Pergamon

Tetrahedron: Asymmetry Vol. 6, No. 12, pp. 3015-3022, 1995
Elsevier Science Ltd
Printed in Great Britain
0957-4166/95 $9.50+0.00

0957-4166(95)00398-3

A SIMPLE METHOD FOR CALCULATING ENANTIOMER RATIO AND EQUILIBRIUM CONSTANTS IN BIOCATALYTIC RESOLUTIONS

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Abstract: A computer programme for determination of equilibrium constant *(K)* and enantiomer ratio *(E)* in biocatalytic resolutions has been developed. The programme utilises experimental data, ee_S and ee_P measured at more than one conversion, and determines both *K* and *E* no matter whether the reaction is irreversible (*K*=0) or reversible (*K*>0). An estimation of errors in the calculations indicates that errors in *E* does not show a Gaussian distribution, while errors in K does. The usefulness of the programme has been tested in a lipase-catalysed transesterification of 1-phenoxy-2-propanol at various concentrations of acyl donor, with different solvents and at different water activities.

INTRODUCTION

Racemate resolution is one of the most popular ways to obtain homochiral building blocks for synthesis of homochiral pharmaceuticals. Maximum chemical yield of a biocatalytic kinetic resolution is 50% of each enantiomer, *i.e.* when one enantiomer has reacted (product) and the other is left untouched (substrate). The result of the process is described by the enantiomeric excess of the product (ee_P), of the remaining substrate (ee_S) and the yield, which is related to the degree of conversion *(c)*.

A parameter that comprises both the enantiomeric excess and the degree of conversion, and thus the yield, is the enantiomer ratio *E*. In biocatalytic resolutions it is defined as the ratio of k_{cat}/K_M for the two enantiomers. While ee is a property of the product, *E* is characteristic of a process. *E* describes the enantioselectivity or better the enantiospecificity, *i.e.* under particular physical conditions (solvent, temperature, pH, etc.), with a certain substrate and a specific enzyme, it is a constant parameter. For an irreversible process, such as a biocatalytic hydrolysis, simple calculations give *E* when ee_S, ee_P and *c* is measured (Eqns. 1 and 2, *K* = 0). (*E* and *K* are defined according to Chen *et al.*[1,2]) It is not strictly necessary to measure c since $c = ee_S/ee_S+ee_P$.

1)
$$\frac{\ln(1-(1+K)(c+ee_s(1-c)))}{\ln(1-(1+K)(c-ee_s(1-c)))} = E$$

2) $$\frac{\ln(1-(1+K)c(1+ee_p))}{\ln(1-(1+K)c(1-ee_p))} = E$$

Since ee_S in this case is constantly increasing as the reaction proceeds towards 100% conversion, it is always possible to reach 100% enantiomeric excess of the unreacted substrate if a low yield can be accepted. It is important to emphasise that as opposed to asymmetric synthesis, in racemate resolution the quality of the product may be improved by sacrificing on the yield. It has been shown that a complete description of an irreversible process includes the equilibrium constant. (Eqns. 1 and 2, K > 0).[2] This relationship depends on the thermodynamic parameter $K = (1\text{-}c_{eq})/c_{eq}$ where c_{eq} is the equilibrium conversion. Since this definition of K does not include all participants in the reaction, it is often referred to as the "apparent equilibrium constant". In order to calculate E in this case, K has to be determined by allowing the faster reacting species to reach equilibrium and measure the concentrations of the product and the remaining substrate.

Calculation of ee_S and ee_P values as a function of c for given values of K and E is not possible by separating the ee_S and ee_P ($ee_S = f(c)_{K,E}$, $ee_P = f(c)_{K,E}$). Previously different methods for calculating ee_S and ee_P have been presented. These include numerical integration of the rate equations for both enantiomers[3] and parametric representation of the equations 1) and 2).[2]

RESULTS AND DISCUSSION

The programme

We have developed a method in order directly to calculate E and K using equations 1) and 2).

In order to evaluate ee_S and ee_P as a function of c with constant K and E the algorithm finds values of ee_S and ee_P that minimises equations 3) and 4).

3) $$\underset{\substack{K,\ E \text{ and } c \\ \text{constant}}}{\min} \left(\frac{\ln(1-(1+K)(c+ee_s(1-c)))}{\ln(1-(1+K)(c-ee_s(1-c)))} - E \right)^2$$

4) $$\underset{\substack{K,\ E \text{ and } c \\ \text{constant}}}{\min} \left(\frac{\ln(1-(1+K)c(1+ee_p))}{\ln(1-(1+K)c(1-ee_p))} - E \right)^2$$

Currently we are doing this by the method of golden sectioning.[4] With the accuracy needed for this application the method is very fast and reliable. Using a 60 MHz PowerMacintosh 6100 more than 100 function-evaluations per second was generated.

Due to the nature of reversible reactions, there will be values of c where ee_S and ee_P is not defined. Inspection of equations 1) and 2) shows that the maximum value of c is constrained by K for both ee_S and ee_P (Eqn. 5).

5) $$c \leq \frac{1}{K+1}$$

Obtaining K and E values from a given set of (c, ee_S) and (c, ee_P) data require a two dimensional minimisation by varying K and E. The function to be minimised is a penalty function (often called "force-field") dependent on different parameters chosen as important for description of "good" values of K and E. We have used four objectives that are necessary for describing a "good fit" of K and E to the data set. The first and second are that the values of K and E must be in the allowed region, $K \geq 0.0$ and $E > 1.0$. If these objectives are violated there will be a large penalty, increasing with the distance from the allowed region. In practice we are using penalties so large that it is impossible to get these disallowed values in the final result. The third objective is not to allow values of c that are not consistent with equation 5. This punishment however, must be small enough to allow minor violations. Otherwise one single outlying point could influence the K value too much. The last objective is to minimise the difference between the experimental and calculated data. Values of K and E that give small differences are best. We have chosen to minimise the root mean square error between experimental and calculated points (Eqn. 6)

6) $$error = \frac{\sum_{i=1}^{N_{ee_s\text{points}}} (ee^i_{s(\text{calc})} - ee^i_{s(\text{exp})})^2 + \sum_{i=1}^{N_{ee_p\text{points}}} (ee^i_{p(\text{calc})} - ee^i_{p(\text{exp})})^2}{N_{ee_s\text{points}} + N_{ee_p\text{points}}}$$

We chose the downhill simplex method[4] for the minimisation of the penalty function. This method is relatively robust, it is easy to use, and it does not require calculation of derivatives. One drawback is that it requires relatively many evaluations of the penalty function. This is not a serious problem since a typical minimisation takes less than five seconds on a 60 MHz PowerMacintosh 6100.

The downhill simplex method requires a N+1 dimensional simplex as a starting point, for a two-dimensional minimisation this is a triangle. Depending on the value of the penalty function at the points describing the triangle, the triangle reflects, contracts and expands towards lower values for the penalty function.

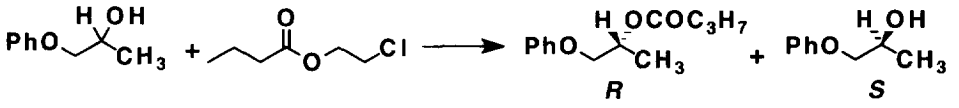
In order to obtain ee_S and ee_P values GLC-integrals of all four enantiomers in question are measured. With this method many systematic errors become self-compensating and thus the standard deviations of the errors smaller. We assume that errors of integral measurements are in the order of 2% which in turn give an absolute standard deviation of 0.01 in the calculated ee_S and ee_P values.

In order to estimate errors in E and K we created one thousand new data sets for each measurement by adding Gaussian noise with a standard deviation of 0.01 to a data set with the same c-value as the original data set, and sets of ee_S and ee_P values according to the calculated E and K values.

Biocatalysis experiments

To illustrate the use of the programme, we have chosen reversible transesterification with lipase B from *Candida antartica* as catalyst.

The substrate, 1-phenoxy-2-propanol, was selected as a continuation of earlier work[5] and on basis of molecular modelling of substrate/lipase interactions.[6] The reaction with 2-chloroethyl butanoate as acyl donor showed reversibility, and was therefore well suited as a model reaction.



The absolute configurations of the products were established by comparison with *(S)*-1-phenoxy-2-propanol which has been described previously.[7] The *(R)*-enantiomer was produced in a hydrolysis of the butanoate also catalysed by lipase B.

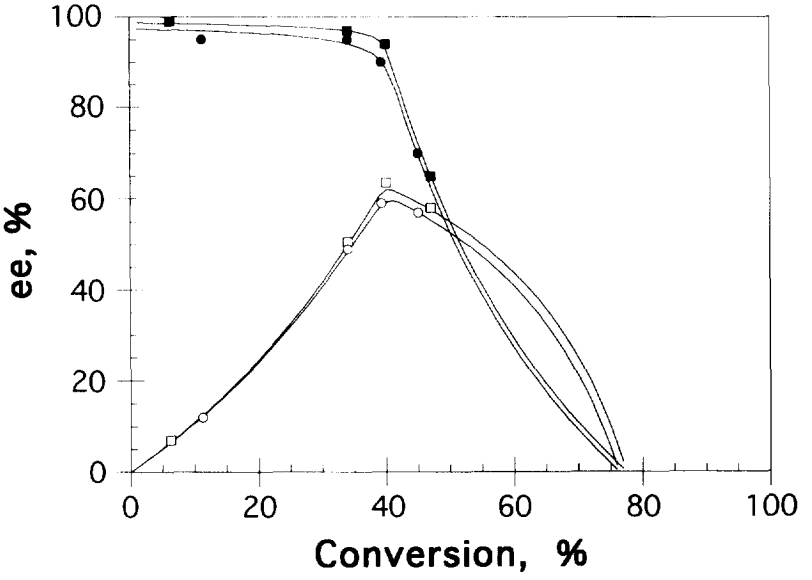


Figure 1. Effect of variation of solvent in transesterification of 1-phenoxy-2-propanol with 2-chloroethyl butanoate (5x excess). Circles toluene, squares hexane, filled symbols product fraction, open symbols remaining substrate fraction.

When the resolution was performed in two different solvents, hexane and toluene, the *K*-values were virtually the same, 0.29 and 0.31 respectively. However, there was a significant change in the enantioselectivity on going from hexane (E=149) to toluene (E=75.3) (Figure 1). It seems reasonable that the two unpolar solvents do not influence the thermodynamics of the reaction in a different way. The catalytic properties of the enzyme, however, may be altered by different solvents.

When the relative molar amount of acyl donor was increased from 1.5 times the amount of substrate, to 3 and finally 5, the *K*-value naturally increased (Figure 2, Table 1). This just reflects the fact that the equilibrium is shifted towards the product side. The *E*-value, however, which was virtually constant for the two reactions with the smallest amount of acyl donor, E= 242 and 254 respectively, decreased drastically in the third experiment. We think that a five times excess of acyl donor may change the properties of the enzyme. We are currently investigating this observation.

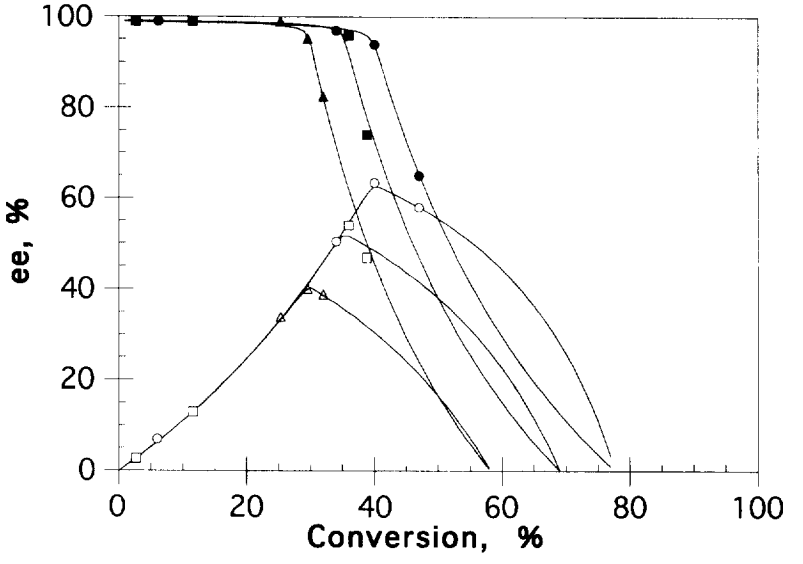


Figure 2. Variation of relative concentrations of 2-chloroethyl butanoate in transesterification of 1-phenoxy-2-propanol. Circles 5x excess, squares 3x excess, triangles 1.5x excess. Filled symbols represent product fraction, open symbols remaining substrate fraction.

[Acyldonor] / [substrate]	[Acyldonor]	*E*	*K*
1.5	0.066	242 (+47, -36)	0.71 (± 0.025)
3	0.131	254 (+240, -79)	0.44 (± 0.014)
5	0.218	149 (+15, -12)	0.29 (± 0.010)

Table 1. Effect of variation of concentration of acyl donor in transesterification. The solvent used was hexane, and the acyl donor 2-chloroethyl butanoate. Figures in parenthesis is standard deviation assuming ±2% inaccuracy in measurements of GLC integrals.

It is well known that the amount of water in the organic solvent, best expressed as the water activity (a_W), may influence the reaction. The water activity may easily be altered and kept at a constant level by introducing pairs of inorganic hydrate salts into the reaction vessel.[8] It has been reported that the water activity both influences the enantioselectivity[9] and that it does not.[10] For the present system we have monitored the reaction at three different levels of a_W and we observe an increase of both *E* and *K* with increasing water activity. (Figure 3, Table 2)

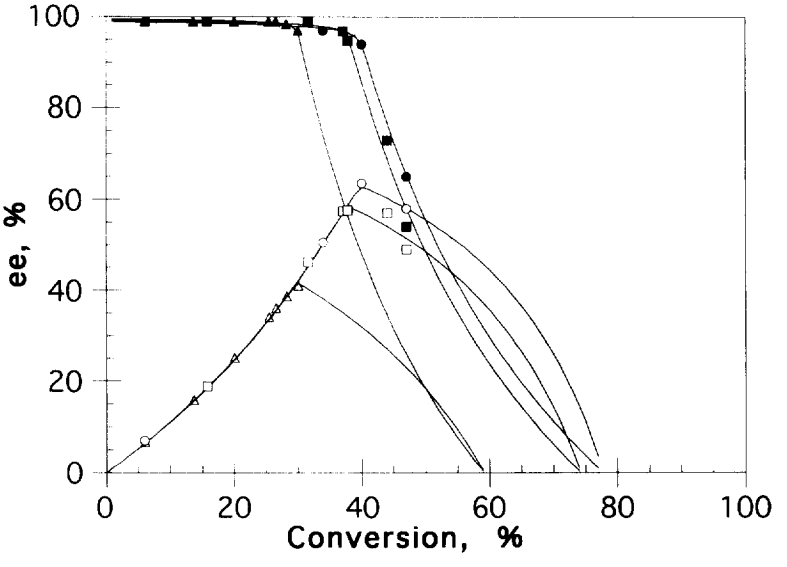


Figure 3. Variation of water activity (a_W) in hexane in transesterification of 1-phenoxy-2-propanol with 2-chloroethyl butanoate (5x excess). Circles a_W=0, squares a_W=0.16, triangles a_W=0.61. Filled symbols represent product fraction, open symbols remaining substrate fraction.

a_W	*E*	*K*
0	149 (+15, -12)	0.29 (± 0.010)
0.16	258 (+37, -27)	0.35 (± 0.0087
0.61	418 (+141, -90)	0.69 (± 0.010)

Table 2. Variation of water activity (a_W) in hexane in transesterification of 1-phenoxy-2-propanol with 2-chloroethyl butanoate (5x excess). Figures in parenthesis is standard deviation assuming ±2% inaccuracy in measurements of GLC integrals.

When the water activity of the reaction medium increases, water will serve as a competing nucleophile and the reverse reaction will be more favoured leading to an increasing *K*. It is very interesting to notice that the enantioselectivity also increases, however, the usefulness of operating at an elevated water activity in order to obtain a high *E*-value is doubtful since *K* also increases.

Concerning the error estimations, the *E*-value, as expected, did not show Gaussian distribution. The error values in Tables 1 and 2 represent a region covering 37% of the data counting in both directions of the calculated *E*. There is no evidence that *K* was not showing a Gaussian distribution, and errors in *K* are given as one standard deviation.

ACKNOWLEDGEMENT

We are grateful to Dr. Martin Barfoed, Novo-Nordisk, Bagsværd, DK for gift of enzymes.

EXPERIMENTAL

General. Chiral analyses were performed using a Varian 3400 gas chromatograph with a CP-Chiracil-DEX-CB from Chrompack. Lipase B (EC 3.1.1.3) from *Candida antarctica* (SP 435) Novo-Nordisk, immobilised on Lewatit, had specific activity of 19000 PLU/g. NMR spectra of 1-phenoxy-2-propanol and its butanoate conformed with their structures.[7]

Transesterification. To solvent (3 mL), was added 1-phenoxy-2-propanol (0.131 mmol), and 2-chloroethyl butanoate (for amount see Tables 1 and 2). The reaction was started by adding the lipase (20 mg) to the reaction mixture. The reactions were performed in a shaker incubator at 30 °C. The samples were filtrated to remove the immobilised enzyme before analysis. The substrate alcohol and product ester were both analysed directly on GLC without derivatisation in the same run. Retention times for 1-phenoxy-2-propanol were 23 - 24 min. and for the butanoates 39 - 40 min., temp. prog. 100 - 142 °C, 1°/min. At various intervals ee_S and ee_P were determined and *c* was calculated from these as shown above. In the experiments with varying water activity a total of 0.4 g of salt hydrate was used for each

experiment, a_W = 0.16 was obtained using equimolar amounts of $Na_2HPO_4 \cdot 2H_2O$ + $Na_2HPO_4 \cdot 0H_2O$ and for a_W = 0.61 was used $Na_2HPO_4 \cdot 7H_2O$ + $Na_2HPO_4 \cdot 2H_2O$.

(R)-*1-phenoxy-2-propanol* was isolated by column chromatography after hydrolysis of racemic butanoate (2. 5g) in phosphate buffer (100 mL) at pH 7.2 using lipase B (50mg), yield 0. 45g), $[\alpha]_D^{22}$ = + 2.8 (c 1.8 EtOH). (For *(S)*-enantiomer see ref. 7)

The programme for calculation of E *and* K was written in ANSI C++ and compiled for the PowerMacintosh type of computers by means of the CodeWarrior gold academic programming environment, version 6. A copy of the program is available on request (E-mail: Thorleif.Anthonsen@avh.unit.no).

Values of *K* and *E* that describe a given (*c*, ee_S) and (*c*, ee_P) data set were calculated by the minimisation of a penalty function. The penalty function was dependent on the root mean square error between experimental points and points calculated on the basis of given *K* and *E* values and also dependent on the values of *K*, *E* and *c* in order to keep the value of these parameters in their allowed region. The initial starting triangle was always at ((*E*,*K*)) (1.1, 0.0), (20.1, 0.0) and (1.1, 1.0), but it was not critical for the result. The minimisation gave *K* and *E* values which were used as input to another program that generated the (c, ee_S, ee_P) curves. Calculation of ee_S and ee_P values given *K*, *E* and *c* was done by minimisation of equations 1 and 2. Visualisation and drawing of the curves was performed by Kaleidagraph™ 3.0.

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(*Received in UK* 14 *September* 1995)