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Journal of Chromatography B, 713 (1998) 245–264

JOURNAL OF CHROMATOGRAPHY B

Review

Determination of biological markers for alcohol abuse

F. Musshoff[a,*], Th. Daldrup[b]

[a]*Rheinische Friedrich–Wilhelms University, Institute of Legal Medicine, Stiftsplatz 12, 53111 Bonn, Germany*
[b]*Heinrich–Heine University, Institute of Legal Medicine, Moorenstrasse 5, 40225 Duesseldorf, Germany*

Abstract

Alcoholism is one of the most frequent addictions and an important subject in forensic medicine and clinical toxicology. Several laboratory abnormalities are associated with excessive alcohol consumption. They are useful in the diagnosis of alcoholism especially during the follow-up of various treatment programs. The biological markers mostly used for diagnosis of alcoholism are presented. Especially, methods for the determination of the following diagnostic tools are reviewed: congener alcohols, γ-glutamyltransferase, aspartate and alanine aminotransferase, β-hexosaminidase, erythrocyte aldehyde dehydrogenase, α-amino-*n*-butyric acid to leucine ratio, macrocytosis, carbohydrate-deficient transferrin, (apo)lipoproteins, fatty acid ethyl esters, blood acetate, acetaldehyde adducts, 5-hydroxytryptophol, dolichol and condensation products. No laboratory test exists that is reliable enough for the exact diagnosis of alcoholism. The combination of physician interview, questionnaire and laboratory markers is necessary for the diagnosis of alcoholism.

Keywords: Reviews; Alcohol

Contents

*Corresponding author.

0378-4347/98/$19.00
PII: S0378-4347(97)00503-3

1. Introduction

Alcoholism is a widespread social, medical and also economical problem in a large section of the population in nearly all ethnic groups. Typical for alcoholics is their ability to hide their addiction; very often they are drinking in secret, so even their families or their colleagues at work have no knowledge of their illness and they do not admit that they are alcoholics. Therefore, it is of a great importance to have diagnostic tools (biological markers) to detect excessive alcohol consumption and alcoholism. Interest in research into these tools has mainly been focused on detection of alcoholism and alcohol consumption in its early phase. Another priority area is the search for biological markers for a predisposition towards alcoholism based on data indicating that this disorder is genetically influenced. Thus, in alcoholism research, a general classification of biological markers is necessary. Markers can be divided into state markers and trait markers of the disease [1–4]. A state-dependent phenomenon, also called episode or state marker, is concomitant with the disease. Such a phenomenon should be absent before and after the illness and is useful for the diagnosis and treatment of illness. A state-independent phenomenon or trait marker is detectable during the entire life span of an individual. It co-exists with the disease state but should also be present in the normal state. Genetic markers are state-independent markers (trait markers). Their identification would permit prediction of genetic vulnerability and of the so-called high risk subjects. Most of the conventional laboratory markers are state markers of alcoholism (for reviews see Refs. [5–14]). They indicate an episode of excessive alcohol consumption and are results of a chronic alcohol intoxication. Trait markers are not discussed in the present paper (for reviews see Refs. [1,15–37]).

2. Alcohols

The most obvious and specific test for heavy drinking is the measurement of blood, breath or urine alcohol. However, this simple test cannot distinguish between acute and chronic alcohol consumption, unless it can be related to increased tolerance toward alcohol. According to the American National Council on Alcoholism (NCA), criteria for the diagnosis of alcoholism, blood alcohol exceeding 1.5‰ without gross evidence of intoxication, over 3‰ at any time, or over 1‰ in routine examination are first level criteria of alcoholism [38].

Methanol is an important congener of most alcoholic beverages [39]. Metabolism of methanol via liver alcohol dehydrogenase is inhibited by ethanol [40,41]. Consequently, excessive and prolonged drinking results in high blood methanol levels [42–45]. Increased blood methanol levels are frequently found in drunken drivers and alcoholics. On the basis of these findings, blood methanol levels exceeding >10 mg/l have been suggested to be indicative of alcoholism (Table 1) [44–53]. To determine methanol, the sensitivity of conventional headspace gas chromatography (GC) for the analysis of blood alcohol has to be improved by special sample preparation or by using a cryofocussing technique with capillary columns [54–59].

Additionally, congener analysis of blood samples from excessive drinkers revealed higher concentrations of acetone and 2-propanol compared to nonalcoholics [60,61]. Iffland et al. [62] proposed the concentration of acetone and 2-propanol as an indi-

Table 1
Laboratory parameters for diagnosis of heavy drinking and alcoholism according to Gilg [14]

	Normal range	Reaction after short-term loading	Diagnostic sensitivity (%)	Diagnostic specifity (%)	Practicability	Normalization after withdrawal
Enzymes						
GGT	<28 U/l	–	50–90	~70	++++	2–5 w
ASAT	<18 U/l	–	30–50	~90	++++	1–3 w
ALAT	<22 U/l	–	20–45	~70	++++	1–4 w
GLDH	<4 U/l	–	5–60	?	+++	?
β-HEX	<6.2 U/l	+	high	?	+	2–4 d
Hematological parameters						
MCV	<92/100 fl	−	40/70–96	~60–90	++++	1–3 m
CDT	<20/26 U/1	−	50–90	90–100	(++)	~2 w
HDL chol.	<50 mg/dl	−	50–90	high	+++	1–4 w
Apolipop. A1/2		−	>45	high	++	~2 w
Metabolites						
Acetaldehyde		(+)	−	−	+	
Acetate	0.75 m*M*	−?	−	−	(++)	?
2-Propanol	<2 mg/l	(+)	−	?	(++)	hs
Acetone	<7 mg/l	−	−	−	(++)	hs
Methanol	<10 mg/l	(++)	~80	high	(++)	hs–1 d
Urine/(blood)						
5-HTOL/5-HIAA	<20	+	60	high	+	hs
Dolichol	4.7 ng/ml	+	low	?	+	ds–ws
TIQ and THBC (SAL)					+	

Sensitivity: % of positive results for alcoholics; specifity: % of negative results for nonalcoholics. +: Special laboratory; ++: special method outside a clinical laboratory; +++: special test in a routine laboratory; ++++: simple routine method in a clinical laboratory.

cation of drinking behaviour, caused by reciprocal formation by the alcohol dehydrogenase system; if the concentrations exceed 9 mg/l, heavy drinking could be suspected (Table 1). However, due to the effects of metabolic disorders (ketosis, diabetes, hunger, physical stress), the significance has been estimated to be very low.

3. Enzymes

3.1. γ-Glutamyltransferase (GGT)

Measurement of the serum level of the enzyme γ-glutamyltransferase (GGT) – a membrane-bound enzyme located in the sinusoidal membranes of the liver cell – is today the most commonly used laboratory marker of heavy drinking and alcoholism. GGTs plasma level correlates purely with the total amount of alcohol consumed and is not effected by acute alcohol consumption. It, however, correlates slightly with the period of time of excessive consumption. In chronic alcoholics or heavy drinkers, serum GGT has been reported to be elevated in 34–85% of cases [7]. During abstinence, GGT returns to normal levels in 1–2 months (Table 1). The mechanisms of GGT increase are not fully understood. The hypothetical possibilities are induction of hepatic GGT in the endoplasmatic reticulum, physicochemical alterations of liver cell plasma membranes, and decreased uptake of GGT by hepatocytes from blood. Increased GGT levels are not specific for heavy alcohol addiction. All sorts of liver diseases, diabetes, obesity, pancreatitis, hyperlipidemia, heart failure, age, smoking and severe trauma should also be taken into account when the

diagnostic significance of increased GGT is considered. In clinical laboratories, GGT is most commonly measured using a kinetic method [63,64]. For the determination in completely haemolysed or frozen blood samples, a method is described by Gjerde and Mørland [65].

3.2. Mitochondrial aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT)

Total aspartate aminotransferase (ASAT) of serum and liver homogenate consists of two isoenzymes [66]. The soluble cytosolic isoenzyme accounts for about 20% of total liver activity and the mitochondrial isoenzyme for the remaining 80%. Increased activities of serum mitochondrial ASAT levels have been found in alcoholic patients with and without alcoholic liver disease (Table 1) [67,68]. The efficiency of the test may be further increased by the determination of the ratio of mitochondrial ASAT to total ASAT [69–71].

The activity of mitochondrial ASAT can be analyzed with a rather simple immunochemical procedure [72,73]. The essential antibody against the soluble aspartate aminotransferase is commercially available.

The alanine aminotransferase (ALAT) is found in the liver cytoplasm and is released in to blood as a consequence of the damage of hepatocytes. A ratio of ASAT to ALAT exceeding two is found in alcoholics and can be used for the differentiation from nonalcoholic liver injury [71,74,75]. A decrease in ALAT is related to the deficiency of pyridoxal 5-phosphate associated with excessive alcohol consumption [76,77].

3.3. β-Hexosaminidase (HEX)

β-Hexosaminidase (HEX) is an acid lysosomal glycosidase. Increased urinary HEX is a sensitive indicator of renal diseases. Increased serum HEX levels are found in liver diseases, hypertension, *Diabetes mellitus*, myocardial infarction and other diseases. In serum, increased HEX levels were found in 69% to 94% and in urine in approximately 81% in cases of alcoholism (Table 1) [78–80]. Especially the isoenzyme HEX B was demonstrated to be highly indicative of alcohol abuse [81,82]. The cause for the increased content is not known but could result from alterations in the rate of clearance/elimination or production/release to the circulation.

Total HEX is measured by a spectrophotometric method using *p*-nitrophenyl-*N*-acetyl-β-glucosamine as substrate [83–85]. The isoenzymes can be determined by an immunochemical method [81,82,86].

3.4. Erythrocyte aldehyde dehydrogenase (ALDH)

Human erythrocyte aldehyde dehydrogenase (ALDH) shows a single band on starch gel electrophoresis and isoelectric focusing in polyacrylamide gel [87,88]. As compared to healthy controls and others, the activity of erythrocyte ALDH was found to be decreased in chronic alcoholics and the test was suggested as a biochemical marker of alcoholism [89]. In man, at least four different ALDH isoenzymes have been identified [90]. Two major forms with high abundance in the liver are the cytosolic ALDH I and the mitochondrial ALDH II with k_m values of about 50–100 μ*M* and 1 μ*M*, respectively, for the toxic ethanol oxidation product acetaldehyde, and mitochondrial ALDH II is considered to be responsible for the oxidation of the acetaldehyde formed during ethanol intoxication. The accumulation of acetaldehyde and associated flushing response frequently observed following alcohol consumption in Orientals is due to a structural mutation of this isoenzyme leading to impaired catalytic activity [91,92].

A number of different methods and aldehyde substrates, which have been used for the assay of ALDH activity in whole blood or separated erythrocytes, were reviewed by Helander [93]. These include determination of acetaldehyde disappearance by GC [94,95] or spectrofluorimetry [96], determination of indole-3-acetic acid (IAA) formation from indole-3-acetaldehyde (IAL) by spectrofluorimetry [97] involving several extraction steps with minor recovery for IAA, and isoelectric focusing followed by densitometry [89]. A high-performance liquid chromatographic (HPLC) method has been described by Helander and Tottmar [98]. The aldehyde of dopamine, 3,4-dihydroxyphenylacetaldehyde (DOPAL) which is not available commercially, was used as the substrate and the 3,4-dihydroxy-

phenylacetic acid (DOPAC) formed was quantified electrochemically. The procedure can be performed on a very small sample volume of whole blood with no time-consuming pretreatments. Most commonly, ALDH activity has been assayed spectrophotometrically with acetaldehyde or propionaldehyde as substrate by monitoring the reduction of NAD^+ to NADH at 340 nm [99–104]. A specific problem when ALDH is assayed in blood with spectrophotometric or fluorometric methods is the separation of samples from haemoglobin which interferes with the measurements due to high background absorbance and problems with light scattering. Also procedures measuring the disappearance of acetaldehyde are not very accurate, since they are made at a relatively high background concentration of aldehydes and aldehydes readily interact with various tissue constituents. Thus, a procedure that measures formation of the product generally offers a more accurate determination of the enzyme activity.

4. Hematological parameters and metabolites

4.1. Plasma α-amino-n-butyric acid to leucine ratio

α-Amino-*n*-butyric acid (AANB) increases following chronic alcohol consumption caused by increased hepatic production. However, protein malnutrition also depresses plasma AANB level and in order to minimize the effect of protein deficiency, AANB was related to leucine and the AANB:leucine ratio was introduced as an empirical biochemical marker of alcoholism [105]. In some studies, plasma AANB to leucine ratio has appeared to be of limited value as a biochemical alcoholism marker caused by the rather low sensitivity and specificity [106–110].

The determination of the AANB to leucine ratio requires an amino acid analyzer, which makes the test expensive and not widely available.

4.2. Macrocytosis (MCV)

Elevated mean corpuscular volume (MCV) is found in 31–96% of alcoholic patients (Table 1) [7]. However, conditions unrelated to alcohol like vitamin B_{12} and folic acid deficiency, liver disease, reticulocytosis, antiepileptics, age, smoking and others may also increase MCV values. The measurement is inexpensive and can be easily performed. Methodical differences have to be taken into consideration in the discussion of critical values.

4.3. Carbohydrate-deficient transferrin (CDT)

Transferrin is a glycoprotein involved in delivery of iron to body tissues. Chronic exposure to alcohol is believed to alter the glycosylation process in transferrin formation. The resulting transferrin is often missing some carbohydrate terminal chains, including sialic acid, galactose and *N*-acetylglucosamine; thus, the term ‘carbohydrate-deficient’ transferrin (CDT) [111]. Several years ago, an abnormal microheterogenity of transferrin was demonstrated in 81% of patients consuming more than 60 g of ethanol daily [112,113]; at least one week of heavy drinking is minimally needed to elevate CDT levels. CDT returns to normal levels during abstinence and seems to have a half-life of approximately 15 days (Table 1) [111,114]. Clinical studies of CDT as a marker of alcohol abuse are reviewed by Stibler [111], Allen et al. [114], Litten et al. [115] and others.

At first isoelectric focusing was the predominant method used to demonstrate abnormal transferrins in alcoholics. Its presence was initially evaluated qualitatively [112,116–118] but it was later followed by quantitative immunological measurements by combining isoelectric focusing with immunofixation [119,120] or zone immunoelectrophoresis [121,122], and by quantifying an isoform with p*I* 5.7 in relation either to total transferrin or to the main isotransferrin with p*I* 5.4. Procedures were too complex and time-consuming for routine clinical purposes. Two new approaches were developed by Stibler et al. [123] and Storey et al. [124,125] based on isocratic anion-exchange chromatography and chromatofocusing, respectively. In the former procedure, iron-saturated whole serum was passed through a microcolumn, and all isotransferrins with p*I* values >pH 5.65 were eluted (Fig. 1) and quantified by a subsequent transferrin immunoassay. The latter method required an initial partial isolation of serum transferrin followed by chromatofocusing on a Mono P column and collection of the isotransferrin with p*I* 5.7. The

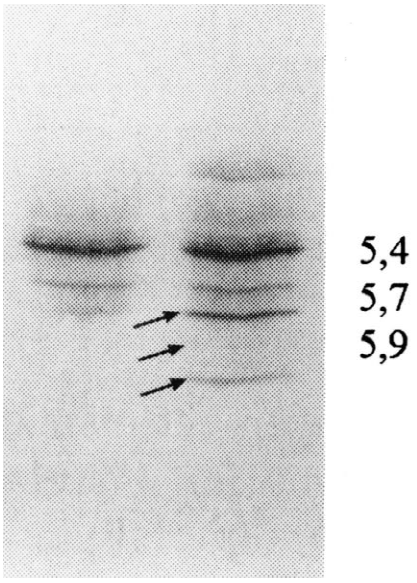

Fig. 1. Serum transferrin purified by immunoaffinity chromatography from a healthy control (left) and an alcohol-abusing patient (right) (Stibler et al., [123]). Isoelectric points are given at the right. Arrows indicate abnormal transferrin forms.

isotransferrin was later quantified with a transferrin radioimmunoassay [126]. Xin et al. [127] described a method for quantitation of carbohydrate-deficient transferrin by isoelectric focusing coupled with Western blotting with a high diagnostic accuracy [128]. Jeppsson et al. [129] developed a fully automated ion-exchange chromatographic method for quantitating carbohydrate-deficient transferrin (CDT) on a Mono Q column. Quantitation relies on the selective absorbance of the iron–transferrin complex at 460 nm, and transferrin isoforms with p*I*s 5.7 and 5.9 can easily be separated and quantitated as a percentage of the total transferrin. According to modified procedures [130–132], today CDT is mostly measured using commercially-available assay kits using a combination of anion-exchange chromatography and immunoassay.

The first commercially-available kit was the CDTect from Kabi Pharmacia Diagnostics. In this assay, transferrin isoforms in serum samples are separated on a microcolumn, and the eluted transferrin fraction, which is deficient in its carbohydrate moieties, is quantitated by a subsequent double antibody radioimmunoassay. Another commercially-available radioimmunoassay kit is the Axis %CDT test, in which serum transferrin is radiolabeled using antibody fragments before separating the low-sialic acid transferrin (%CDT) on an ion-exchange chromatography minicolumn. The amount of labelled antibody–transferrin complexes eluted is expected to be independent of the total transferrin level in the serum, and the relative amount of CDT (%CDT) is obtained by measuring the radioactivity of the eluted fraction. Amounts exceeding 2.5% are considered elevated.

Sorvajarvi et al. [133] compared the two different methods for measuring CDT (CDTect and %CDT) and total transferrin. The overall sensitivity for detecting alcohol abuse was clearly higher for CDTect than for %CDT. However, any alteration in serum transferrin concentration markedly decreased the assay specificity.

Recently, the CDTect method was modified to a commercially-available competitive enzyme immunoassay [134]. Transferrin in the sample is separated by ion-exchange chromatography into different isoforms on a microcolumn. CDT is obtained in the eluate, while the other transferrin isoforms remain on the column. The CDT in the eluate competes with a fixed amount of enzyme-labelled transferrin for the binding sites of monoclonal anti-transferrin antibodies. These antibodies bind to anti-mouse antibodies, coated on the surface of a microplate well. When unbound material is washed away, bound transferrin conjugate is measured by the addition of enzyme substrate to the wells. After hydrolysis, the substrate gives a coloured product and the absorbance, which is inversely proportional to the concentration of CDT in the sample, is measured by a microplate reader. In the same way the %CDT kit was modified to an enzyme immunoassay.

Heggli et al. [135] investigated the sensitivity and accuracy of the test using relative concentrations of different isotransferrins in serum and quantified the different isoforms by HPLC. Including the trisialotransferrin fraction into the definition of %CDT resulted in an increased accuracy.

Simonsson et al. [136] compared the CDTect procedure with the HPLC method. HPLC determination offered the advantage of identifying isoforms of transferrin and thus reducing analytical pitfalls associated with the CDTect method.

Finally, a method based on polyacrylamide gel isoelectric focusing, direct immunofixation with a specific antibody, and measurement by computerized scanning densimetry was described [137]. This easy-to-perform and inexpensive procedure showed very good reproducibility and accuracy and resulted in a specificity of 100% and a sensitivity of 94%. Bean et al. [138] studied the performance of a isoelectric focusing/immunoblotting/laser densimetric (IEF/IB/LD) procedure to evaluate CDT derived from dry blood spots.

First Kilar and Hjerten [139] described a fast and high resolution analysis of human serum transferrin by high-performance isoelectric focusing in capillaries. Recently, a capillary zone electrophoretic-(CZE)-based assay for the analysis of CDTs was evaluated using uncoated capillaries and separation augmented with cationic additives [140]. CZE was also used to resolve the glycoforms of normal transferrin and a set of glycoforms which were prepared by digesting the sugars on the intact glycoprotein with sialidase [141]. These authors suggested that CZE can be used as a rapid diagnostic test for the carbohydrate-deficient-glycoprotein-syndrome group of diseases. In combination with sialidase digestion, the distribution of p*I* and molecular mass of asialo-, mono-, di-, tri- and tetrasialotransferrin variants was obtained from capillary isoelectric focusing–electrospray ionization mass spectrometric measurements [142]. This method represents a potentially useful analytical procedure which can provide information concerning the characteristics of these and perhaps other proteins.

4.4. High density lipoprotein cholesterol and apolipoproteins

High density lipoprotein (HDL) cholesterol increases as a consequence of excessive alcohol consumption (Table 1). Chronic administration of liver-microsomal-system-inducing drugs and vigorous physical exercise are other factors capable of increasing HDL cholesterol. Consequently, HDL cholesterol has been suggested to be a rather specific laboratory marker of alcoholism [143–146]. A daily intake of 75 g of ethanol for 5 weeks was shown to increase HDL cholesterol and it returns to preconsumption levels in 1–2 weeks [147]. A significant decrease in HDL cholesterol was found in alcoholic patients during abstinence [148,149]. Plasma HDL cholesterol was determined after manganese–heparin precipitation (normally a magnesium chloride–heparin precipitation is used) of low density lipoproteins (LDL) and very low density lipoproteins (VLDL) [150]. In addition to HDL cholesterol, alcohol consumption also increases HDL phospholipids and apolipoproteins A-I and A-II [151–155]. Recently, Lin et al. [156] described the known increase in plasma concentration of apolipoprotein A-I and A-II in chronic alcoholics with a simultaneous decrease in the apolipoprotein E concentrations, a major component of VLDL and minor component of HDL. Apolipoproteins were assayed by an immunoturbidometric method. Hu et al. [157] developed a capillary-zone electrophoretic procedure for the separation and characterization of lipoproteins and results showed that capillary electrophoresis (CE) is a very efficient and effective technique for lipoprotein analysis. New methods have also been developed using CE to quantitate plasma levels of apoproteins in HDL samples [158,159]. CE appeared as a method of high-resolution power in the study of apo A-I and apo A-II heterogenity and could lead to the development of an alternative method for the assay of apo HDL. Tadey and Purdy [160] reviewed chromatographic techniques for the isolation and purification of lipoproteins including CE techniques which emerged as important methods due to the high speed and superior resolving power.

The transfer of cholesteryl esters from HDL to VLDL and LDL is mediated by plasma cholesteryl ester transfer protein (CETP). Alcohol drinking reduces plasma CETP activity [161–163], which is evaluated as a marker of alcoholism [164]. The CETP activity was measured by determining the transfer of [^{3}H]cholesteryl esters from HDL to VLDL [164–166].

Recently, an improved method of immunoblotting of plasma onto agarose gel matrix containing antiapolipoprotein A-I was described [167]. Fresh plasma samples were subjected to gradient polyacrylamide gel electrophoresis followed by electrotransfer onto agarose gel layer containing antiapolipoprotein A-I. This method was compared with immunoblotting onto nitrocellulose and was shown to be more convenient and quantitative.

4.5. Fatty acid ethyl esters

Fatty acid ethyl esters (FAEE), a family of ethanol metabolites, are formed by esterification of ethanol with fatty acids and have been detected in human organs commonly damaged by ethanol abuse [168–171]. Post mortem studies evaluated this end product of alcohol ingestion as an useful alcoholism marker in forensic pathology. Results indicated that FAEE are long-lived ethanol metabolites whose persistance and accumulation in adipose tissues may allow an accurate diagnosis of significant alcohol consumption even when ethanol has been completely eliminated from the body [171].

Isolation of FAEE according to Kinnunen and Lange [172] can be performed by extraction of adipose tissue with acetone (10% w/v) with tritiated ethyl oleate as a yield marker, followed by a separation of the lipids by thin-layer chromatography on silica gel using petroleum ether–diethyl ether–acetic acid (75:5:1) as solvent. Using gas–liquid chromatography with a flame ionization detector and methyl myristate as internal standard, the ethyl esters of palmitate (16:0), palmitoleate (16:1), stearate (18:0), oleate (18:1) and linoleate (18:2) were detected in adipose tissue of chronic alcoholics at levels (nmol/g) seven times greater than that of control subjects [171].

Recently, Emrich et al. [173] also detected fatty acid methyl esters (FAME) in post mortem human tissues and found a relationship between various levels of alcohol consumption and the appearance of FAMEs, caused by the accumulation of the congener alcohol methanol during chronic alcohol abuse.

Björntorp et al. [174] and Doyle et al. [175] studied the potential usefulness of FAEEs as markers of alcohol abuse in different collectives. Their short half-lives in adipose tissues – a correct determination of the half-lives was not possible – appeared to limit usefulness. However, it should be noted that this specific metabolite of ethanol clearly has a longer half-life than ethanol in the blood. The induction of FAEE synthase activity appeared to be more promising. FAEE synthase activity was determined with ethanol and C^{14} oleic acid as substrates, and the FAEE produced were isolated by thin-layer chromatography and determined quantitatively.

Recently, a two-step method was developed to purify FAEEs using solid-phase extraction [176]. First the lipid mixtures are applied to an aminopropyl–silica column with simultaneous elution of FAEE's and cholesteryl esters with hexane. Then, the FAEE's can be separated by chromatography on an reversed-phase column and elution with 2-propanol–water (5:1, v/v). Laposata et al. [177] detected FAEEs in serum following ethanol ingestion using a quantitative GC–MS procedure. FAEEs were isolated by thin-layer chromatography.

4.6. Blood acetate

The enhancement of ethanol elimination as a consequence of chronic alcohol abuse is well established. Demonstrated as an inconsistent finding in the first oxidative step, plasma levels of acetaldehyde are increased [178,179], decreased activity of mitochondrial aldehyde dehydrogenase may further increase blood acetaldehyde levels [180]. Because of low sensitivity, these increased blood levels cannot be considered as a suitable biochemical marker of chronic alcoholism. In a second oxidative step, acetate is formed in the liver from acetaldehyde and is oxidized further to CO_2 and water mainly in the peripheral tissues. During ethanol oxidation, blood acetate rises quickly and reaches a plateau which is maintained as long as ethanol is present in blood. During ethanol oxidation, acetate hepatic metabolism is blocked probably due to ethanol-induced change in the hepatic redox state. On the basis of these findings, increased levels of blood acetate during ethanol oxidation was suggested to indicate metabolic tolerance toward alcohol, and it was concluded that it is a sensitive and reliable biological marker of alcoholism and excessive alcohol consumption (Table 1) [181–184].

Blood acetate can be easily analyzed with a commercially-available enzymatic test kit in a routine laboratory. Acetate is first activated to acetyl-CoA with acetyl-CoA synthetase and then reacted with oxaloacetate to form citrate using citrate synthase. The required oxaloacetate is formed from malate and nicotinamide adenine dinucleotide, in the presence of malate dehydrogenase. The consumption of oxaloacetate is stoichiometric with the amount of reduced nicotinamide adenine dinucleotide formed in malate dehydrogenase equilibrium reaction. Persson

et al. [185] described a procedure for the measurement of plasma acetate using automated HPLC. This procedure involves extraction from plasma with diethyl ether, derivatization with bromacetophenone, and separation of the phenacyl derivative on a C_{18} reversed-phase column.

However, it should be emphasized that blood acetate levels can be determined only in the presence of ethanol, i.e. when the liver is oxidizing the alcohol. Therefore, possible candidates for this type of examination are intoxicated patients in hospitals with a suspicion of chronic alcohol-related problems or persons arrested for driving while intoxicated.

4.7. Acetaldehyde adducts

The highly reactive ethanol oxidation product acetaldehyde has been shown in recent years to form stable adducts with several proteins. Protein acetaldehyde adducts (-AAs), e.g. the 37 kDa protein-AA [186] and cyt-P450IIE1-AA [187], have been detected in the liver of rats chronically fed with alcohol. Additionally, two serum protein-AA with molecular masses of 103 kDa and 50 kDa have been detected in some alcoholic patients [188]. These protein-AAs were detected by sodium dodecyl sulphate–polyacrylamide gel electrophoresis plus immunotransblot and by ELISA.

Measurement of Hb-AAs as a diagnostic marker for chronic alcohol abuse has generated a great deal of interest in recent years. Because adduct formation is very proximal to the pathway of alcohol metabolism and is not dependent on an alcohol-induced organ injury, it has been speculated that measurement of Hb-AAs should be superior to other less-direct tests as biological markers of alcohol abuse. Analogous to the use of glycated Hb (HbA_{1c}) or glycosylated albumin in diabetic patients to monitor the degree of glycemia control as a function of time, it was thought that Hb-AA measurement should reflect the presence of elevated blood acetaldehyde levels from alcohol consumption integrated over time. Human adult haemoglobin contains three fractions: HbA (97% of total), HbA_2 (2.5%) and HbF (0.5%). Chromatographic analysis of HbA has revealed a number of minor haemoglobin fractions, HbA_{1a}, HbA_{1b} and HbA_{1c} [189]. Analysis of stable acetaldehyde–haemoglobin adducts has revealed that valine, lysine and tyrosine residues of globin are the sites of reaction. In contrast to the first studies of Stevens et al. [190], Hoberman and Chiodo [191] and Huisman et al. [192] who found elevated levels of haemoglobins eluting earlier than HbA_0 following cation-exchange chromatography in subjects consuming excessive ethanol, several groups have failed to distinguish alcoholics from controls [193–195]. Recently, it became clear that the amount of Hb modified by acetaldehyde from alcohol abuse is very small (approximately 0.2% of total Hb) and thus highly sensitive techniques should be used.

For further studies, Sillanaukee and Koivula [189] developed a cation-exchange liquid chromatographic method for the analysis of acetaldehyde binding with haemoglobin. After centrifugation of blood samples, red cells were washed with isotonic saline, incubated with sodium acetic buffer (pH 5.5) to remove unstable Schiff bases, and finally washed with isotonic saline before haemolizing with phosphate buffer (pH 6.3). A lithium chloride salt gradient was formed by mixing malonate buffers and diluted samples were eluted with a nonlinear gradient to separate the different Hb fractions, which were detected at 405 nm. When human haemolysates were incubated with micromolar concentrations of acetaldehyde without reducing agents, HbA_0 was found to form two new fractions. One of them was eluted with HbA_{1c}. A novel, previously undescribed fraction (HbA_{1ach}) was detected between HbF and HbA_{1c}. Sillanaukee et al. [196] compared this liquid chromatographic assay (separation of HbA_{1a}, HbA_{1b}, HbA_{1ach}, HbA_0, HbA_2, HbA_3, HbF) with an immunological procedure. With both methods, the concentrations of Hb-AAs were found to be significantly higher in red cells of heavy drinkers and subjects with alcoholism when compared with control subjects, and were proposed as a diagnostic marker for alcoholism and (as a ratio of HbA_{1ach}/HbA_{1c}) as an indicator of heavy drinking [197,198].

Hazelett et al. [199] reported an improved separation of HbA_{1ach} and other acetaldehyde-haemoglobin adducts using a polyaspartic acid column (PolyCAT A) and a nonlinear buffer gradient with pH changes from 6.6 to 6.8. HbA_{1ach} and several others, including two peaks in the HbA_{1a+b} cluster, Hb Pre-A_{1c}, and HbA_{1d3} were significantly increased by acetaldehyde incubation. Chen et al. [200] val-

idated a fluorometric HPLC analysis of acetaldehyde in haemoglobin fractions separated by polyaspartic acid cation-exchange chromatography and reported three new peaks after reaction of haemoglobin with acetaldehyde (HbA_{1ach-3}, HbA_{1c-1} and HbA_{0-1}). Spectra obtained by GC–MS and electrospray ionization (ESI) were used for the characterization of the fluorophore and each new peak was found consistent with the expected product from the reaction of acetaldehyde and cyclohexandione in the presence of ammonium ion. Gross et al. [201] analyzed Hb-AAs after HPLC separation by plasma desorption mass spectrometry (PDMS). The tryptic map of heavy drinkers HbA_{1C} indicated two peptides and mass measurements of these peptides were consistent with the existence of acetaldehyde modifications of the alpha and beta N-termini of haemoglobin. These possible acetaldehyde modifications of Hb N-terminal tryptic peptides include 2-methyl imidazolidin-4-one and *N*-ethyl derivatives. A highly sensitive sandwich ELISA technique with different antibodies for the measurement of haemoglobin-acetaldehyde adducts was also developed [202]. Recently, a HPLC-based method was described to distinguish 18 human haemoglobin fractions including a new acetaldehyde-induced fraction HbA_{1ach3} using a Poly CAT A cation-exchange column and a stepwise salt and pH gradient [203].

Further investigations should be necessary. Detailed characterization of acetaldehyde-modified proteins should be helpful in understanding alcohol-related tissue damage, immune responses in heavy drinkers and the identification of markers of alcohol consumption.

5. Urine/(blood)

5.1. 5-Hydroxytryptophol and 5-hydroxytryptophol-3-acetic acid

Serotonin is metabolized to 5-hydroxytryptophol-3-acetic acid (5-HIAA) and 5-hydroxytryptophol (5-HTOL), both of which are normal constituents of human urine. Normally, 5-HIAA is the major metabolite, but alcohol intake produces a shift in serotonin metabolism toward increased formation of 5-HTOL by inhibition of aldehyde dehydrogenase and/or by the increase in the NADH/NAD ratio [204,205]. A reference level of 20 for an abnormal value of the molar ratio (pmol/nmol) between 5-HTOL and 5-HIAA in urine has been proposed (Table 1) [206].

Determination of 5-HTOL in urine involving hydrolysis of conjugates by β-glucuronidase, solvent extraction and preparation of pentafluoropropionyl derivatives, followed by capillary column gas chromatographic–mass spectrometric analysis was described by Voltaire et al. [206]. 5-HIAA in urine can be determined simply by direct injection of urine onto a reversed-phase HPLC system with electrochemical detection [206,207]. Both 5-HTOL/5-HIAA and 5-HTOL/creatinine ratios appear to be clinically useful to reveal recent alcohol drinking. The 5-HTOL/5-HIAA should be preferred, because fluctuations in serotonin intake and/or excretion of creatinine influenced by muscle activity is compensated [208].

5.2. Dolichol

Dolichols are long-chain polyprenols containing an α-saturated isoprene unit. Their main known function is to act as glycosyl carrier lipids in the biosynthesis of *N*-linked glycoproteins. Elevated urinary dolichol levels have been demonstrated in chronic alcoholics (Table 1) [183,209–211]. It has been postulated, that ethanol could interfere with the catabolism of dolichols, since both are thought to be oxidized by alcohol dehydrogenase.

Quantitation of dolichol has usually been performed by HPLC (C_{18} silica-gel column), because of the chemical structure with a relatively high molecular weight of about 1200. Dolichols have a long chain of polyprenol comprising isopreines ($n=18$, 19 and 20 for dolichol-90, -95 and -100) and are monitored with a variable wavelength detector. However, various extraction and clean-up procedures for dolichols in biological material have also been used such as liquid–liquid extraction steps [211,212] or that combined with TLC [209] and various kinds of prepacked cartridge column extraction [183,210,213]. Normally, heneicosaprenol is used as internal standard and urinary dolichol is estimated as the sum of the different chain length isoprenologues calculated by using the internal standard [210,214,215]. In the same way, Roine et al. de-

termined blood dolichols after alcohol ingestion [216].

Kazunaga et al. [217,218] tested various types of extraction columns according to the method of Turpeinen [213] and found good recoveries using BondElut (C_{18}) for the analysis of urine samples. The pH of the sample was adjusted to 3.0, the columns were washed with HCl–sodium acetate buffer (pH 3.0), distilled water and methanol and dolichols were eluted with a mixture of 2-propanol–dichlormethane–methanol (5:3:2, v/v). Recently, Piretti et al. [219] developed a procedure for a complete determination of nonpolar isoprenoid lipids in tissues by reversed-phase HPLC of just two samples. The first, extracted from unaltered tissues and suitably processed by column chromatography, contained free cholesterol, cholesteryl esters, coenzymes Q, free dolichols and dolichyl esters. The second, obtained from saponified tissues, was used to detect both total cholesterol and total dolichols.

5.3. Condensation products

During the past decades, research in the aetiology of alcoholism has focused on the hypothesis that condensation products formed endogenously by reaction of indolalkylamines and catecholamines with aldehydes or pyruvate might be implicated in neurochemical mechanisms underlying addictive alcohol drinking. The formation of 1,2,3,4-tetrahydro-β-carbolines (THBCs) and 1,2,3,4-tetrahydroisoquinolines (TIQs) via the Pictet–Spengler reaction is extensively documented [220–232]. Especially with the identification of dopamine-derived TIQs, such as salsolinol (1-methyl-6,7-dihydroxy-TIQ, SAL) in human brain, urine and cerebrospinal fluid, several studies have been done to improve analytical techniques. Poor assay specificity and possible artifactual formation of these alkaloids during sample work-up and storage have been suggested to be responsible for controversial reports on the detection of these compounds in mammalian tissues and fluids after alcohol intake. The variability of reported levels of SAL might also be a result of variables, including dietary conditions during the experiments or the duration of ethanol ingestion and analytical problems associated with the detectability of the alkaloids. Recently, we found great inter-individual variations

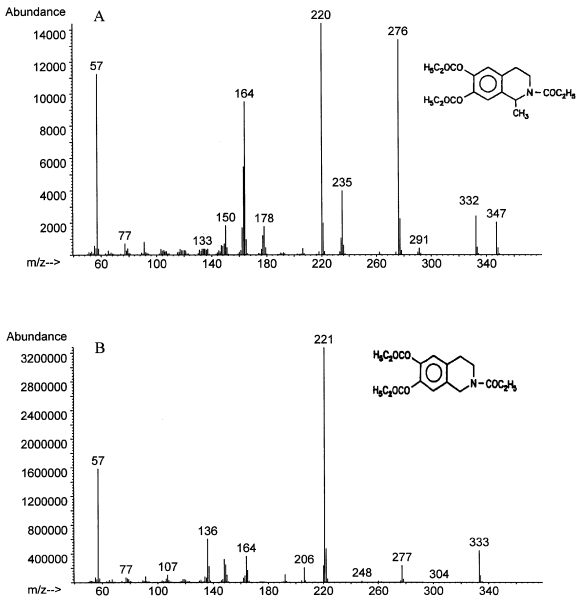

Fig. 2. Mass spectra of salsolinol (SAL) (A) and norsalsolinol (NorSAL) (B) derivatized with propionic anhydride. The concentration ratio of NorSAL and SAL, the so-called dopamine–aldehyde adduct ratio (DAAR) is significantly increased in alcoholics. It could serve as a marker for the processor state of the dopaminergic system ([294]).

in urinary SAL and NorSAL levels in chronic alcoholics and in a group of nonalcoholics (Fig. 2). Thus the levels of individual alkaloids are insufficient markers for distinguishing between alcoholics and nonalcoholics. However, by using the concentration ratio of norsalsolinol and salsolinol, the so-called dopamine–aldehyde adduct ratio (DAAR), significant differences were detected. This concentration ratio could serve as a marker for the processor state of the dopaminergic system [294].

The presence of TIQ and THBC compounds has been established in body fluids and tissues using (radioenzymatic) TLC methods, HPLC coupled with electrochemical or fluorescence detection, or GC procedures mostly combined with MS (Table 2). Especially dopamine-derived alkaloids, such as SAL and NorSAL have been proposed to play a role in alcohol addiction, and it is assumed that biotransformation of alcohol to its active metabolite (acetaldehyde/formaldehyde) induces alterations in the metabolism of dopamine and produces TIQs having addictive properties. Recently, it was considered that

Table 2
Methods for determination of tetrahydrosoquinolines and tetrahydro-β-carbolines

Method	Analyte	Material	Limit of detection	Reference
Radioenzymatic TLC assay	SAL and catecholamines	Plasma and tissues	0.1–0.5 ng/g	[233]
Radioenzymatic TLC assay	SAL and dopamine	Tissues and fluids	10–50 pg/g	[234]
TLC with radiolabeled marker	Tetrahydronorharman and other THBCs	Urine, platelets, and brain	10 ng/sample 5.8 pmol/sample	[235–237]
Radioenzymatic assay	SAL sulfate and dopamine sulfate	Plasma	5–10 pg/ml	[238]
HPLC with electrochemical detection (HPLC/ED)–cation-exchange chromatography	TIQ and others	Food and urine	100 pg/sample	[239]
HPLC/ED-extraction with aluminia; cation-exchange chromatography	SAL, NorSAL and tetrahydropapaveroline	Urine and brain	2 ng/g	[240]
HPLC/ED-extraction with Amberlite; cation-exchange chromatography; ion-pair chromatography	SAL	Brain	40 fmol/sample	[241]
HPLC/ED-extraction columns packed with cation-exchange resin; ion-pair chromatography	SAL and catecholamines	Urine	6 nmol/l	[242]
HPLC/ED-extraction columns packed with cation-exchange resin; ion-pair chromatography	SAL	Urine	1 ng/ml	[243]
HPLC/ED-purification by cation resin and aluminia; reversed-phase chromatography	SAL	Urine		[244]
HPLC/ED–solid-phase extraction over phenylboronic acid cartriges; derivatization with *N*-trifluoroacetyl-L-propylchloride or *S*-1-(1-naphthyl)ethyl isothiocyanate for enantiomeric separation	SAL	Urine and foods	2.25–2.5 ng/sample	[245,246]
HPLC/ED–solid-phase extraction over phenylboronic acid cartridges; cyclodextrin-modified silica gel HPLC column for enantiomeric separation without derivatization	SAL	Plasma	20 pg/ml	[247,248]
HPLC/ED-liquid–liquid extraction and micro centrifugation; cyclodextrin-modified silica gel HPLC column for enantiomeric separation without derivatization	SAL and N-Me-SAL	Brain	0.05–0.08 pmol/sample	[249,250]
HPLC with fluorescence detection–solid-phase extraction with ion-pairing reagent; Reversed-phase chromatography	Various THBCs	Plasma and platelets	pmol range	[251]
HPLC with fluorescence detection–preparative derivatization; reversed-phase chromatography	Various THBCs	Tissues, biological fluids	0.5–1 ng/sample	[252,253]
HPLC with fluorescence detection–ion-exchange chromatography with post-derivatization	SAL	Urine	2 pmol/ml	[254]
HPLC with fluorescence detection-liquid–liquid-extraction followed by fluorescence derivatization and reversed-phase chromatography	TIQ	Brain	1 pmol/g	[255]
HPLC with fluorescence detection–extraction using aluminia; reversed-phase chromatography	SAL	Urine	20 pmol/ml	[256]
HPLC with fluorescence detection–preparative derivatization; reversed-phase ion-pair chromatography	Tetrahydroharman and Tetrahydronorharman	Urine	10–20 pg/sample	[257,258]
HPLC with fluorescence detection–solid-phase extraction; reversed-phase ion-pair chromatography	Harman and norharman	Plasma	20 pg/sample	[259]

Table 2 (Continued)

Method	Analyte	Material	Limit of detection	Reference
HPLC with fluorescence detection or MS–solid-phase extraction with propylsulphonic acid extraction columns; reversed-phase chromatography; LC–MS with thermospray interface	Various β-carbolines	Foodstuffs		[260]
Gas chromatography with electrochemical detection (GC/ED)-aluminia extraction; fluoracylation procedure	Various TIQs and catecholamines	Tissues and body fluids	0.2–50 pg/sample	[261,262]
GC/ED-liquid–liquid reextraction; fluoracylation procedure	SAL	Brain	10 pg/sample	[263]
gas chromatography–mass spectrometry (GC/MS)-liquid–liquid extraction; TMS derivatives	TIQs	Urine		[264]
GC/MS-liquid–liquid extraction; HFB derivatives	Various THBCs	Blood, Platelets and plasma; brain	app. 1 pmol/sample	[265–267]
GC/MS–aluminia extraction with deuterated standards; PFP derivatives	SAL and others	Brain and Biological fluids	1 pmol/sample	[268,269]
GC/MS-liquid–liquid extraction with deuterated standard; PFP derivatives	Various THBCs	Biological fluids; foods	0.3 pmol/ml	[270–272]
GC/MS-extraction with Amberlite; propionyl derivatives	NorSAL	Brain	1 ng/g	[273]
GC/MS-liquid–liquid extraction with deuterated standards; trifluoroacetyl derivatives	SAL and dopamine	Brain	0.05 pmol/sample	[274]
GC/MS-liquid–liquid extraction with deuterated standards; trifluoroacetyl derivatives	SAL and others	Urine and brain		[275]
GC/MS-liquid–liquid extraction; HFB derivatives	TIQ and *N*-methyl-TIQ	Brain and foods	0.25 ng/sample	[276–279]
GC/MS with negative chemical ionization (GC/NICI/MS)-liquid–liquid extraction; pentatluorobenzyl derivatives	Various β-carbolines	Brain and foods	0.1–0.5 ng/sample	[253,280]
GC/MS-liquid–liquid extraction or extraction over phenylboronic acid cartriges; TMS derivatives; EI and CI mode	SAL and others	Brain	$<$1 ng/sample	[281–283]
GC/NICI/MS-liquid–liquid extraction; TFA derivatives	1-Me-THBC	Urine	10 fg/sample	[284]
GC/NICI/MS-solid-phase extraction over phenylboronic acid cartriges; PFP derivatives	SAL	Urine	0.55 pmol/ml	[285]
GC/MS-preparative derivatization (combined with solid-phase extraction)	Various THBCs and TIQs	Urine	100 pg/ml	[286,287]
GC/NICI/MS-liquid–liquid extraction; derivatization with (*R*)-(−)-2-phenylbuturyl chloride for enantiomeric composition	1-Me-THBC	Urine		[288]
GC/MS–solid-phase extraction over phenylboronic acid cartriges; two step derivatization to transform the SAL enantiomers into diastereomers	SAL and others	Plasma and urine	100 pg/ml	[289,290]
GC/MS-extraction and derivatization in one step by Schotten–Baumann two-phase reaction utilizing pentafluorbenzoylchloride	SAL	Urine	10 fmol/ml	[291,292]
GC/NICI/MS-liquid–liquid extraction; TFA derivatives	THBC and 1-Me-THBC	Brain	20 pg/sample	[293]
GC/MS-solid-phase extraction over phenylboronic acid cartridges; derivatization with propionic anhydride	SAL and NorSAL	Urine	100 pg/ml	[294]

the (*R*)-(+)- and (*S*)-(−)-enantiomers of SAL do not exert identical biological activities. Thus methods for determination of the enantiomeric composition of endogenous SAL were developed, which could be helpful in understanding their possible formation.

Further investigations are necessary. Condensation products could be an important topic, because they are involved in mechanism of the limbic system, which is responsible for all kinds of addiction processes.

6. Test combinations

The sensitivities of laboratory markers of alcoholism can be improved by using various combinations [5,295–300]. Even more sophisticated mathematical models have been suggested for differential diagnostic purposes [298,301]. However, no combination has been widely accepted for clinical use. A major problem is that if the different tests are combined to improve sensitivity, specificity falls dramatically and the results cannot be interpreted with confidence in an individual patient.

Additionally, screening questionnaires are used for the detection of alcoholism. Most commonly used are the Michigan Alcoholism Screening Test (MAST) [302], the Cage Questionnaire [303], the Munich Alcoholism Test (MALT) [304], the Brief MAST [305] and the Lesch Alcoholism Typology (LAT) [306].

7. Conclusions and perspectives

Several laboratory abnormalities are associated with excessive alcohol consumption. They are useful in the diagnosis of alcoholism especially in the follow-up of various treatment programs. Sensitivities and specificities of the laboratory markers vary considerably. In addition to alcohol and alcohol-induced tissue injury, they may be influenced by many nonalcohol related diseases, nutritional factors, metabolic disorders, etc.. No laboratory test exists that is reliable enough for the exact diagnosis of alcoholism. The combination of physician interview, questionnaire and laboratory markers is necessary for the diagnosis of alcoholism. In addition to possible diagnostic aims, in the research on biological markers trait markers of the disease should be taken into account to expand the knowledge about pathogenesis and aetiology of alcoholism. Knowledge about genetic markers of increased risk of alcoholism should give us leads to important pathophysiological bases of alcohol drinking behaviour and ultimately to better forms of prevention and therapy.

8. List of abbreviations

AA	Acetaldehyde adduct
AANB	α-Amino-*n*-butyric acid
ALAT	Alanine aminotransferase
ALDH	Aldehyde dehydrogenase
ASAT	Aspartate aminotransferase
CDT	Carbohydrate deficient transferrin
CE	Capillary electrophoresis
CETP	Cholesteryl ester transfer protein
DAAR	Dopamine aldehyde adduct ratio
DOPAC	3,4-Dihydroxyphenylacetic acid
DOPAL	3,4-Dihydroxyphenylacetaldehyde
ECD	Electrochemical detection
ELISA	Enzyme linked immuno assay
ESI	Electrospray ionization
FAEE	Fatty acid ethyl ester
FAME	Fatty acid methyl ester
GC	Gas chromatography
GGT	γ-Glutamyltransferase
Hb	Haemoglobin
HDL	High density lipoprotein
HEX	β-Hexosaminidase
5-HIAA	5-Hydroxytryptophol-3-acetic acid
HPLC	High performance liquid chromatography
5-HTOL	5-Hydroxytryptophol
IAA	Indole-3-acetic acid
IAL	Indole-3-acetaldehyde
IB	Immunoblotting
IEF	Isoelectric focusing
LD	Laser densiometry
LDL	Low density lipoprotein
MALT	Munich Alcoholism Test
MAST	Michigan Alcoholism Screening Test
MCV	Mean corpuscular volume
MS	Mass spectrometry
NCA	National Council on Alcoholism

PDMS	Plasma desorption mass spectrometry
SAL	Salsolinol
THBC	Tetrahydro-β-carboline
TIQ	Tetrahydroisoquinoline
TLC	Thin-layer chromatography
VLDL	Very low density lipoprotein

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