KINETICS AND ISOFORMS OF SERUM GLUTAMATE DEHYDROGENASE IN ALCOHOLICS

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Abstract — Aims: The goal of this paper was to determine Glutamate dehydrogenase’s (GLDH) increased activity and rapid decrease in alcoholics according to last consumption of alcohol as well as to confirm that quick normalization cannot be a sign of hepatocyte necrosis and that GLDH from rough endoplasmic reticulum exists in the serum of alcoholics. Methods: GLDH activity was assed in 238 alcoholics admitted to hospital. A blood sample was taken from every subject three times: on admission to hospital, after 24 hours and after 7 days. We established our own reference activities for GLDH in serum, i.e., up to 124.0 nkat/l in males and 64.5 nkat/l in females. Results: Alcoholics were ascertained to have statistically significantly higher mean GLDH serum activities (men 351.53 nkat/L, women 296.08 nkat/L); the higher the level, the less elapsed time there was after the latest alcohol intake. There was an increased GLDH activity in 63.5% of alcoholics; furthermore, the percentage rose sharply to 72.2% with those who had last consumed alcohol within 48 hours. In the serum of alcoholics, it was found that, on average, it was 32.4% thermo-stable and 67.6% thermo-labile GLDH, which means that almost one third of GLDH serum originates from rough endoplasmic reticulum and rest from mitochondria. This is a completely new finding. Conclusions: A statistically significant fast decrease of GLDH serum activity after a break in alcohol consumption has been confirmed. It is estimated that increased GLDH activity in the sera of alcohol dependents and its fast decrease after total abstinence has been restored are specific for alcohol addiction.

INTRODUCTION

Alcohol is the most misused drug worldwide and its consumption is still on the increase. Alcohol dependence with its variety of symptoms is a disease similar to other mental disorders. It is an interaction between various psychosocial, genetic, biological and behavioural patterns (Gelder et al., 1999).

There are still no reliable biochemical markers for alcohol consumption and dependence whose sensitivity and specificity would be high enough to be relied on, but laboratory findings provide a significant aid in diagnostics. Of the currently available laboratory tests, carbohydrate-deficient transferrin (CDT) offers the best combination of specificity and sensitivity. Less reliable but widely used in clinical practice are: γ-glutamyltransferase (GGT), aspartate-aminotransferase (AST), alanine-aminotransferase (ALT), and erythrocyte mean cell volume (MCV). However, these markers are not sufficiently specific and sensitive for chronic excessive alcohol intake. Various markers combinations increase sensitivity but decrease specificity (Stamm et al., 1984a; Anton et al., 2002; Conigrave et al., 2003; Ropero-Miller and Winecker, 2003).

Glutamate dehydrogenase (GLDH) (EC. 1.4.1.3.) consists only of polypeptide chains. The active compound is the hexamer with a molecular mass between 310,000 and 350,000. The enzyme occurs in two catalytically active forms, determined as soluble (thermo stable) and particulate (thermo labile) (Plaitakis et al., 1984; Thomas, 2005).

GLDH is to be found almost exclusively in the mitochondria. The more recent data show it also occurs in the rough endoplasmic reticulum in thermo stable form (Lee et al., 1999). The distribution among the major organs is very uneven, with the liver being the outstanding one (Schmidt and Schmidt, 1988; Bais and Panteghini, 2006). GLDH catalyzes reversible deamination of glutamate to alpha-ketoglutarate and ammonium ion. More important is its catabolic function (Devlin, 2001; Thomas 2005).

The GLUD1 gene (for housekeeping GLDH) is localised on human chromosome 10 and is expressed as thermo stable, but GLUD2, however, (nerve tissue-specific) is localised on human chromosome X and is expressed as thermo labile isoprotein (Shashidharan et al., 1997; Plaitakis and Zaganas, 2001).

The increased sera GLDH activity is believed to be exclusively the result of liver damage caused by mitochondrial injury at cell necrosis (Schmidt and Schmidt, 1988; Thomas, 2005; Bais and Panteghini, 2006). Its release indicates a profound and probably irreversible disturbance of cellular integrity. The release of GLDH in serum is much lower than the release of cytosolic enzymes (Schmidt and Schmidt, 1988; Panteghini 2006). The occurrence of cellular nonmitochondrial GLDH activity (Mastorodemos et al., 2005) and alcohol-induced enhanced mitochondrial membrane permeability (Morales-Gonzalez et al., 2004) suggest that one part of GLDH might be released by plasma membrane blebbing (Gores et al., 1990).

Excessive consumption of alcohol can lead to hepatocyte injury and enzyme release in serum even before any symptoms can be noticed. Researchers quote contradictory findings about increased GLDH activity being used as an alcohol dependence marker; its value is denied by the majority (Worner and Lieber, 1980; Ghiese-Beer and Grafe, 1986; Schmidt and Schmidt, 1988; Weill et al., 1989; Goldberg and Kapur, 1994; Salaspuro, 1999, Thomas, 2005; Bais and Panteghini, 2006). However, only Weill found a fast decrease of serum GLDH activity during short-term alcohol withdrawal (median 35.8% in 24 hours) and higher GLDH specificity and sensitivity than GGT’s in alcoholics (Worner and Lieber, 1980), without sufficient explanation.

AIMS

The aim of this study was to clarify counter-evidence. The goal was to determine the diagnostic value of GLDH’s increased activity.
activity and rapid decrease in alcohol dependent persons according to the period since last consumption of alcohol, to confirm the hypothesis that quick normalisation of increased serum GLDH activity cannot be a sign of hepatocyte necrosis and to confirm the hypothesis about the existence of GLDH from the rough endoplasmic reticulum in serum of alcohol dependents.

SUBJECTS AND METHODS

Subjects
In the study, those inpatient alcohol addicts who met diagnostic criteria for alcohol addiction according to ICD X (WHO 1993) and DSM IV (American Psychiatric Association, 1994) were included. The control group was selected from general-practice healthy patients and blood donors; both sexes, aged from 18 to 65 years, were included. The exclusion criteria for both groups were: acute right heart failure, toxic circulatory failure, obstructive jaundice and severe respiratory insufficiency, acute viral hepatitis, severe acute intoxication and hypovolemic shock.

Materials
A blood sample was taken from every subject three times: on admission to hospital, after 24 hours and again after seven days. Blood was taken from the healthy control group only once, because no further changes were expected. That is why recurring blood tests would be considered to be ethically disputable.

The serum samples not analysed four hours after blood withdrawal were stored in a refrigerator at a temperature between 4 and 8°C. Analysis was carried out 16 hours after the withdrawal of blood at the latest.

Methods
By means of taking histories and clinical evaluation, the number of days passed between the last consumption of alcohol until the first blood test were recorded.

GLDH, AST, ALT and GGT were defined in serum, but MCV in blood. According to the recommendations of the International Federation of Clinical Chemistry (IFCC) AST, ALT and GGT were ascertained with reference procedures at 37°C (Committee on Reference Systems for Enzymes) (IFCC 2002 a,b), MCV was determined with the blood test with Abott Cell-DYN 610 and Mellet Schloesing Laboratories MS4 equipment. The catalytic GLDH activity was defined with new Deutsche Gesellschaft fuer Klinische Chemie (DGKC) method and Dialab kit (No authors listed, 1992).

The thermal inactivation of GLDH in serum was performed by serum heating at 47.5°C for one hour (Grossman et al., 1987).

Statistical analysis was carried out by using SPSS 12.0.1 for Windows. Frequencies, arithmetic mean, median, variance, standard deviation and standard error of the arithmetic mean, highest and lowest values, specificity and sensitivity, t-test, nonparametric Wilcoxon W-test, Mann–Whitney U-test, Komolgorov–Smirnov Z-test, Spearman’s ρ, Friedman’s and Cochran’s tests were used. Reference values were determined by using the nonparametric method (90%).

RESULTS

General data
The control group consisted of 141 healthy persons (107 men (75.9%) and 34 women (24.1%)) and 238 alcohol dependents ((199 men (83.6%) and 39 women (16.4%)). There were no statistically significant differences between the groups, $P (c^2\text{-test}) = 0.631$. The healthy persons’ mean age was 44.9 (SD 11.9), that of alcohol-dependent ones was 44.3 (SD 8.8). There were no statistically significant differences between groups, $P (t\text{-test}) = 0.520$.

The abstinence period since last consumption of alcohol in healthy subjects (6.92 ± 7.77 days) was significantly longer than that of alcohol-dependent subjects (3.3 ± 4.81 days) with Mann–Whitney $U = 2378.0$, Wilcoxon $W = 29639$, Komolgorov–Smirnov $Z = -4.635$, $P < 0.005$. Thirty-eight healthy and 233 alcohol-dependent subjects drank alcohol within last 30 days; even between them there was a remarkable difference in the time of restraint from alcohol ($U = 2378.000$, $W = 29639.000$, $Z = -4.635$, $P < 0.005$).

GLDH reference activities
So far, IFCC has not accepted the reference procedure for determining serum GLDH activity. Dialab, the producer of equipment and reagents, recommends reference activities up to 117 nkat/l for men and 83.3 nkat/l for women. However, Ti-etz’s Laboratory medicine recommends reference activities up to 133 nkat/l for men and 100 nkat/l for women (Bais and Panteghini, 2006), their method being the same as ours.

Reference values were determined in the group of 141 healthy subjects and blood donors, who had also AST, ALT, GGT, and MCV values within reference intervals. Using the nonparametric statistical method for determining reference ranges, the interval of 5- and 95-percentiles was chosen. The reference interval of up to 124.0 nkat/l for men ($n = 107$) respectively 64.5 nkat/l for women ($n = 34$) was thus calculated. The differences between sexes were statistically significant with $P = 0.005$ ($U = 1241.500$, $W = 1836.500$, $Z = -2.788$).

GLDH activity in healthy subjects and alcohol dependents
The GLDH and GGT serum activity in both groups has been presented in Table 1 and Fig. 1, with mean GLDH activities of 2M . KRAVOS AND I. MALEŠIČ

### TABLE 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean GLDH Activity</th>
<th>SD GLDH Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy subjects</td>
<td>4.635</td>
<td>0.520</td>
</tr>
<tr>
<td>Alcohol dependents</td>
<td>3.3 ± 4.81</td>
<td>3.3 ± 4.81</td>
</tr>
</tbody>
</table>

There were also statistically significant differences between groups ($U = 1139.000$, $W = 1842.000$, $Z = -7.188$, $P < 0.005$).

The GLDH specificity in healthy subjects was 89.8%. Increased GLDH was found in 65.5% (156) of alcohol dependents, but the percentage rose to 72.2% (83 out of 115) among those who had consumed alcohol within 48 hours before blood test. However, 65.6% (64 out of 96) of those who had last drunk alcohol three to seven days before blood test, and only 37%
Table 1. Statistical data on GLDH activity of Healthy Subjects in 1st testing and Alcoholics in all three testings

<table>
<thead>
<tr>
<th>n</th>
<th>GLDH [H]</th>
<th>GLDH (I) [A]</th>
<th>GLDH (II) [A]</th>
<th>GLDH (III) [A]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum (nkat/l)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Maximum (nkat/l)</td>
<td>185</td>
<td>336</td>
<td>2768</td>
<td>1651</td>
</tr>
<tr>
<td>Mean activity (nkat/l)</td>
<td>34.37</td>
<td>342.45</td>
<td>276.56</td>
<td>125.77</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>34.26</td>
<td>455.36</td>
<td>437.41</td>
<td>205.83</td>
</tr>
<tr>
<td>Median</td>
<td>27.24</td>
<td>183.00</td>
<td>132.00</td>
<td>64.00</td>
</tr>
</tbody>
</table>

Note: denotations H (healthy), A (alcoholic) and I (at admission), II (after 24 h), III (after 7 days) withdrawal blood.

Figure 1. Differences in GLDH and GGT activity between Healthy Subjects and Alcoholics.

(10 out of 27) of those who had last drunk alcohol eight days or more before blood test had elevated GLDH activity.

Spearman’s correlation coefficient between GLDH and GGT was 0.59 and signifying high correlation between them.

GLDH activity statistical data—three tests
For statistical data about GLDH activity in all three tests, see Table 1. The mean GLDH activity in alcoholics was 6.05 (median 5.38) times higher than in healthy subjects. After total abstinence from alcohol, the mean GLDH activity in alcoholics dropped to 80.76% (median 72.13%) within the first 24 hours and to 36.73% (median 34.97%) after seven days.

With Friedmann’s nonparametric test, it was found that mean GLDH and GGT serum activity diminished significantly by ($P < 0.001$) after the resumption of abstinence (Fig. 2). Only data of those alcoholics whose blood had been taken in all three tests ($n = 191$) has been included.

For changes of GLDH activity in course of time according to sex and time after the resumption of abstinence see Tables 2 and 3. The nonparametric Wilcoxon Signed Ranks Test was used.

The change of rate of increased GLDH activity from research at admission to Day 7 was tested with the nonparametric Cochran test ($Q = 57.855$, df = 2, $P < 0.0005$). Gradually, the level of GLDH increased statistically significantly from 34.5% (at admission) to 47.0% (after 24 hours) to 65.6% (after seven days). Only data of alcoholics who had participated in all three blood tests ($n = 191$) has been included.

Spearman’s nonparametric $\rho$ showed a statistically significant correlation between GGT and GLDH activities in all three tests. The high correlation ranged from 0.363 to 0.869 ($P < 0.01$).

Temperature GLDH inactivation
Grossman (Grossman et al., 1987) ascertained in his work, that he was able to inactivate approximately 17% of soluble (thermo stabile) and approximate 49% of particulate (thermo labile) GLDH.

Thermal GLDH inactivation from 205 alcohol dependents was determined, with mean thermo stabile GLDH activity at...
Figure 2. Changes of GLDH and GGT activity as regards time. GLDH (nkat/l) and GGT (µkat/l) where is 1st box = at admission, 2nd box = after 24 h, 3rd box = after 7 days. The distribution is displayed with “box-and-whiskers plot” (central line indicates median, boxes 1st and 3rd third part, handles 1st and 9th tenth part).

Table 3. Course of time of changes of GLDH activity after the resumption of abstinence as regards time

<table>
<thead>
<tr>
<th>Marker</th>
<th>(n = 66)</th>
<th>Z</th>
<th>P (2-tail)</th>
<th>(n = 71)</th>
<th>Z</th>
<th>P (2-tail)</th>
<th>(n = 74)</th>
<th>Z</th>
<th>P (2-tail)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLDH (I)–GLDH (II)</td>
<td></td>
<td>-4.287</td>
<td>0.001</td>
<td>-3.914</td>
<td>0.001</td>
<td>-4.138</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLDH (II)–GLDH (III)</td>
<td></td>
<td>-4.921</td>
<td>0.001</td>
<td>-3.719</td>
<td>0.001</td>
<td>-3.582</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLDH (I)–GLDH (III)</td>
<td></td>
<td>-5.419</td>
<td>0.001</td>
<td>-4.214</td>
<td>0.001</td>
<td>-4.616</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: denotations I (at admission), II (after 24 h), III (after 7 days) withdrawal blood.

104.73 (±182.43) nkat/L. The mean percentage of thermal inactivation was 67.57% (median 70.62%, standard deviation 26.12%, lowest 0% and highest 100%).

Among them, 129 (62.93%) had more than 20% thermostable GLDH. The number of alcoholics with 20% thermostable form was 10 (4.88%). There was only one case without thermostable form in the serum, but its overall GLDH was very low, only 3 nkat/L. Over 95% thermostable GLDH was found in 36 (17.56%) of cases, 24 (66.67%) of them had total GLDH activity in the reference range.

Spearman’s nonparametric ρ test shows a relatively high correlation between thermostable GLDH and total GLDH (correlation coefficient is 0.691, P < 0.0005, n = 212) and a moderate one between thermostable GLDH and GGT (correlation coefficient is 0.395, P < 0.0005, n = 212).

We did not find any GLDH activity in 31 (21.99%) of healthy persons, which makes determination and unbiased statistical analysis of thermostability impossible. The very low serum GLDH activity of healthy persons is derived exclusively from the liver because of cell apoptosis and belongs to thermostable GLDH of mitochondria (Thomas, 2005; Panteghini et al., 2006). This is also confirmed by our data in the group of alcoholics with 95–100% of thermostability where the majority had serum GLDH activity within the reference range.

On the grounds of the results, it can be concluded, that besides thermostable (mainly from mitochondria) one can find also thermostable GLDH (mainly from rough endoplasmic reticulum) in the serum of alcoholics.

DISCUSSION

The mean age of healthy subjects as well as alcohol dependents was anticipated, because of the long term needed for the development of disease.

We determined our own reference GLDH activities. The almost two times higher GLDH activity in men than women can presumably be related to their more intensive metabolism.

We discovered that GLDH activity was significantly higher in alcohol dependents than in healthy subjects. Furthermore, GLDH activity in moderate drinkers was lower than in alcohol dependents; yet it was not compared by Weill et al. (1989) and Worner and Lieber (1980) and therefore it can not serve as an example. GLDH was increased in both sexes.
Our investigation showed a 65.5% mean sensitivity, which increased to 72.2% with those who had drunk alcohol within 48 hours before first test. The sensitivity of GLDH as a marker of alcoholism was remarkable, particularly if compared with other markers in Stamm’s et al. (1984b) and Roppero-Miller’s and Winecker’s (2003) quotations.

Weill et al. (1989), who is the only one to has noticed prompt change in GLDH activity thus far, yet he overlooked the connection between time interval of last alcohol intake and increased GLDH activity and its regression.

There was a high correlation between GLDH and GGT activities and inversely proportionally with period from last alcohol consumption.

The less time passed since last drink of an alcohol dependent, the greater likelihood there was for increased GLDH activity. Mean GLDH activity was six times higher in alcohol dependents than in healthy subjects, but after seven days it dropped to 36% of the primary level.

When comparing median activities, we found similar results. In our study similar to Weill et al. (1989), median activities were found to be more reliable than mean activities because of three extremely elevated ones. The fall in serum GLDH activity, as regards the time passed since last alcohol intake, was statistically significant. After one week, GLDH activity in men has decreased to the upper reference level.

A 24-hour interval of alcohol abstinence is sufficient enough for a reliable evaluation of the fall in GLDH activity, or even more when alcohol dependents had not drunk alcohol for three to seven days. The rapid dynamic of fall in serum GLDH activity is a response to a break in drinking and proceeds for a relatively long time, for at least 10 days.

After the cessation of drinking alcohol, the level of normal GLDH activities in alcohol dependents increased quickly.

We strongly believe that watching changes in activity of laboratory markers of alcoholism, after cessation of drinking, is an effective yet overlooked aid in diagnostics. The kind of injury, half-life time and cell enzyme localization determine its activity in the plasma. After 24 hours, we found that normalisation of GLDH activity was faster than that of GGT and the kinetics of GLDH is more applicable than GGT kinetics after a week’s cessation of drinking.

To conclude, serum GLDH activity is equally important as other recognized markers of alcoholism, which have been found to be less specific to other diseases.

Rapid GLDH activity is only possible when alcohol abuse has been given up in case of reversible injury of mitochondrial membranes in hepatocytes but definitely not because of hepatocytes necrosis, as proved until now. Steady excessive alcohol abuse leads to permanent change in alcohol metabolism but probably only in alcohol dependent subjects. Alcohol weakens cytoskeleton of hepatocytes and increases mitochondrial membranes permeability causing plasma membrane blebbing and the increase in serum GLDH activity. The development of blebbing results from breakdown of cytoskeleton-membrane interactions between microvilli core structures and the plasma membrane. GLDH could be linked to amorphous material of blebs. MEOS produces toxins and free radicals during alcohol metabolism, which may additionally weaken the permeability of hepatocytes plasma membranes. In contrast to cell necrosis, all described pathological processes are functional and reversible.

We conclude that rapid and highly explicit regression in serum GLDH activity in the first days of abstinence is so interesting that it could be generally accepted as a specific marker of alcohol dependence.

Almost two thirds of alcohol dependents had more than 20% thermo stable GLDH, mainly bound to rough endoplasmic reticulum. According to Grossman’s et al. (1987) findings, the fact that a certain proportion of thermo stable GLDH is also inactivated, means there are almost two thirds of alcoholics with serum thermo stable GLDH, but slightly more than 17% of the examined subjects had exclusively thermo labile (more than 95% of total GLDH) to mitochondria linked GLDH. The distribution of both isoproteins is uneven. There is a moderate correlation between thermo stable GLDH in the rough endoplasmic reticulum and GGT inducted by elevated MEOS activity. The presence of thermo stable GLDH in the serum of alcoholics is a clear proof that one part of GLDH originates from hepatocytes rough endoplasmic reticulum. Alcohol has a toxic effect on the plasma and mitochondrial membrane structure, but there are no data available so far whether it can damage membranes of endoplasmic reticulum as well. GLDH is probably released by membrane blebbing.

The fact there are two different isoforms of GLDH in the serum of alcohol dependent subjects is an absolutely new discovery.

The determination of thermo-stable and labile GLDH isoforms could be applied as an additional marker for alcohol dependency, possible liver injury or even as prognostic factor for the possibly (ir)reversible damage. The GLDH activity in serum persists at liver necrosis but it decreases very quickly after cessation of drinking with reversible damage of mitochondria and endoplasmic reticulum. The kinetics and determination of two different GLDH isoforms give us important data about alcohol toxicity on liver tissues.

CONCLUSION

Time aspect considered serum GLDH activity is an ideal marker of alcoholism since it is elevated in alcohol abuse but its activity declines promptly after the last alcohol intake in alcohol dependent subjects and the change lasts long enough to be able to be evaluated. With other standard markers included, it presents an effective biochemical method for diagnosing alcohol dependence. GGT, AST, ALT, MCV, and GLDH are simple, yet effective and reasonably cheaper than CDT or some more recent and not yet generally accepted ones. Two different GLDH isoforms offer a wide range of new opportunities for future investigations. We are convinced that GLDH is a toxic and indirect marker of chronic alcohol consumption. The GLDH diagnostic value in comparison with other markers is our further research focus.

ABBREVIATION

ADH, alcohol dehydrogenase; ALDH, aldehyde-dehydrogenase; ALT, alanine-aminotransferase; AST, aspartate-aminotranferase; CDT, carbohydrate-deficient transferrin; DGKC, Deutsche Gesellschaft fuer Klinische Chemie; DSM, Diagnostic and Statistical Manual of Mental Disorders;
system.

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