Application Note

Simultaneous Determination of Bile Acids Utilizing an Immobilized Enzyme Column

Introduction

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

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Keywords

Bile acids, NAD, NADH, Enzymepak 3a-HSD, Bilepak-II, Fluorescence detector

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Experimental

Equipment

Pump:	PU-2080
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 °C
Wavelength:	Ex. 345 nm, Em. 470 nm, Gain 100x
Injection Volume:	10 µL
Standard Sample:	15 Bile acids (50 µmol/mL each)

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Fig. 1 shows the enzyme reaction for the oxidation of bile acids and reduction of NAD.

Fig. 2 shows a flow diagram for the system used to analyze the bile acids.

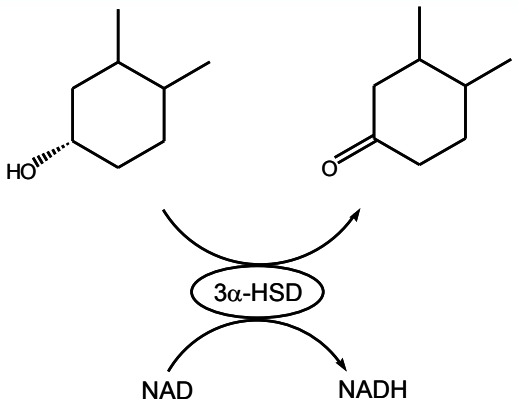


Fig. 1. Mechanism of Enzyme Reaction

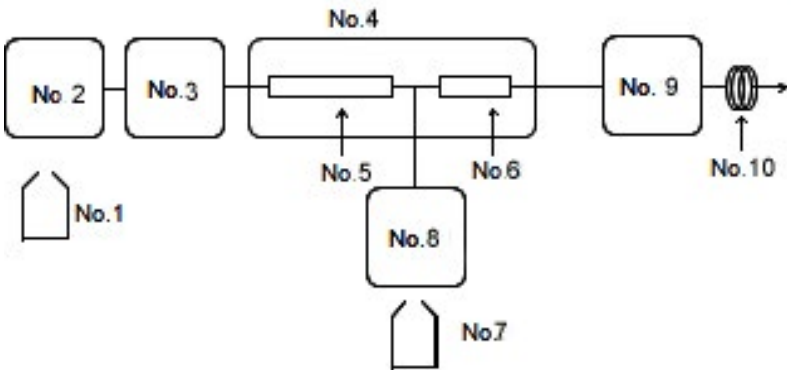
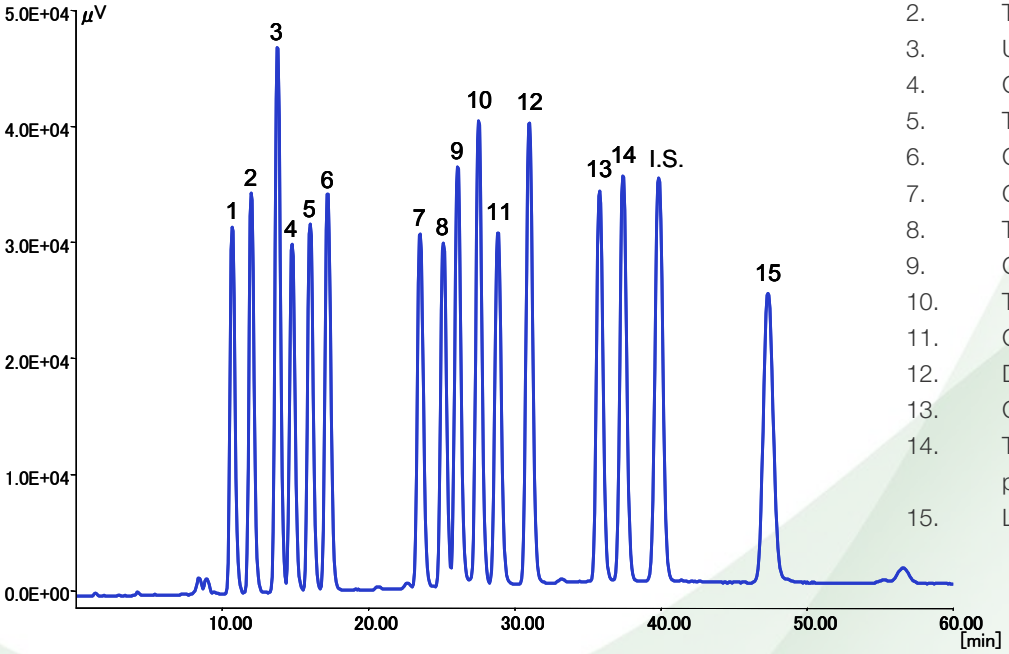


Fig. 2. Flow Diagram

No.1: Eluent
No.2: PU-2089
No.3: Cooled Autosampler (AS-2057)
No.4: Column oven (CO-2060)
No.5: Column (Bilepak II)
No.6: Enzyme column (Enzymepak 3a-HSD)
No.7: Reagent
No.8: Reagent pump (PU-2080)
No.9: Fluorescence Detector (FP-2020)
No.10: Backpressure coil

Results

Fig. 3 shows the chromatogram of a standard mixture of 15 bile acids and an internal standard (I.S.), which were well separated in under 50 minutes.



1. Glycoursodeoxycholic acid (GUDCA)
2. Tauroursodeoxycholic acid (TUDCA)
3. Ursodeoxycholic acid (UDCA)
4. Glycocholic acid (GCA)
5. Taurocholic acid (TCA)
6. Cholic acid (CA)
7. Glycochenodeoxycholic acid (GCDCA)
8. Taurochenodeoxycholic acid (TCDCA)
9. Glycodeoxycholic acid (GDCA)
10. Taurodeoxycholic acid (TDCA)
11. Chenodeoxycholic acid (CDCA)
12. Deoxycholic acid (DCA),
13. Glycolithocholic acid (GLCA)
14. Taurolithocholic acid (TLCA), I.S.: 5 β pregnan-3α,17α,20α-triol
15. Lithocholic acid (LCA)

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

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Table 1. Shows the retention time and peak area reproducibility of each bile acid when a 0.5 nmol standard mixture of bile acids (injection volume: 10 µL) (n=10). The %RSD of retention time and peak area for each component obtained was 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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