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Detection of Fluorescently Labeled Carbohydrate-Deficient Transferrin, a Marker of Alcohol Dependence, in Dried Blood Spots

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Abstract. A fluorescence-based (HPLC-FLD) method was developed for the detection of carbohydrate-deficient transferrin (CDT), a marker of alcohol dependence, in dried blood spots (DBS). Dried blood spots were cut and immersed in hydrochloric acid to leach out proteins, labeled with terbium ions, and then sent to high-performance liquid chromatography coupled with a fluorescence detector for detection. The levels of CDT in the dried blood spot samples of 10 clinically alcohol-dependent individuals were in general agreement with the results of the assayed levels of CDT in serum samples from the same source. The method can effectively amplify the detection signal of micro samples with high sensitivity and specificity, and the sampling method is simple and convenient to ensure the accuracy of the results, which is suitable for the qualitative and quantitative analysis of CDT in clinical micro samples.

Keywords: alcohol dependence marker; carbohydrate-deficient transferrin (CDT); HPLC; fluorescence; dried blood spots (DBS).

1. Introduction

Alcohol predisposes people to dependence, and chronic alcohol abuse can alter brain structure and function, leading to impaired cognitive function as well as other behavioral changes. [1] Early diagnosis of alcohol dependence is not only a major topic of concern for society as a whole, but also an urgent need to meet the demands of building a healthy, stable and safe society.

For short-term occasional drinking or excessive drinking behavior, it can be detected by some alcohol metabolism-related indexes or organ damage-related indexes [2], such as blood-blood (and/or breath) ethanol content [3], acetaldehyde dehydrogenase (ALDH) [4], ethyl glucuronide (EtG) [5], ethyl sulfate (EtS), liver function indexes (GGT, ALT and AST) [6], fatty acid ethyl ester (FAEE) [7], mean corpuscular volume (MCV) [8], and phosphatidylethanol (PEth).

Studies in the mid-1970s found11 that, after sustained heavy alcohol consumption, serum transferrin became abnormally glycosylated. [9] This elevated transferrin fraction after abnormal glycosylation was named carbohydrate-deficient transferrin (CDT) [10-13] and is used as a biomarker to identify persistent heavy drinking and to monitor abstinence during treatment.[15], [16]

Compared with the use of conventional human serum samples, the use of Dried blood spots (DBS) samples from blood cards for CDT screening has many advantages [14], [15] , including a simple and minimally invasive sampling procedure, easy storage of the samples, minimal transportation requirements, and reduced risk of infection with some of the most risky blood-borne infections (HIV, hepatitis, etc.) [16] .

It was found that the addition of the metal terbium ion with fluorescent properties to human serum samples displaces the originally transferrin-bound iron ions complexed with transferrin. [17-19] Such complexes show strong specific fluorescence (λexc = 298 nm and λem = 550 nm).[20] Due to the sensitivity of the HPLC co-fluorescence detection, it allows for a more amplified signal to be detected,

which is more sensitive for the results. Advances in the quality and availability of highly sensitive analytical instruments and methods have led to increased interest in the use of trace samples.

Herefore, in this study, CDT was selected as the research object to respond to the degree of alcohol dependence, and clinical serum and dried blood spot (DBS) samples were detected by high-performance liquid chromatography-fluorescence (HPLC-FLD) method [21] , in order to evaluate and validate the effective performance of this method for the detection of CDT, and to establish a qualitative and quantitative assay for micro samples which is more convenient and sensitive, and has a good accuracy.

2. Materials and Methods

2.1. Main instruments and reagents

HPLC (chromatograph: U-3000, DAD-3000 UV detector, FLD fluorescence detector, Thermo Fisher Scientific, USA), Sapex SAX anion-exchange columns (4.6 mm × 100 mm, 5 μm; proteomix SAX-NP 5U, Sepax technologies, USA).

Terbium (III) chloride anhydrous (Innochem, chromatographic purity), Dextran sulfate (Solarbio, chromatographic purity), Calcium chloride (Innochem, chromatographic purity), Concentrated hydrochloric acid (Macklin), Tris (hydroxymethyl) aminomethane hydrochloride Tris (Macklin, chromatographic purity), Sodium chloride (Macklin, chromatographic purity), Blood collection filter paper, 0.22 μm membrane.

2.2. Preparation of reserve solution

2.2.1. Liquid chromatography separations, column equilibrium and column cleaning with solutions

Mobile phase A: the concentration of Tris was 20 mmol/L, adjust the pH = 8.0.

Mobile phase B: the concentration of Tris was 20 mmol/L, the concentration of NaCl was 1 mol/L, and adjusted pH=8.0.

Mobile phase C: the concentration of NaCl was 1 mol/L.

After preparation, all mobile phases were filtered through 0.22 μm filter membrane and degassed by ultrasonic before use, and could be stably stored at 4℃ for 3 months.

2.2.2. Other solution preparation

Dextran sulfate solution: the concentration of dextran sulfate was1 mmol/L.

Calcium chloride solution: the concentration of calcium chloride was1 mmol/L.

The prepared dextran sulfate solution was mixed with calcium chloride solution in equal parts (v:v=1:1) to make degreasing solution.

Terbium chloride solution: the concentration of $TbCl_3$ was 0.5 mmol/L.

2.3. Sample collection and preservation

Venous blood was collected using red storage tubes without anticoagulant, centrifuged at 3000 r/min for 5 min. After centrifugation, the upper layer of serum was taken in a centrifuge tube and the serum was stored in a refrigerator at -20°C until processing.

Dried blood spot (DBS) samples were collected using blood collection filter paper, dried for 1-2h at room temperature to obtain dried blood spot (DBS), and stored in a sealed container at room temperature.

2.4. Sample pre-treatment

2.4.1. Pre-treatment of serum samples

Take 1 mL of serum sample, add 1 mL of degreasing solution, shake it and put it in the refrigerator at 4℃ for 30~60 min, take out the mixture and centrifuge it at 3000 r/min for 5 min to remove the lipoproteins in the serum, and then add Terbium Chloride solution, and incubate it at room temperature for 4h or put it in the refrigerator at 4℃ for overnight. The samples were diluted five times and ultrasonicated to remove air bubbles, then filtered through 0.22 μm microporous filter membrane and sent to HPLC-FLD for determination.

2.4.2. Pre-treatment of DBS

Dried blood spot (DBS) was cuted and put it into a 1.5mL centrifugal tube, add hydrochloric acid solution, and leave it to stand at room temperature for 4h or refrigerate at 4℃ overnight. The mixture was removed and centrifuged at 3000 r/min for 5 min. Terbium chloride solution was added and incubated at room temperature for 4 h or refrigerated at 4℃ overnight. The samples were filtered through 0.22 μm microporous filter membrane and sent to HPLC-FLD for determination.

2.5. HPLC-FLD detection conditions

Mobile phase A: 20 mmol/L Tris solution, mobile phase B: 20 mmol/L Tris solution (1 mol/L NaCl solution), mobile phase C: 1 mol/L NaCl solution; a weak anion exchange column (5 μm, 4 6 × 100 mm) was used. The gradient elution program is shown in Table 1. The injection volume was 20 μl; the flow rate was 1 mL/min;the column temperature set at 37°C. At the end of detection, the column was cleaned up with mobile phase C for 5 min, and re-equilibrated with mobile phase A before the next injection. The fluorescence detector section was set at an excitation wavelength of 298 nm, an emission wavelength of 550 nm, and a flow cell temperature of 45°C.

Table 1. Solvent gradient for HPLC separation of isotransferrins

Time/min	%A	%B	%C
0[a]	100	0	0
25	70	30	100
25	0	0	100
30	100	0	0

[a] Equilibrate the column for 10 minutes before each sample run so that the mobile phase in the column is 100% A at time=0.

2.6. HPLC-FLD methodology validation

2.6.1. HPLC-FLD assay for DBS samples

One blood cardboard sample from each of the drinkers was taken and processed according to the method in section 1.5.2. Then the samples were injected and detected according to the conditions in section 1.6.1 to examine the possible interfering peaks and carry out the examination of method specificity, which requires that the treated samples are free of impurity interference and have obvious peaks of the substance to be measured and better separation.

2.6.2. Comparison between DBS and serum samples

The processed 10 cases of alcohol dependent persons with serum samples from the same source were subjected to HPLC-FLD, and the fluorescence assay was plotted with the CDT content of the serum samples as the horizontal coordinate and the CDT content of the DBS samples as the vertical coordinate, and the curves were plotted and statistically analyzed.

3. Results and Discussion Literature References

3.1. HPLC-FLD methodology validation

3.1.1. HPLC-FLD assay for DBS samples

In the experimental conditions of HPLC-FLD method, impurities in the processed DBS did not interfere with the sample peaks, the baseline was stable, transferrin had distinct peaks and good separation (1, 2, 3, 4, 5 and 6 correspond to single, two, three, four, five and six salivary acid transferrin), and the retention time of di-sialic acid transferrin was 12.49 min. Fig. 1 shows the HPLC-FLD of DBS from non-drinkers.

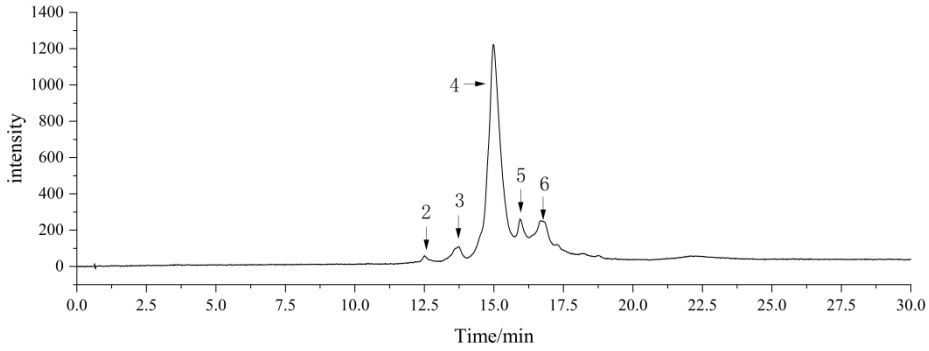

Fig. 1 HPLC-FLD of DBS from non-drinkers

3.1.2. Comparison between DBS and serum samples

There was a good linear relationship between the assay results, and the linear regression equation was $y = 0.938x + 0.1746$ ($R^2 = 0.9399$, $P>0.5$), and the difference between the two groups of data was not statistically significant, so it can be concluded that the results of the assay of the DBS samples and the content of CDT in the serum by using HPLC-FLD were consistent with each other.

4. Conclusion

The results of this paper show that the method is simple, fast and easy to collect samples, as well as having high detection specificity. Therefore, the use of dried blood spot (DBS) combined with fluorescence detection of CDT can realize a simpler and more sensitive detection of the degree of alcohol dependence, provide a scientific basis for its changing level, and play an active role in clinical detection, with a view to providing a simpler and quicker reference method for the assessment of the changes in the level of CDT in the pre-treatment and follow-up process of patients with alcohol dependence in the clinic.

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