoaffective disorder ($n = 2$), persistent delusional disorder ($n = 4$), organic delusional (schizophrenia-like) disorder ($n = 2$), recurrent depressive disorder with psychotic symptoms ($n = 1$), unspecified psychosis ($n = 2$). All subjects demonstrated an extremely aggressive behavior, in the form of a violent rape ($n = 15$), homicide ($n = 3$) or attempt of homicide ($n = 18$). In 4 subjects comorbid alcohol dependence was diagnosed, and in one a sedatives dependence. The control group comprised of 22 male subjects, without any mental disorder, matched by age (mean 39.7; *SD* 12,89) to the cohort of subjects who completed the trial.

The study protocol was approved by the Bioethical Commission of the Institute of Psychiatry and Neurology.

Methods

Reagents and standards

A weighing of hydroxylactam of hemopyrrole (3-ethyl-1,5-dihydro-5-hydroxy-4,5-dimethyl-2H-pyrrol-2-one, HPL) was made from the neat material synthesized by the Technology of Pharmaceutical Product Department, University of Warsaw. The chemical structure and purity of HPL were confirmed by H-NMR and MS methods. Creatinine and papaverine were purchased from Sigma. Acetonitrile was obtained from Merck (Darmstadt, Germany), water was prepared at the laboratory by distillation in the quartz system and formic acid purchased from Ubichem (Hampshire, UK). Kryptopyrrole (2,3-dimethyl-4-ethylpyrrole) also synthesized appeared to be very unstable even during the first hours after the preparation of its methanol solution.

Instruments

The Agilent Technologies HP-1100 Series liquid-chromatograph (LC) interfaced with a mass selective detector (MSD) and equipped with an autosampler was used for the analysis. An atmospheric pressure electrospray ionisation (ESI) was applied. The ions were monitored in SIM mode at programmed fragmentor voltage in CID zone m/z 156, 138, 94 for HPL, 114 and 340 for creatinine and papaverine, respectively.

LC

The LC gradient program was elaborated to get symmetrical peaks. Gradient program was as follows: A – 0,09% formic acid in water; B – 0.09% formic acid in acetonitrile: 0 min-5%(A):95%(B), 10 min-95%(A):5%(B), 12 min-95%(A):5%(B), 13 min-5%(A):95%(B), 18 min-5%(A):95%(B). Mobile phase flow rate was 0.4 ml/min. Separation of the analyte and internal standard from urine matrix components was performed using a chromatographic column LiChroCART 125 x 2 filled with RP-select B stationary phase (Darmstadt, Germany). The stationary phase ensured retention of analytes (HPL: $TR = 6.42$ min; creatinine: $TR = 2.41$ min; papaverine: $TR = 7.51$ min).

MS

The optimization of ionization and fragmentation parameters was performed using a flow injection analysis (FIA). Total ion current (TIC) was recorded but plots were constructed only for most abundant ions: m/z 156, 138, 94. The tested solution consisted of HPL at 10 mg/l concentration in a mixture of acetonitrile and water (1:1) with an addition of formic acid in the final concentration 0.09%. The injected volume was 10 μ l and injections were performed every 1 min. Optimized MS parameters were: fragmentor voltage 60 V for m/z 156, 340, 114; 90V for m/z 138; 150V for m/z 94, capillary voltage 4000 V, vaporizer temperature 320°C, drying gas temperature 300°C, nebulising gas flow 10 l/min, nebulising gas pressure 40 psi.

Validation

Stability studies during freeze and thaw cycles

For the freeze-thaw cycle testing urine spiked with HPL to 500 ng/ml was froze three times and after thawing on different days its concentration was determined. The following results were obtained: I) 460 ng/ml; II) 502 ng/ml; III) 482 ng/ml.

Matrix effect

Three urine samples containing only trace amounts of HPL (< 20 μ g/l) were spiked with analyte to achieve 500 ng/ml. Identical samples were prepared using water. To correct any variation of the instrument papaverine was added at 2000 μ g/l. Contribution of undesired compounds, coextracted from biological matrix, significantly reduced signal (intensity). Though its influence on the signal the intensity was not higher than 20%.

Precision and accuracy

Precision and accuracy was determined for three levels: 20, 100 and 500 ng/ml each in 5 replicates analysis of spiked urine. Relative standard deviation calculated

for precision were respectively 4%, 3% and 1 %. Accuracy ($n = 5$, $\alpha = 0.05$) was 20%, 11% and 4%.

Linearity

Linearity was calculated based on six levels: 0, 20, 50, 100, 200, 500 ng/ml. The equation of regression line and correlation coefficient for the pseudomolecular ion were $y = 0.0241x + 0.0001$; $R = 0.993$.

Limits of quantitation and detection

The limit of detection (LOD) was defined as the concentration yielding a signal-to-noise ratio $S/N = 3$. The limit of quantitation (LOQ) was defined as the lowest concentration of the calibration standards with a precision of $\pm 25\%$. It was 10 and 20 ng/ml respectively.

The elaborated procedure appeared to be effective in determination of HPL in urine at levels reported by inventors of HPU diagnostics. The case with the highest concentration of HPL (539 ng/ml) was used to prove the correctness of analyte identification. For that purpose Waters and Micromass LC-MS/MS system was used. Appropriate chromatograms were shown on Figure 1 (chromatogram for sample G-2 with the highest HPL concentration, and chromatogram that demonstrates the analysis of HPL standard).

Three MRM transitions (156 \rightarrow 138, 138 \rightarrow 123, 138 \rightarrow 110) were applied for that purpose. Nevertheless, the MRM method of ion transitions recording was not sufficiently sensitive (LOD approx. 100 ng/mL) to detect low concentrations of HPL. More sensitive MS systems or other sample preparation methods could resolve this problem. SIM mode was utilized in the presented research (most abundant ion $M+H^+$ of m/z 156 was used for quantitation).

Sample preparation

Urine spot samples were collected at the same day time (morning). To prevent analyte decomposition, thawed urine samples were not extracted but diluted with a standard mixture. 100 μ l of urine was mixed with 400 μ l of papaverine solution in water that gave its final concentration of 2 mg/l. 10 μ l of solution was injected directly without extraction. Papaverine was used rather to correct eventual detector variability than to control any analytes loss.

Results

In order to verify the normality of investigated variables several tests were carried out.

All of them showed that the distribution of HPL differs significantly from the normal distribution (K-S $d = 0.22826$; $p < 0.01$; Lilliefors $p < 0.01$; Shapiro-Wilk $W = 0.72303$; $p = 0.00000$). Similarly, the distribution of a variable defined as the quotient of HPL and creatinine differs from the normal distribution (K-S $d = 0.23043$; $p < 0.01$; Lilliefors $p < 0.01$; Shapiro-Wilk $W = 0.73821$; $p = 0.00000$). However, for the distribution of creatinine two tests did not show deviations from normality (K-S $d = 0.11337$, $p > 0.20$; Lilliefors $p < 0.10$) but the third one showed significant differences (Shapiro-Wilk $W = 0.94944$; $p = 0.01714$). For this reason, a nonparametric Mann–Whitney U test was used. Despite the HPL level was not significantly different between groups (Figure 1), the variable of HPL/creatinine quotient differs significantly between the study group and the control group (Figure 2). Results of the Mann–Whitney U test showed significant differences ($p = 0.037964$) between HPL/creatinine quotient: 1192.000 vs. 519.0000 ($U = 266.0000$, $Z = 2.075257$).

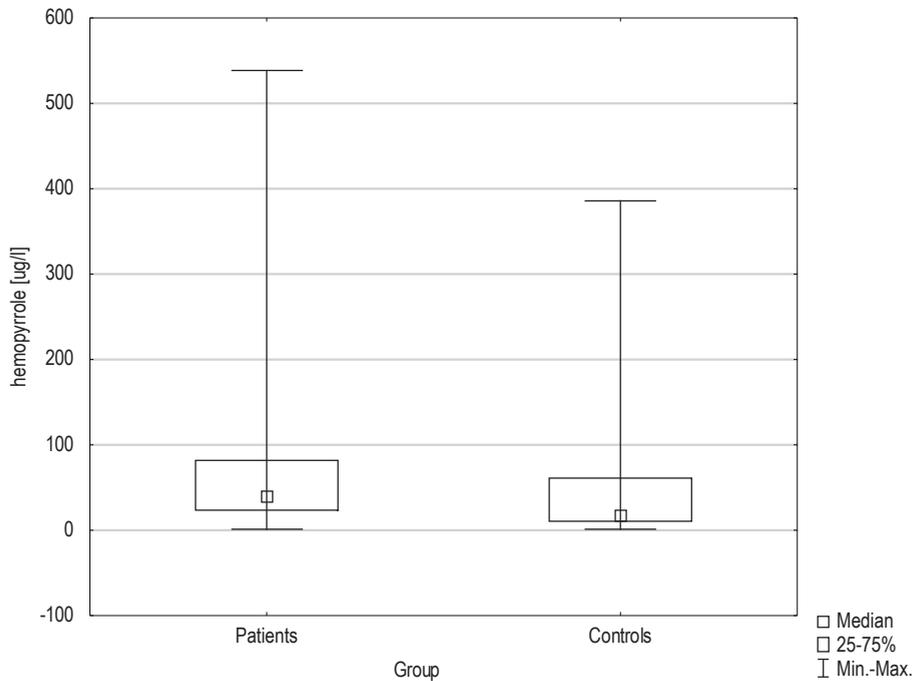


Figure 1. HPL level in the group of patients and healthy controls

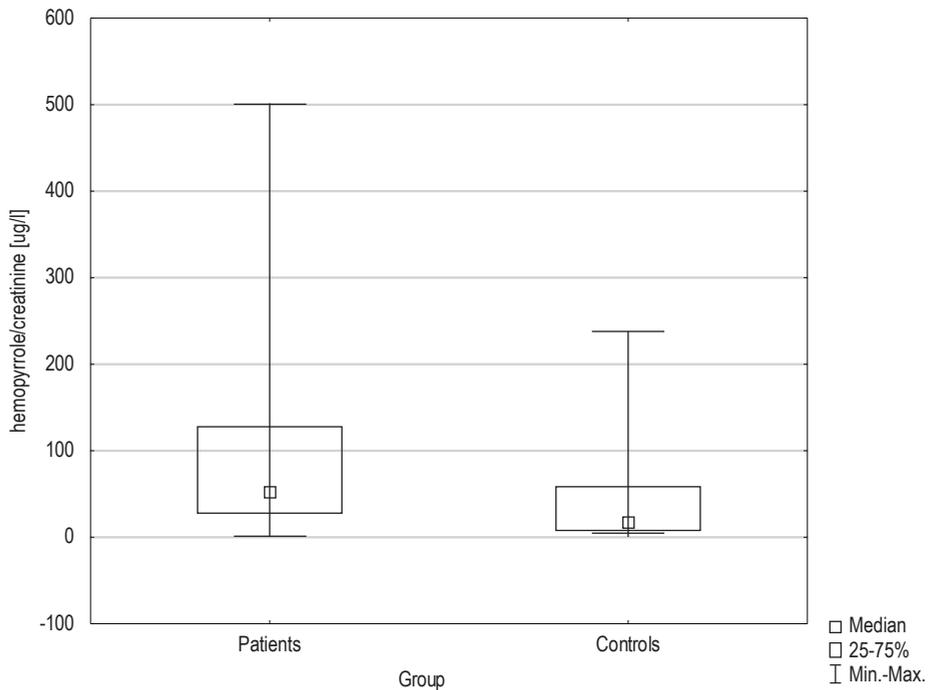


Figure 2. Differences of HPL/creatinine quotient in the group of patients and healthy controls ($p = 0.037964$)

Discussion

Two different LC-MS systems were used for the analysis of HPL. A more selective mode (MRM in the LC-MS/MS system) was perfect to show the correctness of HPL identification. Three transitions and relative retention time could be used to establish HPL presence. SIM mode (HP-1100 LC-MS system) appeared to be more sensitive and was used for quantitation. Some of the previous researches claimed the absence of HPL in urine based on a sensitive and specific method like GC-MS [17]. This could have been the result of an improper choice of the substance being determined, which was due to ambiguous naming in the early years of the research on this compound. LC-MS method, comparing to colorimetric or HPLC-UV methods applied formerly, is more convenient and sensitive in application, especially when HPL at normal concentrations have to be quantitated. The direct analysis of diluted urine could be performed so any losses caused by oxidation or low extraction efficiency were avoided.

Despite the obvious limitations described below, results of our study indicate that in the group of subjects with a history of an extremely aggressive behavior the level of HPL may be elevated. Our study does not address the direct question on reasons for HPL elevation. One possible explanation of elevated hydroxylactam of hemopyrrole

may be, as postulated by Walsh et al. [19], that pyroluria is common in the group of mentally ill patients and is not specific for any disorder. Our control group comprised of subjects without any severe mental disorder, therefore we may not exclude, that elevation of HPL level in the urine have been the result of mental disorders not correlated with the aggressiveness. On the other hand, it was known to be associated with a poor stress control and explosive anger [4], therefore in our group of extremely aggressive individuals the HPL level might be *a priori* elevated. Further studies of HPL urine concentration in the groups of subjects without any mental diagnosis demonstrating an aggressive behavior would verify these hypotheses.

Another possible explanation of the elevation of HPL is, according to Irvine [22], the stress induced mechanism associated with an involuntary embedment in psychiatric institutions as a consequence of an aggressive behavior. Additional psychosocial stressors may assume deprivation of social contacts, long-lasting hospitalization, and involuntary pharmacological therapy. According to such a theory in the whole group of involuntarily treated patients the level of HPL should be elevated. We were not able to find any research on hydroxylactam of hemopyrrole in involuntarily treated subjects.

The elevated level of HPL observed in our group may have an important clinical correlation. The treatment of pyrrole disorder involves supplementation of vitamins and microelements, including pyridoxine, zinc, and vitamins C and E. A pharmacological intervention with supplements may lead to the normalization of imbalanced pyrroles and to a mitigation of an aggressive and violent behavior. Further studies are needed to confirm the clinical effectiveness of such an experimental approach.

Our study protocol does not consider any evaluation of duration of time from committing an aggressive act to urine samples collection. In the further research of a reliable link between HPL elevation and aggression, a sample may need to be collected as close in time as possible to committing an aggressive act. Implementation of genetic approach in identification of possible abnormalities in metabolic pyrrole pathways seems to be reasonable.

The obvious limitation of this trial is the sample size and heterogeneity of psychiatric disorders in our study group. The occurrence of specific psychopathological symptoms in the study group have not been estimated.

Considering whether elevated levels of HPL in urine may be the predictor of aggressive behavior in the population of mentally ill people, requires further research and verification of results, among others, in a population of patients with no aggressive behavior.

Conclusions

Statistically significantly elevated corrected level of HPL in a group of subjects with a history of an extreme aggressive behavior observed in this study indicates the need for further studies to evaluate the reasons of HPL elevation, its role in the ethiopathogenesis

of aggressive behavior and its clinical implications – including possibilities of pharmacological interventions. We successfully used proprietary method for marking HPL level in urine, developed for the purposes of the project, which it is worthy of further verification.

Statement of Interest

The authors report no conflict of interest regarding the study.

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