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MODELLING OF PEAK PROFILES

APPLICATION TO THE PREPARATIVE LIQUID CHROMATOGRAPHY OF STEROIDS

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SUMMARY

The solution of the mass balance equations in liquid chromatography describes the propagation of signals of finite concentration through the column. The general numerical solution requires the prior determination of the partition isotherm. For low solute concentrations, when the isotherm equation can be replaced with a two-term expansion, an analytical solution for the peak profiles is obtained.

The theory is applied to predict, as a function of solute concentrations, the elution profiles of two steroids of similar structure, *cis*- and *trans*-androsterone, with organic solvents as the mobile phase and buffered silica gel as the stationary phase. At infinite dilution both steroids are well resolved, the *trans* isomer being eluted before the *cis* isomer. At high concentrations their adsorption isotherms intercept each other and, for large amounts injected, their elution order is reversed, with marked differences in the elution peak shapes of both steroids.

INTRODUCTION

The modelling of peak profiles obtained at finite concentration is of prime importance in optimizing separations in preparative liquid chromatography¹. Generally, separations are optimized by a trial and error method², a procedure which may be tedious and prolonged, as it involves many experiments. Modelling leads to a better understanding of the phenomenon and may indicate the experimental parameters that will give the best separations in preparative chromatography^{3,4}.

In a previous study¹, we established the mass balance equations in liquid chromatography that describe the propagation of solutes of finite concentration. A general numerical solution of the system exists^{5,6} that takes into account the injection profile and requires a knowledge of the sorption isotherm. An analytical expression of the peak profile is derived for moderate concentrations when the sorption isotherm can be replaced by a two-term expansion^{1,7}. The aim of this work was to compare the advantages and possibilities of both models and to discuss their applicability in preparative liquid chromatography of lipid-soluble compounds.

For the preparative liquid chromatography of organic lipid-soluble compounds normal-phase liquid chromatography is generally preferred, as the use of an organic solvent allows both increased sample solubility in the mobile phase and easy purification by evaporation of the collected fractions⁸.

One of the disadvantages of untreated silicas in liquid-solid chromatography is the dramatic influence of the water content on the solute retention times⁹, which leads to uncontrollable retention behaviour. In this work, in order to obtain reproducible results, independent of trace amounts of water, we used a buffered silica gel¹⁰, which has several other advantages for preparative liquid chromatography: it reduces the tailing of the elution peaks of polar compounds and permits the adsorption chromatographic separation of isomers. On an analytical column, packed with a buffered silica gel, we have studied the problem of the separation of *cis*- and *trans*-androsterones. To test the ability of the theoretical model to fit the experimentally overloaded profiles, an analytical chromatographic system was used in this study but, as is well known, analytical equipment may serve to develop separations for preparative liquid chromatography¹¹.

THEORY

We have shown previously¹ that the propagation of the concentration profile for one solute and a mobile phase is described by the mass balance differential equation of liquid chromatography:

$$\frac{\partial(c+k)}{\partial t} + \frac{\partial(uc)}{\partial z} = D \cdot \frac{\partial^2 c}{\partial z^2} \quad (1)$$

where

- c = concentration of the solute in the mobile phase;
- t = time elapsed since injection;
- z = abscissa along the column;
- u = velocity of the mobile phase;
- D = dispersion coefficient;
- k = function describing the partition equilibrium.

$$n_A/V_L = k(c) \quad (2)$$

where n_A is the number of adsorbed molecules and V_L the volume of the liquid phase.

Moderate overload peak model

An expression of the chromatographic elution peak can be derived as a solution of eqn. 1 when k is expanded to the second term:

$$\frac{\partial n_A}{\partial n_L} = k' = k'_0 + k''_0 V_L c \quad (3)$$

where k' is the capacity factor and n_L the number of moles in the liquid phase.

In quasi-pulse injection, the algebraic expression of the elution profile is

$$c = \frac{2 \left(\frac{D'}{\pi t} \right)^{1/2}}{\lambda U} \cdot \frac{\exp(-\xi^2/4 D' t)}{\coth(\mu/2) + \operatorname{erf}[\xi/2(D' t)^{1/2}]} \quad (4)$$

where

$$U = L/t_R \text{ and } \xi = L - U t \quad (5)$$

$$D' = D/(1 + k'_0) \quad (6)$$

$$\lambda = k''_0 V_L/(1 + k'_0) \quad (7)$$

$$\mu = a\lambda U^2/2D' \quad (8)$$

and a is the peak size parameter.

Only four parameters are necessary to define the chromatographic peak:

(1) the peak size parameter, a , equal to the peak area, A ;

(2) the slope at origin of the isotherm, k'_0 , which is related to the retention time at infinite dilution, t_R :

$$t_R = (1 + k'_0)t_0 \quad (9)$$

where t_0 is the hold-up time;

(3) the global apparent dispersion coefficient, D' ; and

(4) the curvature of the isotherm at the origin, k''_0 , which is related to the peak slant coefficient, λ (eqn. 7).

A simple relationship exists between the peak slant coefficient and the locus of the maximum of the elution profile:

$$c_M = (t_M - t_R)/\lambda t_M \quad (10)$$

This equation is a hyperbola that reduces to a straight line for small values of c_M .

Numerical calculation of the chromatographic peak

To perform an approximate integration of eqn. 1 we first neglect the value of D , which would lead to intricate problems of numerical stability, and thus we have to solve

$$\frac{\partial(c + k)}{\partial t} + u \cdot \frac{\partial c}{\partial z} = 0 \quad (11)$$

where we assume u to be independent of z , as is usual in liquid chromatography.

The numerical procedure is easier in the present instance than in gas chromatography^{5,6}, as the mobile phase velocity is kept constant and the second differential equation is not needed because volume effects can be neglected¹. Therefore, the numerical procedure based on Godunov's scheme^{5,6} is not needed in order to solve eqn. 11.

To solve eqn. 11, we have

$$\frac{\partial c}{\partial z} = -\frac{1}{u} \cdot \frac{\partial(c+k)}{\partial t} \quad (12)$$

We start with a set of values $c(t, 0)$ corresponding to the injection signal ($z = 0$). Eqn. 12 is converted to

$$\frac{\Delta'c}{\Delta'z} = -\frac{t_0}{L} \cdot \frac{\Delta[c+k(c)]}{\delta t} = -\frac{t_0}{L} \cdot \frac{\Delta c + \Delta k(c)}{\Delta t} \quad (13)$$

Here Δ is used for constant z and Δ' for constant t . Finally,

$$c(t, z + \Delta'z) = c(t, z) - \frac{t_0}{L} \cdot \frac{\Delta'z}{\Delta t} \left[c(t + \Delta t, z) - c(t, z) + k(t + \Delta t, z) - k(t, z) \right] \quad (14)$$

As one uses discrete steps for integration, this will result in a smoothing of the theoretical signal with numerical "dispersion". The step in z , $\Delta'z$, is equal to H_{\min} , the smallest value of the height equivalent to a theoretical plate (HETP), and $\Delta t = 2t_R$. $\Delta'z/L$ is chosen so that the best stability conditions are realized according to the Friedrich, Lévy and Courant criterion^{5,6}.

In addition to the parameters of the isotherm function $k(c)$, the peak profile is defined by two other parameters, the peak area and the global apparent diffusion coefficient, related to the HETP (H) by

$$\Delta'z = H = 2D' t_R/L \quad (15)$$

Therefore, as with the moderate overload peak model, the numerical procedure needs four parameters to describe an elution peak in the case of a parabolic sorption isotherm.

EXPERIMENTAL

The HPLC system consisted of a Model 110 B (Beckman, Berkeley, CA, U.S.A.) and a Model 7125 sampling valve (Rheodyne, Berkeley, CA, U.S.A.) with a 40- μ l loop. The detector employed was a Model R401 differential refractometer (Waters, Assoc., Milford, MA, U.S.A.).

The analogue outputs of the detectors were connected to a Model 3497 digital voltmeter (Hewlett-Packard, Palo Alto, CA, U.S.A.), which was interfaced with a CBM 4032 microcomputer (Commodore, Santa Clara, CA, U.S.A.) that actuated the injection valve and controlled the data acquisition rate. The data, collected to a precision of four decimal places, were stored on a floppy disk. The theoretical calculations were carried out in Fortran with a Hewlett-Packard 21 MX computer and with the computer of the Centre Inter Régional de Calcul Electronique (Orsay, France), using a non-linear least-squares fit program.

The eluent was dichloromethane-acetonitrile (90:10, w/w; 99% purity) (Carlo Erba, Milan, Italy). The solutes used were *cis*- and *trans*-androsterones (98% purity) (Fluka, Buchs, Switzerland).

The solid phase was a buffered silica gel. It was prepared by Schwarzenbach's method¹⁰ and the silica gel surface (LiChrosorb Si-100, particle size 10 μm) (Merck, Darmstadt, F.R.G.) was coated with a phosphate buffer (pH 6.8).

The adsorbent was packed into a 25 \times 0.46 cm I.D. stainless-steel column by using the slurry technique. The column was placed in a water-bath at 20 \pm 0.1°C. The dead-volume of the column ($V_L = 3.55$ ml) was determined from the differences between the weights of the column filled with carbon tetrachloride and methanol¹².

Good reproducibility in the retention behavior, independent of the trace water content in the liquid and stationary phases, is observed when buffered silica gel is used as an adsorbent and organic solvents in which steroids are soluble are used as the mobile phase.

The refractive index detector had a non-linear response at concentrations higher than 3 $\mu\text{mol/ml}$ for both steroids. The detector was calibrated and the output voltage, s , was related to the concentration through an equation optimized by the least-squares fit method:

$$c = 2.237 \cdot 10^{-3} \cdot s \cdot e^{0.034 \cdot s}$$

for *cis*-androsterone and

$$c = 2.341 \cdot 10^{-3} \cdot s \cdot e^{0.034 \cdot s}$$

for *trans*-androsterone, where the output voltage, s , is in volts and the concentration in $\mu\text{mol/ml}$.

RESULTS AND DISCUSSION

Determination of the adsorption isotherm by frontal chromatography

At high concentrations the displacements and distortions of the elution peak are mainly due to the non-linearity of the adsorption isotherm. It is therefore useful to measure it by an independent method in order to select the appropriate adsorption isotherm model.

The adsorption isotherms of *cis*- and *trans*-androsterones on silica gel were measured by the frontal analysis technique¹³. The experimental data are well fitted by Langmuir's equation:

$$\frac{n_A}{V_L} = \frac{\alpha c}{(1 + \beta c)} \quad (16)$$

The coefficients α and β , determined for both compounds, are given in Table I. The corresponding first and second derivatives of the adsorption function at zero surface coverage are related to α and β parameters by

$$k'_0 = \left(\frac{\partial n_A}{\partial n_L} \right)_{n_L = 0} = \alpha \quad (17)$$

TABLE I

COMPARISON OF THE CHARACTERISTICS OF ADSORPTION ISOTHERMS OF *cis*- AND *trans*-ANDROSTERONES BY DIFFERENT METHODS

Method	<i>cis</i> -Androsterone			<i>trans</i> -Androsterone		
	α	$10^3 \beta$ (ml/ μ mol)	$10^3 k_0''$ (μ mol) ⁻¹	α	$10^3 \beta$ (ml/ μ mol)	$10^3 k_0''$ (μ mol) ⁻¹
Frontal analysis	1.53	20.3	-17.0	1.37	7.0	-5.2
Elution peak numerical model	1.52	15.7	-13.0	1.41	7.0	-5.4
Moderate overload model:						
Elution peak model	1.52		-12.8	1.36		-5.2
Apex locus	1.53		-14.6	1.41		-5.6

and

$$k_0'' = \left(\frac{\partial^2 n_A}{\partial n_L^2} \right)_{n_L = 0} = \frac{-2\alpha \beta}{V_L} \quad (18)$$

Both isotherms intercept, with a more negative curvature and a larger slope at the origin for *cis*-androsterone. From Fig. 1 it is possible to predict that *cis*-androsterone,

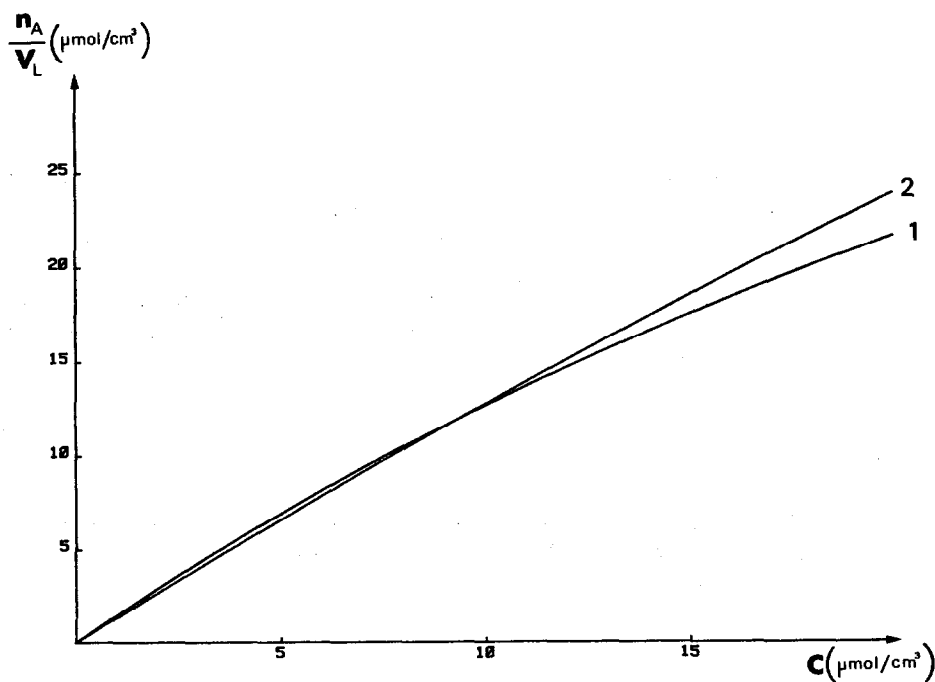


Fig. 1. Liquid-solid adsorption isotherms determined by frontal analysis. 1 = *cis*-Androsterone; 2 = *trans*-androsterone. Solvent, dichloromethane-acetonitrile (90:10, w/w).

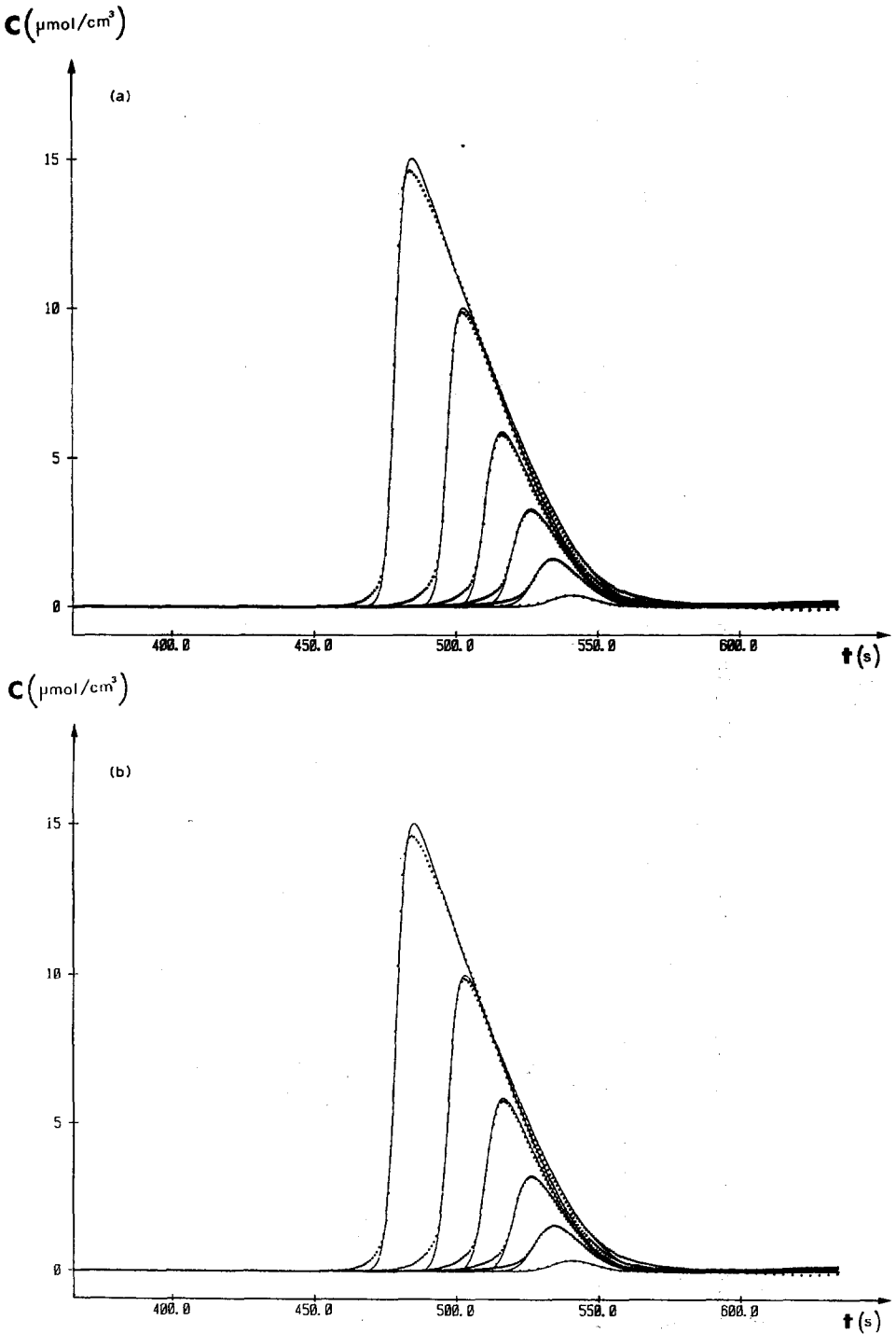


Fig. 2. Best fit of the moderate overload model. Adsorbent, buffered silica gel, 10 μm ; solvent, dichloromethane-acetonitrile (90:10, w/w); flow-rate, 0.98 ml/min. (a) *cis*-Androsterone; (b) *trans*-androsterone. Full lines, theoretical model; broken lines, computer data-acquisition experimental points.

TABLE II
ELUTION PEAK PARAMETERS OBTAINED BY MODEL FITTING METHODS

Compound	m (μmol)	A ($\mu\text{mol} \cdot \text{s/ml}$)	a ($\mu\text{mol} \cdot \text{s/ml}$)	$10^4 D'$ ($\text{cm}^2 \cdot \text{s}$)	k'_0	$10^3 k'_0$ (μmol) ⁻¹	$10^3 \beta$ ($\text{ml}/\mu\text{mol}$)	Method
<i>cis</i> -Androsterone	0.089	6.3	5.9	1.00	1.56	-23.4		Moderate overload model
	0.182	12.9	11.8	1.22	1.55	-24.9		
	0.932	67.7	63.0	1.22	1.52	-17.4		
	1.848	153.0	144.0	1.30	1.51	-13.1		
	3.246	217.5	210.1	1.32	1.51	-13.0		
	11.705	846.4	881.7	3.40	1.48	-10.4		
	16.698	1130.1	1175.6	4.20	1.51	-11.1		
<i>trans</i> -Androsterone	11.705	846.4	809.1	2.6	1.48	-11.4	14.1	Numerical model
	16.698	1130.1	1119.0	4.1	1.52	-12.8	15.3	
	0.079	6.8	7.1	1.10	1.42	-6.7		Moderate overload model
	0.533	16.7	32.7	1.24	1.43	-8.1		
	1.098	75.3	73.3	1.32	1.42	-6.6		
	2.200	149.2	147.8	1.50	1.41	-5.4		
	4.931	305.4	302.7	1.75	1.40	-4.8		
	8.893	591.6	604.6	2.60	1.41	-5.0		
4.431	305.4	295.1	1.70	1.40	-4.9	6.4	Numerical model	
8.893	591.6	568.1	2.20	1.41	-5.5	7.1		

which is the more retained compound at infinite dilution, will be the one less retained at high concentrations.

Fitting of the moderate overload peak model

The modification of the shape of the elution peaks, as a function of the steroid mass injected, are shown in Fig. 2a and b for both steroids.

The plot of the maximum concentration, C_M , versus the retention time at the peak maximum, t_M , is well fitted (Fig. 3) by the apex locus hyperbola (eqn. 10) over a limited concentration range ($< 6 \mu\text{mol/ml}$). From the adjustment of eqn. 10 to the experimental points we determined the values of the retention time at infinite dilution, t_R , and the peak slant coefficient, λ , and calculated the slope and curvature at the origin, k'_0 and k''_0 (Table I).

The parameters k'_0 and k''_0 , determined by the apex locus method provide a good estimation for further adjustment of the moderate overload peak model (eqn. 4) to the experimental data points of the peak profile collected by the computer. The adjustment of the theoretical model is good (Fig. 2) and the four parameters necessary to define the peak profile are listed in Table II (a , D' , k'_0 , k''_0). The parameters k'_0 and k''_0 are in good agreement with those obtained from the apex locus method.

The analytical expression of the peak profile (eqn. 4) is valid over only a limited range of concentration, as shown by the apex locus method. Nevertheless, the adjustment of the moderate-overload elution peak model is satisfactory, even for con-

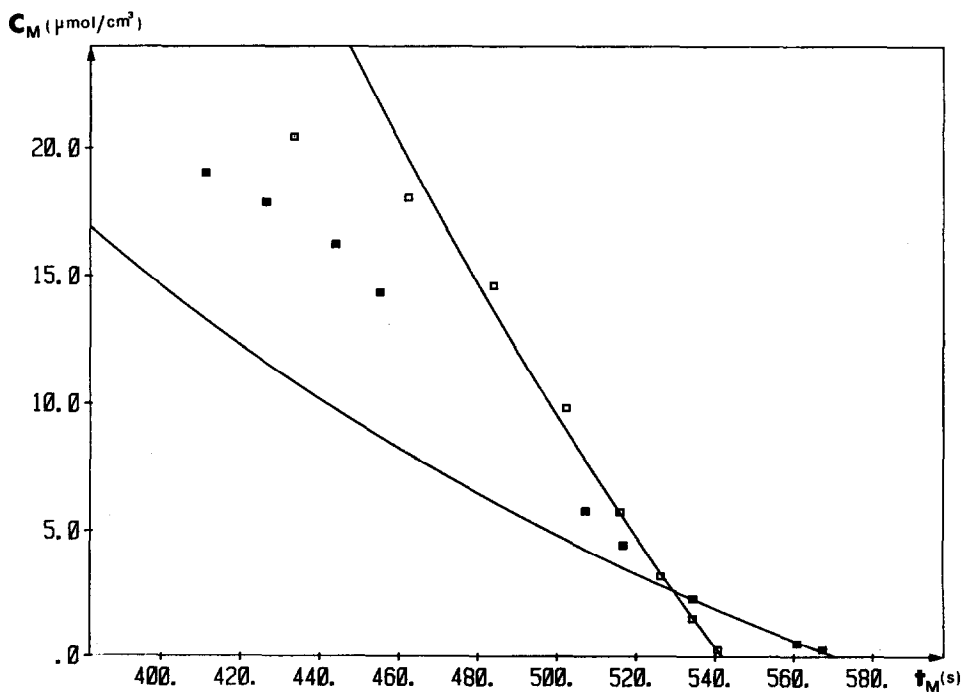


Fig. 3. Variation of the retention volume of peak maximum with the maximum concentration. ■, *cis*-Androsterone; □, *trans*-androsterone. ■, □, Experimental results; —, theoretical curve for C_M vs. t_M (eqn. 10).

concentrations higher than 6 $\mu\text{mol/ml}$ (Fig. 2) and leads to the values of the four parameters (a , D' , k'_0 , k''_0) for defining the chromatographic profile as listed in Table II. In this instance, the peak size parameter, a , is lower than the experimental peak area, A . The dispersion coefficient, D' , is increased by a factor of 4 at the largest amount injected, but k'_0 and k''_0 remain close to the values given by the apex locus method.

Simulation of the peak profile with the numerical scheme

The experimental peak profiles were correlated with the theoretical ones simulated according to the numerical scheme previously described. Adjustments were carried out only for the highest concentrations (Fig. 4) because of the long calculation times involved with this procedure. We did not try to fit the lower concentration peaks because the dispersion coefficients were lower than those at high concentration, and the number of calculation steps is proportional to the dispersion coefficient (eqn. 15).

The parameters α and β of the Langmuir isotherm, the dispersion coefficient, D' , and the peak size parameter, a , introduced into the numerical scheme were allowed to vary until the best adjustment was obtained between the experimental and theoretical profiles. The corresponding k'_0 and k''_0 values (Table III), calculated from α and β , are in good agreement with those measured by frontal chromatography or those determined by the moderate overload peak model.

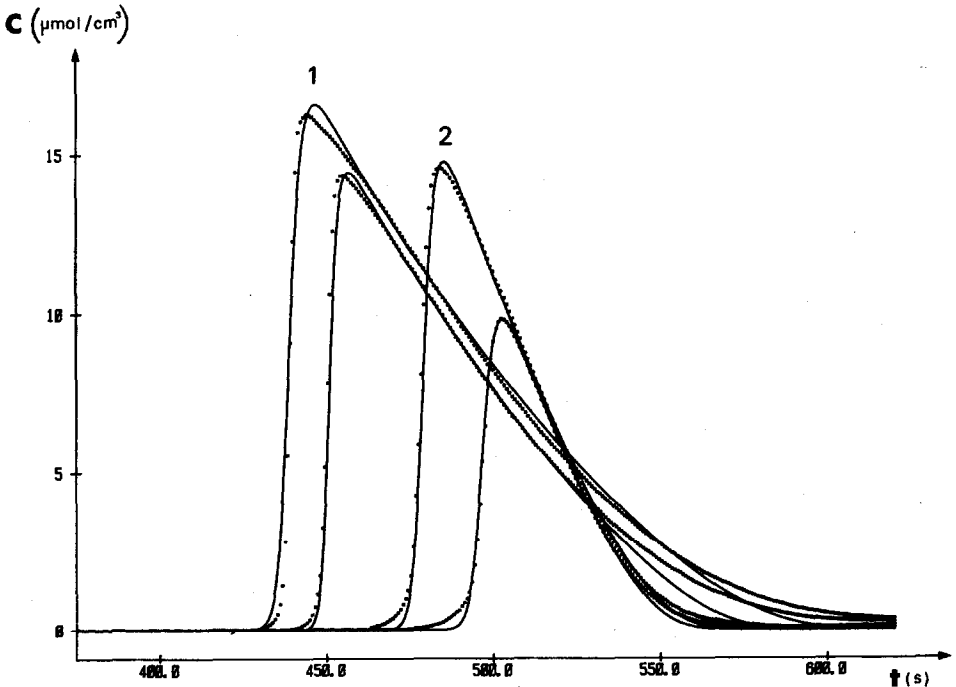


Fig. 4. Best fit of elution peak numerical model. 1 = *cis*-Androsterone; 2 = *trans*-androsterone. Full lines, theoretical model; broken lines, computer data-acquisition experimental points. Experimental conditions as in Fig. 2.

It is interesting to compare the values of the parameters obtained by numerical simulation methods with those of the moderate overload peak model (Table II) for the higher concentrations when the peak analytical expression (eqn. 4) is no longer valid, but the adjustment is still good. The peak area parameter, a , is in good agreement with the experimental value, A , only in the case of numerical simulation.

The dispersive coefficients estimated obtained by the two methods are fairly close (Table II), as a dispersive term was taken into account in the mass balance equation of the moderate overload model. Evidence is provided that dispersion arising from the discrete steps of the numerical integration method (eqn. 15) is equivalent to dispersion through the column.

Comparison of peak-profile modelling methods

There are several advantages in the numerical simulation method: any kind of adsorption isotherm may be assumed, there is no restriction on the concentration range and only one injection is necessary to obtain the parameters of an isotherm equation if a theoretical model can be assumed.

In this work, the numerical simulation of the peak was based on the assumption of a Langmuir-type adsorption isotherm, and the parameters α and β of eqn. 16 were introduced into the simulation program to obtain the best fit between the model and the experimental elution profile. The validity of this hypothesis is demonstrated in Table I, as α and β determined by this method are in good agreement with those measured by frontal chromatography.

The numerical simulation method requires long calculation times on a micro-computer, as peak adjustments imply many trials. The moderate overload peak model, which approximates the isotherm as a parabola, allows a quick determination of the first and second derivatives at the origin, k'_0 and k''_0 , which may be obtained from a plot of the retention time *versus* peak height at the peak maximum. In the special case of a Langmuir isotherm, both α and β , which characterize the adsorption over the whole concentration range, can be calculated from the values of k'_0 and k''_0 (eqns. 17 and 18). The α and β values (Table I) obtained from the moderate overload peak model are in good agreement with Langmuir parameters determined by numerical peak simulation or by frontal chromatography.

CONCLUSION

Despite the similar structures of the steroids studied, both of which exhibit an isotherm of the Langmuir type, the curvature of *cis*-androsterone isotherm is three times lower than that of *trans*-androsterone. This causes *cis*-androsterone, which is the more retained compound at infinite dilution, to be the less retained when large concentrations are injected. A careful determination of the adsorption isotherm is therefore important, as a simple look at the chromatogram in Fig. 5 might lead to mistakes if one attributes the same retention order at finite concentration and at infinite dilution.

For optimization in preparative liquid chromatography, modelling is essential in similar instances, as only one product, that with the lowest retention time at high concentrations, could be obtained in a pure form. In order to know how the composition of the binary solute mixture will vary at the column outlet, it is useful to

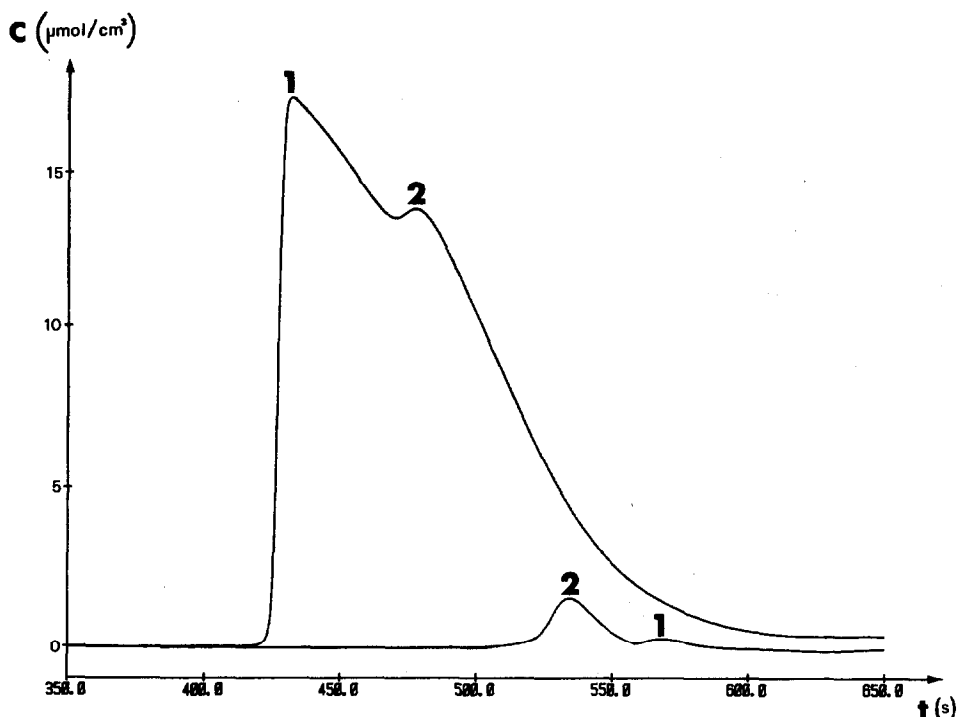


Fig. 5. Experimental elution peak of mixtures of androsterone isomers. 1 = *cis*-Androsterone; 2 = *trans*-androsterone. Small amounts injected: 0.026 mg *cis*-, 0.155 mg *trans*-. Large amounts injected: 5.22 mg *cis*-, 1.80 mg *trans*-.

build a theory involving mutual influences on adsorption equilibrium. Therefore, a two-compound elution model should be considered. Work along these lines is now in progress.

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