Flow-Injection Determination of a Primary Bile Acid (Cholyl Glycine) Using Co-immobilized Bacterial Luciferase and Oxidoreductase.

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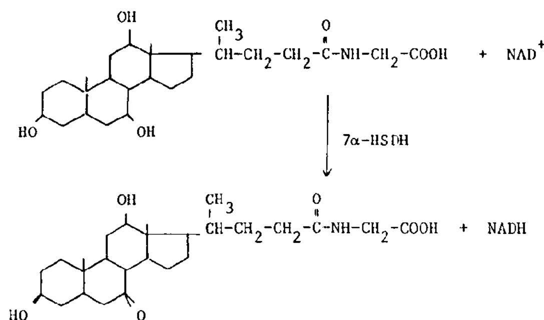
Summary: A flow-injection procedure for the indirect determination of a primary bile acid (cholyl glycine) using immobilized 7α-hydroxysteroid dehydrogenase and co-immobilized bacterial luciferase and oxidoreductase is described. The linear range is $1x10^{-6}$-$1x10^{-3}$ M cholyl glycine. The reproducibility is less than 3.4% (n = 5) and the sample throughput is 60 h^{-1}.

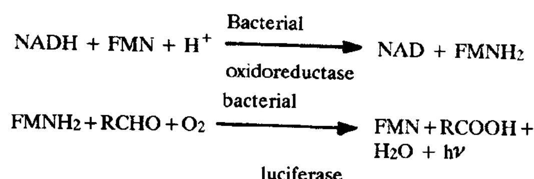
Introduction

The predominant bile acids in humans are the glycine and taurine conjugates of chenodeoxycholic acid, deoxycholic acid and cholic acid[1]. Elevated bile acid levels in serum are related to certain liver and intestinal diseases[2], and the determination of bile acids is therefore of importance in clinical chemistry. This can be done by oxidation of the 7α-hydroxyl group (which is common to all bile acids) with 7α-hydroxysteroid dehydrogenase (7α-HSDH), as shown below for cholyl glycine:



The NADH produced can be determined spectrophotometrically[3] or fluorimetrically[4] and is directly related to cholyl glycine concentration under appropriate reaction conditions.

If a more sensitive assay is required, the NADH produced in the above reaction can be coupled with the bioluminescent bacterial luciferase/oxidoreductase reaction as shown below:



where FMN and $FMNH_2$ are the oxidized and reduced forms of the co-factor flavin mononucleotide. The intensity of the blue light emitted can then be related to cholyl glycine concentration[5].

To reduce reagent costs the bacterial oxidoreductase and luciferase enzymes can be immobilized on a solid support. Using immobilized enzymes Roda et al.[5] reported a detection limit of 0.5 pmol with a photomultiplier detector and Green and Kricka[6] reported a limit of 50 pmol with photographic film. An automated method based on air-segmented continuous flow analysis has also been reported[7].

Nabi and Worsfold have shown that immobilized bioluminescent enzymes can be incorporated within unsegmented flow-injection manifolds to provide rapid, reproducible and sensitive analytical procedures for a range of enzymes and metabolites[8-10]. This paper describes a flow-injection procedure incorporating immobilized 7α-HSDH and co-immobilized bacterial luciferase and oxidoreductase for the indirect determination of a primary bile acid (cholyl glycine).

Materials and Method

All reagents were obtained from Sigma. Stock solutions of cholyl glycine (0.1 M), NAD (0.01 M), FMN (0.001 M) and dithiothreitol (10^{-4} M) were prepared in phosphate buffer (0.05 M) at pH 7.0. An emulsified decanal solution (0.01% v/v) containing Triton X-100 (0.001% v/v) was also prepared in phosphate buffer.

Bacterial luciferase and oxidoreductase were extracted and isolated from *vibrio harveyi* and co-immobilized on cyanogen bromide activated Sepharose 4B as described previously[10]. 7α-HSDH was separately immobilized using the same procedure. A 1 mg amount of 7α-HSDH (0.64 U mg^{-1}) was dissolved in 4 ml of sodium hydrogen carbonate solution (0.1 M) at pH 8.0. Cyanogen bromide activated Sepharose 4B beads (0.5 g) were added and the mixture was stirred gently for 16 h at 4°C. the immobilized 7α-HSDH was washed with buffer and stored at 4°C.

The merging-zones flow-injection manifold used for the determination of cholyl glycine is shown in Fig. 1. Aqueous cholyl glycine standards 30 μl) covering the range $1x10^{-6}$ - $1x10^{-2}$ M and $1x10^{-6}$ M FMN (30 μl) were simultaneously injected into separate carrier streams using a dual-injection rotary value; cholyl glycine into a 0.05 M phosphate buffer stream at pH 7.5 containing NAD (5 x 10^{-4} M) and dithiothreitol (1 x 10^{-4} M) and FMN into a 0.05 M phosphate buffer stream at pH 7.5 containing emulsified decanal solution (0.001% v/v).

The carrier streams were pumped at 0.4 ml min^{-1} using a peristaltic pump (Ismatec Mini S840) and PTFE tubing (0.5 mm i.d.) was used throughout the remainder of the manifold. The two zones were merged 12.5 cm downstream and then travelled 2.2 cm before passing into a glass coil (60 x 2.5 mm) containing immobilized 7 HSDH (0.1 g beads) and co-immobilized bacterial luciferase and oxidoreductase (0.1 g beads). The detector was an end-window photomultiplier tube (Thorn EMI 9789QB) located in a light-tight housing[11].

Results and Discussion

Optimization of the flow-injection manifold

The flow rate was shown to be inversely related to sensitivity over the range 0.4 - 1.5 ml min^{-1} per channel due to lower sample dispersion at low flow rates and a relatively slow turnover rate for luciferase[12]. A flow rate of 0.4 ml min^{-1} was therefore used for all subsequent experiments. The concentration of FMN ($1x10^{-6}$ M), NAD ($5x10^{-4}$ M) and decanal (0.001% v/v) used were the rate limiting concentrations for cholyl glycine over the concentration range studied.

Calibration data for cholyl glycine

Fig. 2. shows a log-log calibration graph of cholyl glycine concentration ($1x10^{-6}$ - $1x10^{-2}$ M) versus light intensity. The response is linear over the range $1x10^{-6}$-$1x10^{-3}$ M. The sensitivity is shown in Table 1 and relative standard deviations were all less than 3.4% (n=5). The sample throughput was 60 h^{-1}. The limit of detection is determined by the magnitude of the blank signal which arises from the presence of contaminating dehydrogenases in the bacterial extract[13].

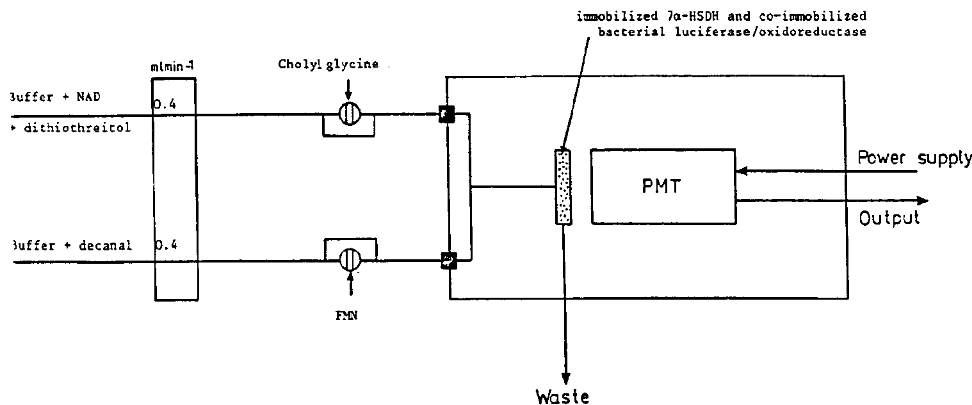


Fig. 1: Merging-zones flow-injection manifold for the determination of cholyl glycine using co-immobilized bacterial luciferase and oxidoreductase and immobilized 7α-HSDH.

TABLE-I - Calibration data for cholyl glycine

with immobilized 7α-HSDH			with soluble 7α-HSDH		
Conc. (M)	Output (V)	r.s.d. (n=5) (%)	Conc. (M)	Output (V)	r.s.d. (n=5) (%)
0	0.100	0.0	0	0.070	0.0
$1x10^{-6}$	0.120	0.0	$1x10^{-5}$	0.100	0.0
$1x10^{-5}$	0.220	0.0	$1x10^{-4}$	0.295	3.9
$1x10^{-4}$	0.600	2.3	$1x10^{-3}$	0.546	1.3
$1x10^{-3}$	0.900	3.1	$1x10^{-2}$	0.640	0.0
$1x10^{-2}$	0.990	3.4	$1x10^{-1}$	0.674.	1.4

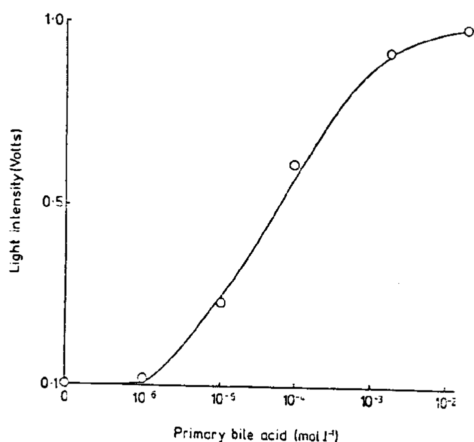


Fig. 2. Calibration graph of cholyl glycine concentration versus light intensity.

The sensitivity of the procedure using immobilized 7α-HSDH was compared with the sensitivity obtained using free 7α-HSDH by including the enzyme (1.3 U 1^{-1}) in the NAD carrier stream. The results given in Table 1 show that the sensitivity and reagent cost of the immobilized system are much better than those of the free system under the conditions used.

Conclusions

The results show that bacterial luciferase/oxidoreductase co-immobilized on Sepharose 4B within a flow-injection manifold can be coupled with an NADH producing reaction (using 7α-HSDH) for the determination of a primary bile acid, cholyl glycine.

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