# Determination of Kinetic Parameters for 3α-Hydroxysteroid Dehydrogenase Using the Five Major Bile Acids and their Conjugates as Substrates and Correlation with their Structure and Solubility*

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Kinetic parameters for 3α-hydroxysteroid dehydrogenase (3α-HSD) using the five major free bile acids and their glycine and taurine conjugates as substrates and β-nicotinamide adenine dinucleotide (β-NAD+) as coenzyme were determined from initial rate measurements. Four different mathematical methods were used for the evaluation of the results: (a) the double reciprocal plot, (b) the direct linear plot, (c) the Woolf plot and (d) the Scatchard plot methods. The determined kinetic parameters were correlated with the structure of the substrates (number of free hydroxy groups) and their absolute aqueous solubilities.

**Keywords**: *Fluorimetric method; kinetics; enzymes; bile acids; 3α-hydroxysteroid dehydrogenase*

In recent years there has been increasing interest in the bile acids (BA) field.[1] The determination of the three main bile acids [cholic acid (CA), chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA)] in biological fluids has medical significance for the diagnosis of hepatobiliary diseases.[2] CDCA and ursodeoxycholic acid (UDCA) are used clinically for gallstone dissolution as an alternative to surgery.[3] Lithocholic acid (LCA) is the main bile salt constituent of gallstones and studies concerning its properties and determination have increased recently because of its presence in urinary precipitates and its hepatoxicity.[4] Hence there is increasing interest in studying the physicochemical properties and in developing analytical methodology for BA.[1,5]

The kinetics of BA with 3α-hydroxysteroid dehydrogenase (3α-HSD) have been partially studied.[6,7] Similar kinetic studies with 7α-HSD have led to the simultaneous determination of primary BA based on the differences in their kinetic parameters.[8]

This paper describes a more systematic study on the kinetics of the enzyme-catalysed oxidation of the five major BA and their glycine (GCA, GCDCA, GUDCA, GDCA, GLCA) and taurine (TCA, TCDCA, TUDCA, TDCA, TLCA) conjugates. We have determined fluorimetrically the kinetic parameters for 3α-HSD using BA as substrates and β-NAD+ as coenzyme. The determined kinetic parameters were calculated using four different mathematical methods: the double reciprocal plot,[9] the direct linear plot,[10] the Woolf plot and the Scatchard plot methods.[11,12] The results show that the number and positions of free hydroxy groups play a significant role in the affinity of the substrate for the enzyme and that there is a good correlation between their Michaelis - Menten constants, $K_m$, and the absolute aqueous solubilities of the BA. The relationship between the physicochemical properties of BA and their biological properties may explain many of the effects of BA.[5]

The reported kinetic differences may be used for the development of differential kinetic methods for the determination of bile acids in aqueous mixtures and real samples.

## Experimental

### Apparatus

All measurements were performed using a Perkin-Elmer M 512 fluorescence spectrophotometer with a 1.000-cm path length with continuous stirring and at constant temperature (25.0 ± 0.1 °C). The NADH fluorescence was monitored using an emission wavelength of 455 nm and an excitation wavelength of 340 nm. The fluorescence signals were recorded and the initial slope, $\Delta F/\Delta t$, was taken as a relative measure of the reaction rate.[8] The instrument was calibrated against NADH standard solutions under conditions similar to those of the reaction and the initial reaction rate was calculated as $\Delta[NADH]/\Delta t$ (l mol$^{-1}$ s$^{-1}$).

### Reagents

All solutions were prepared in doubly distilled, de-ionised water from analytical-reagent grade materials.

*3α-HSD*. 3α-HSD (E.C. 1.1.1.50). Obtained from Millipore as a powder from *Pseudomonas testosteroni* with an activity of about 0.6 U mg$^{-1}$. A stock solution of 0.6 U ml$^{-1}$ was prepared in 0.020 M Tris - 12.0 mM EDTA buffer (pH 7.2). This enzyme was stored at −10 °C and was stable for 1 week. Working enzyme solutions (0.03 U ml$^{-1}$) were prepared fresh daily by appropriate dilution of the stock solution with the Tris buffer and kept in an ice-bath when in use.

*β-NAD+*. A 55 mM solution was prepared by dissolving 0.0796 g of β-NAD+ (Sigma) (*ca.* 98.7% pure enzymatically) in 2.00 ml of water.

*BA solutions*. BA and their glycine and taurine conjugates were obtained from Calbiochem. Stock solutions of BA (1 mM) were prepared by dissolving appropriate amounts of their sodium salts in water. The stock solutions of LCA and its conjugates (50 μM) were prepared in buffers of pH 9.5, because of the insolubility of this bile acid in water.[13,14] The stock solutions were standardised enzymatically using standard NADH solution in the same buffer (Calbiochem, A grade, 101.78% pure enzymatically, disodium salt tetrahydrate). Working BA solutions were prepared by appropriate dilution of the stock solutions in water and at concentrations depending on the $K_m$ of the BA. The working concentration must be in the range 0.3–2 $K_m$.[9]

*Buffer solutions*. Pyrophosphate, glycine and ethanolamine buffer solutions, each 0.1 M and pH 9.5, containing 5 mM EDTA were used for the determination of kinetic parameters.

### Procedure

A 2.00-ml volume of buffer solution, 0.100 ml of enzyme solution and 0.100 ml of β-NAD+ solution were injected into the cell. The stirrer was started and, after the fluorescence signal of the mixture had stabilised, the reaction was initiated by injecting 0.100 ml of BA solution. The signal was recorded for about 2 min and the initial reaction rate was calculated from the slope of the initial linear part of the reaction curve ($\Delta F/\Delta t$). Absolute reaction rates, $\Delta[NADH]/\Delta t$ (l mol$^{-1}$ s$^{-1}$), were calculated using NADH standards.

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## Results and Discussion

General considerations concerning optimum pH, temperature, effect of β-NAD$^+$ concentration on the reaction rate, NADH monitoring and further details of the reaction mechanism were similar to those reported previously.[6,7,15,16] Further, the reaction of bile acids with the 3α-HSD was also studied using the commercially available NAD analogue thionicotinamide-DPN, 3-acetylpyridinedeamino-DPN and 3-acetylpyridine adenine dinucleotide (APAD) (Sigma), but none of them reacted as 3α-HSD coenzyme.

### Determination of $K_m$ Values for 3α-HSD

The $K_m$ values of LCA, TLCA and GLCA were determined in the three buffers (pyrophosphate, glycine and ethanolamine) each 0.100 M and pH 9.5. Table 1 shows that the nature of the buffer does not have a significant effect on the initial reaction rate and on the kinetic parameters of GLCA. However, pyrophosphate buffer was chosen for all subsequent studies because of its low background fluorescence. An increase in the concentration of the buffer, and hence an increase in the ionic strength, causes a decrease in $K_m$.[7]

Four different mathematical methods were used to determine the kinetic parameters: (a) the double reciprocal plot,[9] (b) the direct linear plot,[10] (c) the Woolf plot and (d) the Scatchard plot[11] methods. The double reciprocal plot is severely affected by experimental errors, whereas the procedure for obtaining estimates of $K_m$ and $V_{max.}$ by direct linear plots is based on distribution-free statistics and is much less dependent on assumptions than the least-squares approach to data fitting.[17] The Woolf plot performs well on both well behaved data and scattered data; it is preferable to the double reciprocal plot[12] and is clearly superior to the Scatchard plot for well behaved data.[11]

Table 2 shows the $K_m$ values of the fifteen bile acids calculated using these four methods. The calculations were carried out using suitable computer programs written in Fortran.

In Table 3, $K_m$ values obtained in this work are compared with those cited in the literature.[6,7,16] Even though the conditions (temperature, buffer, ionic strength and measuring techniques) were very different, many of our $K_m$ values are of the same order of magnitude as those published previously. The differences that do occur may be due to the different experimental conditions used. $K_m$ values for LCA and UDCA and their conjugates are given here for the first time.

### Kinetic Parameters of 3α-HSD in Relation to Structure and Solubility of BA

Fig. 1 shows the stereo-formula of DCA[18] and the structure of the five bile acids. The hydrophobic and hydrophilic character of each bile acid molecule and its aqueous solubility depend mainly on the number and position of free hydroxy groups. An increase in the number of hydroxy groups results in an increase in hydrophilic character.[19]

According to our data the number and position of hydroxy groups in the bile acid molecule influence the kinetic parameters of 3α-HSD. Similar observations concerning both 3α-HSD and 7α-HSD have been reported previously.[6,20,21] This is the first systematic work to attempt to correlate the structure of the substrate with the kinetic parameters of 3α-HSD. The $K_m$ values increase as the number of hydroxy groups increases and a linear increase from LCA through CDCA to CA is observed ($y = 22.6x - 25.8$; $r = 0.91$, $n = 8$). LCA, which is a monohydroxy bile acid and the most hydrophobic and least soluble of all, shows the greatest affinity as substrate to the enzyme. When the $K_m$ values of the dihydroxy bile acids are compared, it is apparent that DCA, in which the second hydroxy group (12a-) is far from the 3α-position, has the lowest $K_m$ value and the greatest affinity

**Table 1.** Effect of buffer solutions (0.100 M) on the kinetic parameters from initial rate measurements of GLCA at pH 9.50. [β-NAD$^+$] = 2.39 mM; 25 °C

| Buffer | $K_m$/μM | $V_{max.}$/l$^{-1}$ nmol s$^{-1}$ | $K_{obs}$/s$^{-1}$ | Background fluorescence, relative units |
|---|---|---|---|---|
| Pyrophosphate .. | 1.28 | 8.36 | 0.653 | 2.0 |
| Glycine .. .. | 1.75 | 8.54 | 0.488 | 17.0 |
| Ethanolamine .. | 1.22 | 8.99 | 0.737 | 10.0 |

**Table 2.** $K_m$ values (μM) for the 3α-HSD using the five major bile acids and their glycine and taurine conjugates as substrates in 0.1 M pyrophosphate buffer, pH 9.50, 25 °C

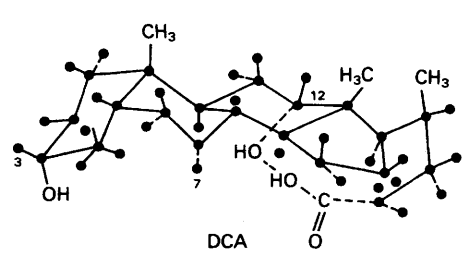
| | Michaelis - Menten constant, $K_m$/μM | | | | |
|---|---|---|---|---|---|
| Bile acids | LCA | DCA | UDCA | CDCA | CA |
| Free .. .. .. | 0.55* | 1.92 | 4.45 | 7.7 | 40.5 |
| | 0.39† | 1.79 | 4.53 | 9.5 | 56.3 |
| | 0.40‡ | 1.82 | 4.79 | 16.9 | 54.2 |
| | 0.64§ | 1.85 | 4.90 | 12.7 | 54.9 |
| Glycine conjugates | 1.28 | 1.37 | 10.2 | 23.9 | 153 |
| | 0.97 | 1.81 | 10.9 | 19.2 | 154 |
| | 0.87 | 1.75 | 10.1 | 19.2 | 171 |
| | 1.29 | 1.64 | 10.3 | 20.4 | 180 |
| Taurine conjugates | 0.60 | 2.53 | 4.76 | 6.4 | 38.2 |
| | 0.58 | 2.25 | 4.60 | 6.5 | 39.3 |
| | 0.59 | 1.73 | 3.46 | 8.0 | 36.9 |
| | 1.04 | 2.24 | 4.03 | 7.9 | 38.9 |

* Double reciprocal plot method.
† Direct linear plot method.
‡ Woolf plot.
§ Scatchard plot.

**Table 3.** Comparison of $K_m$ values (μM) for 3α-HSD using bile acids as substrates with published values

| Bile acid | This work* | Ref. 6* | Ref. 7* | Ref. 16* |
|---|---|---|---|---|
| LCA .. | 0.55 | | ≪5 | |
| GLCA .. | 1.28 | | | |
| TLCA .. | 0.60 | | | |
| DCA .. | 1.92 | | 4.0 | 4.2 |
| GDCA .. | 1.37 | 14 | | |
| TDCA .. | 2.53 | 36 | | |
| UDCA .. | 4.45 | | | |
| GUDCA .. | 10.2 | | | |
| TUDCA .. | 4.76 | | | |
| CDCA .. | 7.7 | | 22 | |
| GCDCA .. | 23.9 | 30 | | |
| TCDCA .. | 6.4 | 36 | | |
| CA .. .. | 40.5 | | 208 | |
| GCA .. | 153 | 89 | 251 | |
| TCA .. | 38.2 | 205 | 63 | |

* Determined by the double reciprocal plot method.




| Bile acid | Position and orientation of OH groups |
|---|---|
| LCA | 3α |
| DCA | 3α,12α |
| UDCA | 3α,7β |
| CDCA | 3α,7α |
| CA | 3α,7α,12α |

**Fig. 1.** Stereo-formula of DCA and position of hydroxy groups of the five major bile acids in man

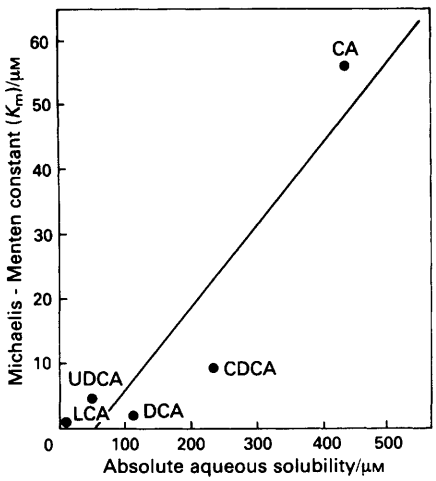



**Fig. 2.** Michaelis - Menten constants ($K_m$) of the major free bile acids with 3α-HSD and NAD as a function of their absolute aqueous solubilities. Linear function: $y = 0.127x - 6.43$; $r = 0.92$, $n = 5$

to the enzyme. In UDCA the $C_7$ hydroxy group is β-oriented and the two hydroxy groups lie 8 Å apart with the 7-OH function oriented towards the opposite side of the hydrocarbon ring system, whereas in its epimer CDCA the two hydroxy groups are α-oriented, lying about 5 Å apart.[19] Therefore, UDCA reacts three times faster that CDCA. The lowest affinity as substrate to 3α-HSD is shown by CA, which is the most hydrophilic of all, having three hydroxy groups (Fig. 1).

No substantial kinetic difference was observed among the free glyco and tauro conjugates of the same bile acid, as they have the same number of hydroxy groups in the same position. The only exception is GCA, which according to our experimental results gives a very high $K_m$ value and was not included in the calculation of the linear function.

Fig. 2 shows that a reasonable correlation ($r = 0.92$) exists between the $K_m$ values and the absolute aqueous solubilities of the free bile acids,[19] which depends mainly on the hydrophilic and hydrophobic properties of the molecules. LCA, which is the most hydrophobic of all, is the least soluble and the best substrate for the enzyme, whereas CA, which is the most hydrophilic, is the most soluble and has the smallest affinity to the enzyme. Solubility data are not available for all the conjugated bile acids. Our findings suggest that the number and position of hydroxy groups in BA (polarity) are the major factors that differentiate the kinetic parameters of 3α-HSD with BA as substrates and play a significant role in their affinity to the enzyme. Similarly, correlation of the polarity of BA with their affinity constants for albumin has been reported recently.[22]

Differences in kinetic parameters have already been used for the development of differential kinetic methods for the determination of primary BA using 7α-HSD.[8,23] However, the kinetic determination of one BA in the presence of others with the available purity of 3α-HSD does not seem so easy, although LCA reacts about 100 times faster than CA, because CA exists in serum in higher concentrations than LCA.

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