Application Note

220052HRE

Simultaneous Determination of Bile Acids Utilizing Immobilized Enzyme Column

Introduction

Bile acids have a hydroxyl group in common in 3α position of their steroidal backbone and 3α-HSD (3α-Hydroxysteroid Dehydrogenase) is known as an enzyme that allows the hydroxyl group to be selectively oxidized in the presence of coenzyme, NAD (Nicotinamide Adenine Dinucleotide). In this enzyme reaction, when one molecule of bile acid is oxidized to 3-ketosteroid, one NADH molecule, the reduced form of NAD, is generated and it has the intense fluorescence (Ex=340 nm, Em=470 nm). Here, the standard mixture of bile acids was measured by using post-column derivatization for detecting NADH generated when mixing continuously the reaction solution containing NAD with effluent from column for the separation and letting this mixed solution go through 3α-HSD enzyme-immobilized column.

Keyword: Bile acids, NAD, NADH, Enzymepak 3α-HSD, Bilepak-II, Fluorescence detector

Experiment

[Equipment]

Eluent Pump:	PU-2080
Degasser:	DG-2080-54
Gradient unit:	LG-2080-04
Reagent Pump:	PU-2080
Column oven:	CO-2060
Autosampler:	AS-2057
Detector:	FP-2020

[Conditions]

Column:	Bilepak-II (4.6 mmI.D. x 125 mmL, 5 μm)
Enzyme column:	Enzymepak 3α-HSD (4.0 mmI.D. x 20 mmL)
Eluent A:	30 mM Ammonium acetate buffer (pH 6.8)/Acetonitrile/Methanol (60/20/20)
Eluent B:	30 mM Ammonium acetate buffer (pH 6.8)/Acetonitrile/Methanol (40/30/30)
Gradient condition:	(A/B), 0 min (100/0)→32 min (0/100)→60 min (0/100) →60.1 min (100/0) 1 cycle; 80 min
Flow rate:	1.0 mL/min
Reagent:	0.3 mM NAD, 1 mM EDTA-2Na, 0.05% 2-mercaptoethanol, 10 mM potassium dihydrogenphosphate, pH 7.8 (adjusted with potassium hydroxide)
Reagent flow rate:	1.0 mL/min
Column temp.:	25 ºC
Wavelength:	Ex. 345 nm, Em. 470 nm, Gain 100x
Injection volume:	10 μL
Standard sample:	15 Bile acids (50 μmol/mL each)

Fig. 1 shows a mechanism of enzyme reaction such as oxidation of bile acids and reduction of NAD and Fig. 2 shows a flow diagram for analysis system for bile acids.

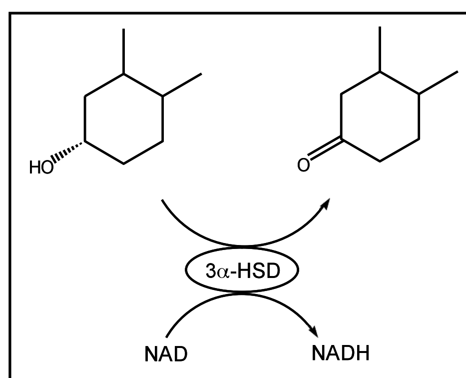
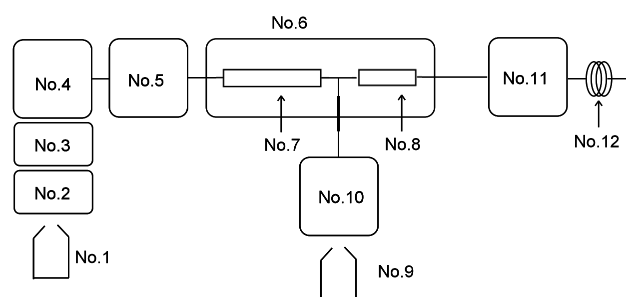


Fig. 1. Mechanism of Enzyme Reaction



No.1:	Eluent	No.7:	Column (Bilepak II)
No.2:	Degasser (DG-2080-54)	No.8:	Enzyme column (Enzymepak 3α-HSD)
No.3:	Low Pressure gradient unit (LG-2080-04)	No.9:	Reagent
No.4:	Pump for eluent (PU-2080)	No.10:	Pump for reagent (PU-2085)
No.5:	Autosampler (AS-2057)	No.11:	Fluorescent detector (FP-2020)
No.6:	Column oven (CO-2060)	No.12:	Backpressure coil

Fig. 2. Flow Diagram

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Results

Fig. 3 shows chromatogram of standard mixture of 15 components of bile acids and a internal standard (I.S.), which was successfully separated into 16 components within 50 minutes.

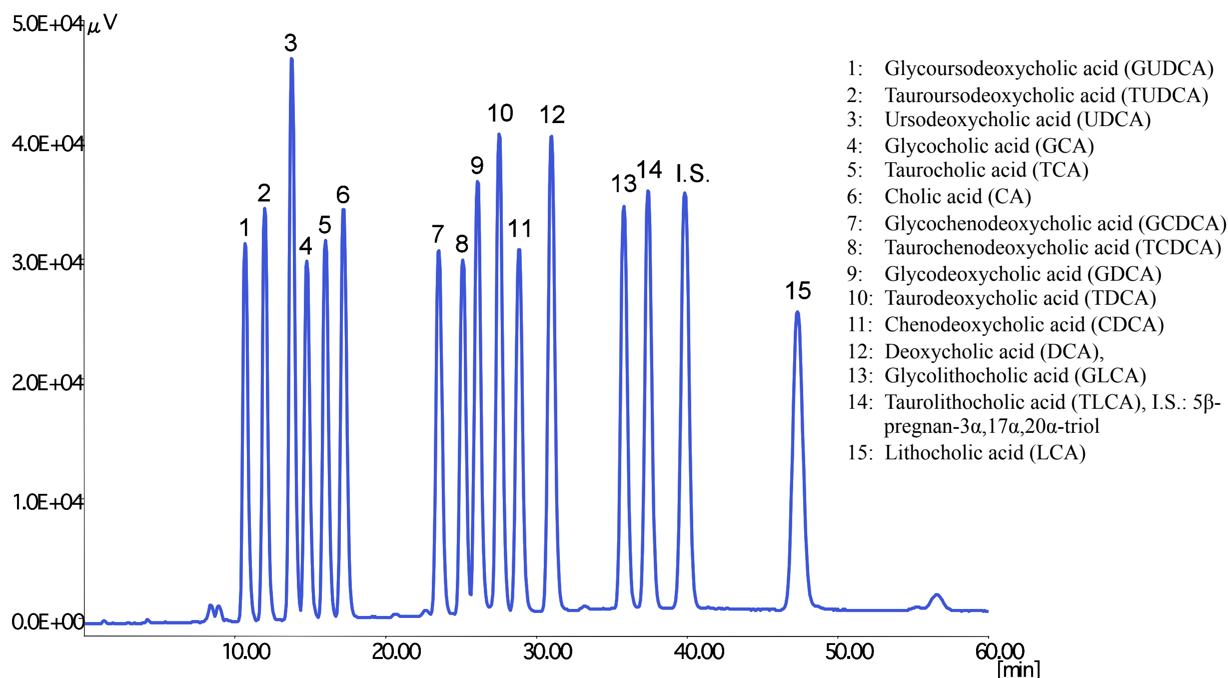

Fig. 3. Chromatogram of Standard Sample of Mixed Bile Acids

Table 1 shows the reproducibility of retention time and peak area of each result when measurement of standard mixture containing 0.5 nmol bile acids (Injection volume: 10 μL) was implemented ten times repeatedly. The relative standard deviation (%RSD) of retention time and peak area of each component obtained was as excellent as 0.2 ~ 0.34 and 0.8% ~ 2.13% respectively.

Table 1. Reproducibility (n=10)

Bile acid	%RSD	
	Retention time	Peak area
GUDCA	0.34	1.43
TUDCA	0.33	1.25
UDCA	0.28	1.47
GCA	0.33	1.36
TCA	0.33	1.16
CA	0.26	1.5
GCDCA	0.24	2.04
TCDCA	0.23	2.13
GDCA	0.24	1.8
TDCA	0.22	1.17
CDCA	0.2	2.11
DCA	0.2	1.16
GLCA	0.22	0.8
TLCA	0.23	0.89
LCA	0.29	1.38

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