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LINEAR RELATIONSHIP BETWEEN THE LOGARITHM OF THE EQUILIBRIUM CONSTANTS AND THE LOGARITHM OF THE LIQUID CHROMATOGRAPHIC SEPARATION FACTORS FOR TAUTOMERS OBTAINED IN DIFFERENT SOLVENTS

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SUMMARY

The linear relationship between the logarithm of the separation factor, α, in liquid–solid chromatography (silica gel) using a less-polar solvent, such as a *n*-hexane–ethyl acetate, and the logarithm of the equilibrium constant, K, for a pair of tautomers in a different solvent such as ethanol was confirmed experimentally using seventeen pairs of tautomers of steroid ketoximes and their corresponding O-methylketoximes. Theoretical considerations using a static model of the adsorption–desorption equilibrium in chromatography afforded the equation

$$\log \alpha = (\Delta\Delta G^0/\Delta G^0) \log K + (\Delta\Delta G^0/\Delta G^0) \log m$$

where m is a constant relating the equilibrium constants of a pair of tautomers in two different solvent systems, namely, the solvent used in the chromatographic separation and that used in the equilibrium constant determination. The correlation coefficient obtained with 40 mol/mol % ethyl acetate in *n*-hexane as the chromatographic solvent system was 0.9804. The two constants of the above equation were determined as $\Delta\Delta G^0/\Delta G^0 = 0.3898$ and $\log m = 0.3999$ with the same solvent system.

INTRODUCTION

As is well known, the free energy change between two tautomers P and Q is proportional to the logarithm of their equilibrium constants in solution. In chromatography, one can obtain the capacity ratios, k'_P and k'_Q, from the difference in distribution constants on the stationary and mobile phases, yielding the separation factor, α, of the two solutes, which is defined as k'_P/k'_Q. The logarithm of the separation factor and the difference in the free energy changes between P and Q in distribution equilibrium between two phases are known to be related. Consequently, if the same solvent system could be applied to obtain the equilibrium constant of P and Q in solution and the separation factor in liquid chromatography, the logarithm of these parameters would be directly proportional to the coefficient of $\Delta\Delta G^0/\Delta G^0$. From this relationship it would be possible to calculate either of the parameters K or α, if its

counterpart is available. These considerations induced us to confirm this hypothetical equation experimentally.

THEORETICAL

For a pair of tautomers P and Q in equilibrium in an appropriate protic solvent A, the following thermodynamic equation holds:

$$\Delta G^0 = -RT \ln K^A \tag{1}$$

To resolve these tautomers by liquid chromatography, it is necessary to use an aprotic solvent B in which the rate of attainment of equilibrium is minimized. In such a system, the separation factor may be expressed by:

$$\alpha^B = k'_P/k'_Q \tag{2}$$

Since the separation factor represents the ratio of distribution constants, K_P and K_Q, of the tautomers P and Q, respectively, between the stationary and mobile phases, the corresponding difference in free energy changes can be expressed by:

$$\Delta\Delta G^0 = -RT \ln \alpha^B \tag{3}$$

A static model of chromatographic separation and equilibrium is illustrated in Fig. 1. The thermodynamic expression of the equilibrium between two tautomers P and Q in the mobile phases B is:

$$\Delta G^0 = -RT \ln K^B \tag{1a}$$

From eqns. 1a and 3 the following relation can be obtained:

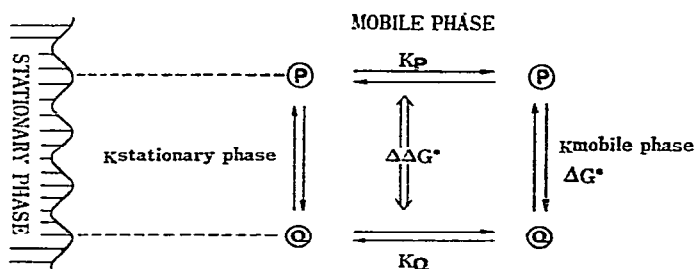$$\ln \alpha^B = (\Delta\Delta G^0/\Delta G^0) \ln K^B \tag{4}$$

Fig. 1. Static model of the chromatographic separation of the isomers P and Q. P and Q are the solutes in the mobile phase, and - - - P and - - - Q are those on the stationary phase. K_P and K_Q represent the adsorption–desorption equilibrium constants of the solutes; $K^{mobile\ phase}$ represents the equilibrium constant between P and Q in the mobile phase. Of course, these isomers do not undergo equilibration during the chromatographic operation. $\Delta\Delta G^\circ$ is the difference between the adsorption–desorption free-energy changes of both solutes P and Q; ΔG° is the free energy change of the isomers in the mobile phase.

This equation represents the linear relationship between the equilibrium constant and separation factor in a chromatographic system. However, because it is difficult to obtain the value of K^B experimentally in less-polar solvents owing to the high stabilities and low solubilities of the tautomers, we applied an approximation by means of the coefficient m, which assumes that the equilibrium constants are dependent upon the dielectric constants of the solvents:

$$K^B = mK^A \tag{5}$$

Then, we obtain from the eqns. 4 and 5 the following relation:

$$\ln \alpha^B = (\Delta\Delta G^0/\Delta G^0) \ln K^A + (\Delta\Delta G^0/\Delta G^0) \ln m \tag{4a}$$

This equation expresses the linear relationship between the two parameters α^B and K^A. To confirm this experimentally, we need to obtain the capacity ratios using a large number of tautomeric pairs under common chromatographic conditions, and to obtain the equilibrium constants using a common solvent system for all pairs of tautomers.

EXPERIMENTAL

Chromatography

A KP-9H reciprocating pump (Kusano Scientific, Tokyo, Japan) and a glass column were connected to a R 401 differential refractometer (Waters Assoc., Milford, MA, U.S.A.). The glass columns (CIG, Kusano), 30 cm × 4 mm I.D. for analytical work and 30 cm × 8 mm I.D. for preparative work, were packed with a silica gel slurry prepared in a mixture of chloroform, carbon tetrachloride and dioxan (2:1:2, v/v) using irregularly shaped silica (10 μm, pore size 70 Å) (Wakogel LC-10H; Wako, Osaka, Japan). 4500 Theoretical plates per 30 cm were obtained using diethyl phthalate as sample and n-hexane–ethyl acetate (9:1 v/v) as mobile phase.

Various mixtures of n-hexane and ethyl acetate were used as the solvent system. Solutions of 300 mg of the equilibrated tautomers in 1 or 2 ml of chloroform were injected into preparative columns by the on-column technique so that the K values could be obtained by weight. Capacity ratios, $k' = (t_s - t_m)/t_m$, for each pair of isomers were obtained using an analytical column system at a constant temperature of 20°C and three different solvent compositions. The hold-up volume, t_m, was obtained using cyclohexane, and flow-rates were 0.3 ml/min for analytical columns and 3 ml/min for preparative columns.

NMR spectroscopy

Structural assignments of pure, isolated tautomers were performed with a JEOL PS-100 spectrometer in deuterochloroform solution at 22°C. Equilibrium constants of mixtures which could not be separated by chromatography were determined by NMR analysis.

Samples

3-Oxosteroid oximes were prepared in 95% ethanol with $NH_2OH \cdot HCl$ and

sodium acetate at 20°C overnight. The crude products were allowed to stand in anhydrous ethanol at 20°C for 24 h. O-Methyloximes were prepared in pyridine with $NH_2OCH_3 \cdot HCl$ at 20°C overnight. The crude products were allowed to stand in anhydrous ethanol containing catalytic amounts of p-toluenesulphonic acid at 20°C for 2–20 days to obtain the equilibrium mixtures. Evaporation of the solvent at 20°C *in vacuo* gave the analytical samples.

RESULTS AND DISCUSSION

The proposed scheme requires the chromatographic separation of a pair of tautomers without isomerization. For this purpose, we have chosen seventeen pairs of geometrical isomers consisting of steroidal ketoximes and O-methylketoximes, some of which had been previously resolved by silica gel liquid–solid chromatography[1–4]. To obtain equilibrated mixtures of these oximes, the crude products, prepared in the usual manner, were allowed to stand in ethanol at constant temperature of 20°C. In the case of O-methylketoximes, equilibration was very slow, requiring 2–20 days, and p-toluenesulphonic acid was added as a catalyst. Although the constitutions of equilibrium mixtures could be determined by NMR spectroscopy[5] for the compounds which involve olefinic protons, accurate values required preparative separation on silica gel (n-hexane–ethyl acetate) and weighing of each isolated isomer. Thus the equilibrium constants, $K_{Z/E}^{EtOH}$, for all pairs of isomers were obtained with minimal experimental errors.

The configurations of the geometrical isomers were assigned according to chemical shifts in their NMR spectra; however, in order for these assignments to correspond with results of chromatographic separations, further considerations are required. If one draws a plane bisecting the angle O–N–lone pair (see Fig. 2), whose lone pair of electrons is the driving force in retention, the larger part of the hydrocarbon moiety is on the same side of the plane as the lone pair of electrons in the Z-form, while polar substituents in addition to the oxime group are located on the same side as the
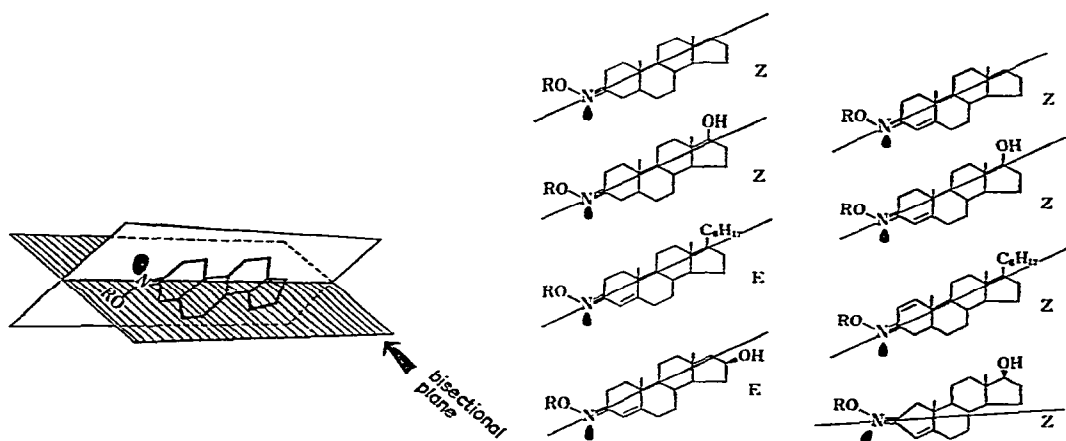

Fig. 2. Symbolism for Z- and E-isomers of steroid oximes, showing the bisectional plane (the lone pair of electrons and alkoxyl group are exchangeable in accordance with the geometrical isomerism) for 5α-androstan-3-one E-oxime.

lone pair of electrons in the E-form. For such compounds the hydroxyl group shows a distinct contribution to adsorption, the olefinic group shows a lesser contribution and bulky hydrocarbon groups show negative effects. These principles are not completely consistent with the stereochemical symbolism of the Cahn–Ingold–Prelog system; however, they may be more flexible and reliable for the retention sequence in liquid–solid adsorption chromatography. Thus we assigned the geometrical isomerism of the solutes as illustrated in Fig. 2.

A chromatographic system to obtain the separation factor, α^B, was designed, using silica gel as the stationary phase and a binary solvent involving n-hexane and ethyl acetate as the mobile phase. Since the steroid molecules contained various D-ring substituents such as hydrocarbon, hydroxyl and acetoxy groups, along with the oxime and methyloxime groups on the A-ring, it is very difficult to obtain the capacity ratios or separation factors using binary solvents of the same concentration. To overcome this difficulty we applied the interpolation and/or extrapolation using experimentally obtained data for binary solvent mixtures of three different compositions.

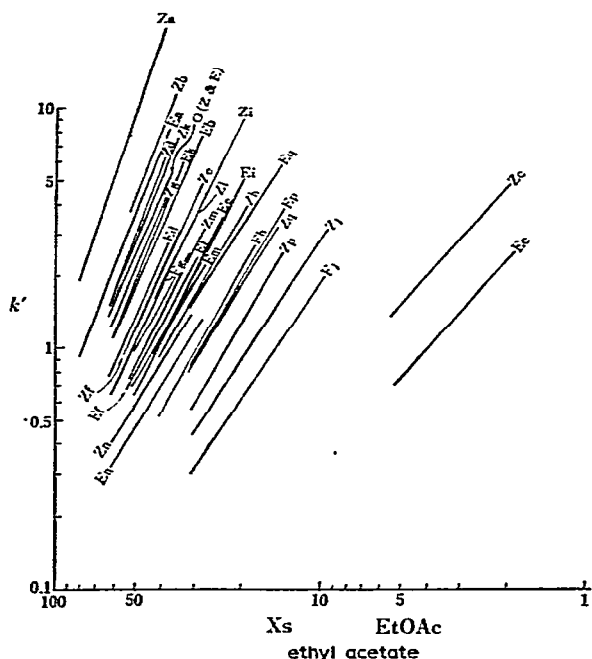In adsorption–desorption chromatography using binary solvent systems, the

Fig. 3. Graph of the logarithm of the capacity ratio *versus* the logarithm of the mole fraction of ethyl acetate in n-hexane. Compounds: a = A-nortestosterone oxime; b = testosterone oxime; c = androst-4-en-3-one O-methyloxime; d = 17β-hydroxy-17α-methyl-4-androsten-3-one oxime; e =testosterone acetate oxime; f = testosterone O-methyloxime; g = 19-nor-17β-hydroxy-17α-ethyl-4-androsten-3-one oxime; h = androst-4-en-3-one oxime; i = testosterone benzoate oxime; j = 17β-acetoxy-5α-androstan-3-one O-methyloxime; k = 17β-hydroxy-5α-androstan-3-one oxime; l = 17β-acetoxy-5α-androstan-3-one oxime; m = 17β-hydroxy-5α-androstan-3-one O-methyloxime; n = 5α-androstan-3-one oxime; o = 16β-hydroxy-4-androsten-3-one oxime; p = 5α-cholest-1-en-3-one oxime; q = cholestenone oxime.

relation between the retention of the solute and the composition of the solvent is given by[6-11]

$$\log k' = c - n \log X_s \tag{6}$$

where c and n are constants and X_s is the mole fraction of the stronger solvent component. Thus we obtained the capacity ratios of 34 compounds using appropriate solvent constitutions at a constant temperature of 20°C. The plot of the logarithms of the capacity ratios *versus* the logarithms of the mole fractions of ethyl acetate was linear for each compound (Fig. 3).

Interpolation and/or extrapolation of the straight lines in Fig. 3 gave the ratio of the capacity ratios of each E- and Z-isomer ($\alpha^B = k'_E/k'_Z$) at a desired solvent composition for all pairs of isomers. Solvent compositions selected for these experiments were 1, 10, 40, 80 and 100 mole per mole % of ethyl acetate. Plots of the logarithms of the separation factors thus obtained *versus* the logarithms of the equilibrium constants in ethanol solutions are shown in Fig. 4a–e. The correlation coeffi-
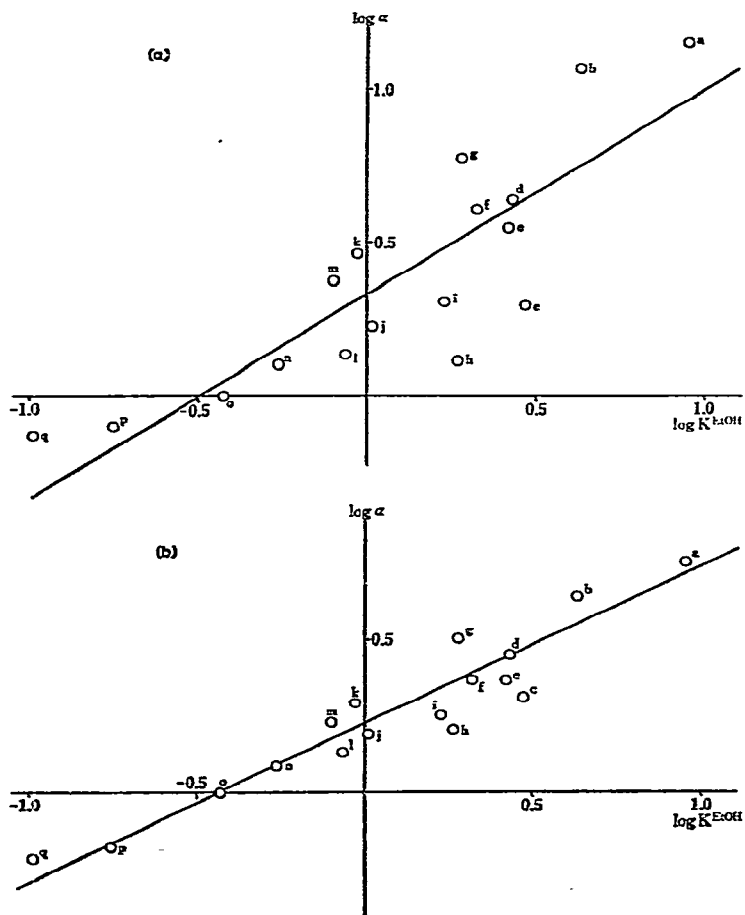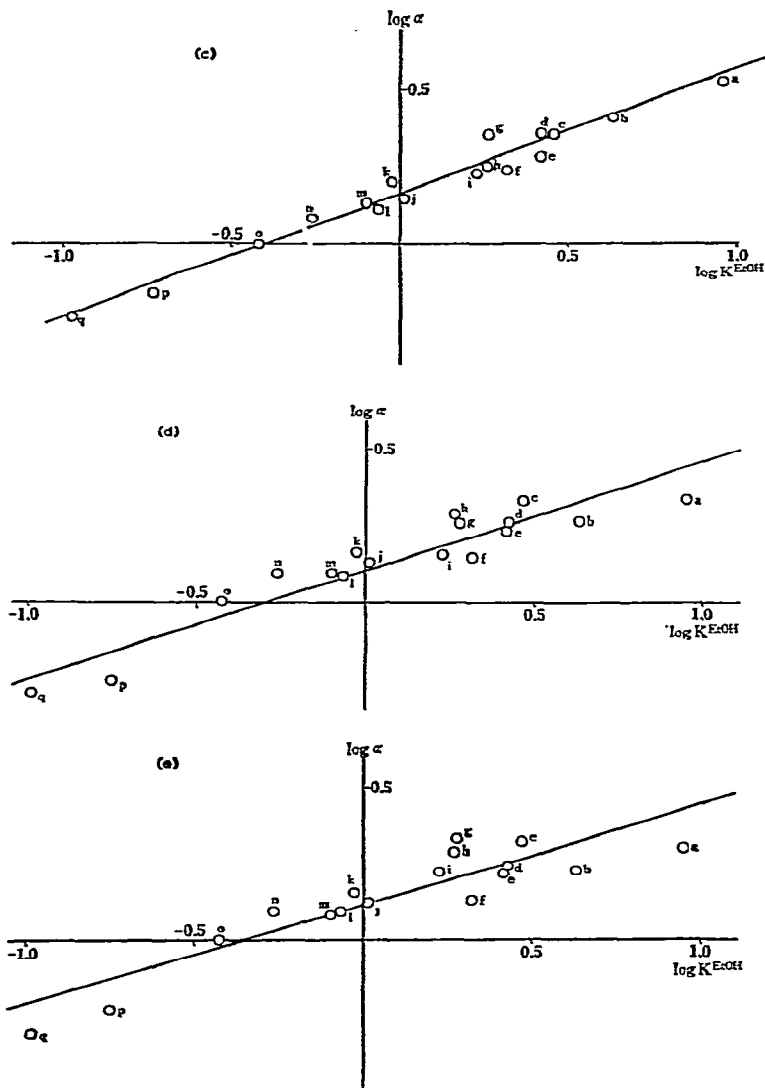

Fig. 4.

Fig. 4. Linear relationship between logarithms of separation factors and equilibrium constants. The logarithms of separation factors were obtained using a silica gel column and a mixture of n-hexane and ethyl acetate while the logarithms of equilibrium constants were determined in ethanol at 20°C. (a) log α = 0.6698 log K^{EtOH} + 0.3310, 1% EtOAc in n-hexane, R = 0.8511; (b) log α = 0.4905 log K^{EtOH} + 0.2232, 10% EtOAc in n-hexane, R = 0.9401; (c) log α = 0.3898 log K^{EtOH} + 0.1559, 40% EtOAc in n-hexane, R = 0.9804; (d) log α = 0.3549 log K^{EtOH} + 0.1040, 80% EtOAc in n-hexane, R = 0.9346; (e) log α = 0.3441 log K^{EtOH} + 0.1082, 100% EtOAc, R =0.9060.

cients, R, based on the least-squares method varied from 0.8511 for 1% to 0.9060 for 100% ethyl acetate taking the maximized R value of 0.9804 for 40% ethyl acetate. The two constants in eqn. 4a at 40% ethyl acetate were $\Delta\Delta G^0/\Delta G^0$ = 0.3898 and log m = 0.3999.

The R values in Fig. 4a–e suggest that the linear relations given by eqn. 6 are reliable for medium-range solvent concentrations, less trustworthy for high concentrations of the stronger component and unreliable for low concentrations of the stronger component.

From the above results, a highly reliable estimation of $\Delta\Delta G^0/\Delta G^0$ and m values can be achieved by the use of capacity ratios at medium concentrations of the binary solvent and by extrapolations and interpolations based on eqn. 6. These results also demonstrate the linear relationship between the equilibrium constants, in appropriate solvents, and the separation factors in different solvent systems as in eqn. 4a. This physicochemical relationship may also exist for many isomer pairs besides the present steroid oximes. If the equilibrium constant of a pair of isomers is known, a chromatographic separation can be performed and if their equilibrium constants and/or free-energy changes are not known, they can be calculated by simple chromatographic techniques. Thus the relationship expressed by eqn. 4a may provide a useful connection between chromatographic separations and the properties of solutes and provide wide application to both analytical and physical chemistry.

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