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Binding of Anabolic Steroids to Human Serum Albumin and Possible Interactions with Other Drugs

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Abstract

Binding of testosterone, epitestosterone and metandienone to human serum albumin (HSA) was studied by the gel filtration method. The experimental results obtained are presented graphically using the method of Scatchard and calculated by linear and non-linear regression: (a) for testosterone and epitestosterone there are two independent and nonequivalent binding sites on the HSA molecule; (b) the binding constants calculated were of the same order of magnitude for the higher affinity binding site (K_1/n for epitestosterone was 3.7×10^5 L mol^{-1}, and for testosterone was 9.7×10^5 L mol^{-1}, for the concentration range from 0.64 to 1.84×10^{-5} mol L^{-1});
(c) differences estimated between the higher affinity binding site constants were statistically significant ($p<0.05$);
(d) differences between the low affinity binding constants (for concentration range between 1.84 to 7×10^{-5} mol L^{-1}) estimated for testosterone ($K_2/n=0.22 \times 10^5$ L mol^{-1}) compared with the constants evaluated for epitestosterone ($K_2/n=0.05 \times 10^5$ L mol^{-1}) could not be statistically evaluated because of high sd values obtained for the low affinity ligand epitestosterone;
(e) for metandienone data for Scatchard plot could be best approximated by a single straight line in the whole range of concentrations tested ($K_1/n=0.18 \times 10^5$ L mol^{-1}) indicating only one set of low affinity binding sites; (f) both isomers (testosterone and epitestosterone) as well as metandienone are bound into a low extent to the HSA molecule (i.e. at a ligand concentration of 1.04×10^{-5} mol L^{-1} the percent of binding ranged from about 23% to 40% for the compounds tested, and higher extent binding was obtained for testosterone when compared to epitestosterone (40% and 23%, respectively). Displacement experiments performed using stanozolol revealed that in the presence of the displacement agent, the percent of binding decreased, however, the differences between estimated binding constants were not statistically significant. When binding of metandienone was tested in the presence of stanozolol the results obtained did not allow calculation of the binding parameters by the Scatchard method. Stanozolol affects the binding of the (-)-isomer of etomoxir at both high and low affinity binding sites. For the (+)-isomer in the presence of stanozolol, data for Scatchard plot could be best approximated by a single straight line indicating that stanozolol affects the binding of (+)-isomer predominantly at its high affinity sites. The effects of stanozolol can be explained by both competitive displacement and by displacement caused by conformational changes in the HSA molecule.

2. Introduction

The significance and the role of human serum albumin as a principal binding protein in plasma for drug's and endogenous compounds (i.e. bilirubin, fatty acids, tryptophan, etc.) was recognised very early (1-5). It is generally accepted that only unbound (or so called "free") fraction of a drug in plasma is responsible for the pharmacological and/or toxicological effects. Besides other functions HSA acts as solubilizer and transport macromolecule for drugs and endogenous compounds which are otherwise sparingly soluble in the water portion of the plasma (3). The complete amino acid sequence of human and bovine albumins was published in 1975 (6, 7, 8). According to these reports albumin is a single peptide chain of over 580 residues (9). Recently, the three-dimensional structure of human serum albumin has been determined crystallographically. The HSA contains three homologous domains that form a heart-shaped molecule, each of domains is a product of the two subdomains. The protein consists 585 amino acids (Mr 65 K) and contains 17 disulfide bridges. The principal regions of ligand binding to human serum albumin are located in hydrophobic cavities in subdomains IIA and IIIA, which exhibit similar chemistry, Table 1 (10).

Table 1. Ligand binding location to HSA according to ref. 10.

Ligand	Observed location
Aspirin	IIA IIIA
Warfarin	IIA
Diazepam	IIIA
Digitoxin	IIIA
Clofibrate	IIIA
Azidothymidine	IIIA

The ligand spectrum of compounds bound to HSA consists of chemically diverse molecules which include endogenous and synthetic steroids as well (11). Comparing to other steroid binding plasma proteins (sex steroid binding protein, SBP, and corticosteroid binding globulin, CBG), binding of steroids to HSA is a low affinity binding and, therefore, it could be expected that the binding to HSA will be of therapeutical/toxicological

significance only at high concentrations of the compounds in plasma (i.e. following high doses applied in doping). For example, binding affinity of SHBG, CBG, and HSA for testosterone are about 1.6×10^9, 5.3×10^6, and 4×10^4 L mol^{-1}, respectively (12). Following high doses of endogenous drugs, and natural and synthetic steroids displacement of the endogenous steroids might occur from their high affinity, specific binding protein SBP. In such a case, in spite of steroid's low affinity binding to HSA, the binding might predominate and become important determinant for activity and distribution of the endogenous steroids. This is even more obvious if we take into account that unbound plasma steroid concentrations, regulated by steroid-to-HSA binding, are more or less constant during high plasma fluctuations of total steroid plasma concentrations (11). Testosterone was, for instance, displaced from the SBP by drugs and synthetic steroids such as methyl testosterone, fluoxymesterone, danazol and norgestrel (13, 14). However, similarly to binding of other drugs to HSA (5), also the binding of steroids might be affected by different factors such as: presence of competitive agents, changed concentrations of endogenous compounds and of HSA, physiological status etc. For instance, free fraction of testosterone in plasma can rise following increased concentration of fatty acids (11, 15). Furthermore, it can well be expected that administration of natural and synthetic steroids might result in displacement of endogenous and exogenous compounds bound to HSA depending on the binding affinity of the compounds. Such displacement can occur as a result of both competitive and noncompetitive interaction depending on the mechanism and location of the binding site. The exact location of binding of steroids on the HSA molecule is until now unknown, however, it was reported that the primary binding site for both testosterone and pregnenolone is at the second domain of the HSA molecule (15). In addition, it was reported that testosterone and stanozolol alter the helical content of HSA by inducing conformational changes in the protein molecule. The changes observed were dependent on the drug: protein molar ratios (16). Our results on the effect of stanozolol on binding of etomoxir isomers (sodium 2/6-(4-chlorophenoxy)-hexyl/-oxiran-2-carboxylate dihydrat) to HSA allowed as to propose that stanozolol is reducing the affinity of the HSA for the isomers by both competitive displacement and displacement caused by conformational changes in the protein molecule. Particularly significant changes have been observed when binding of the (+)-isomer was investigated in the presence of stanozolol suggesting stereoselective interaction of the isomers with the HSA (17).

In this work we examined the binding properties of testosterone, its isomer epitestosterone, and metandienone to HSA by gel filtration method (see structures). Anabolic hormone stanozolol was chosen for displacement study because it is misused by sportsmen, frequently in combinations with other drugs and steroids. Possessing the pyrazole ring in the structure stanozolol interacts with cytochrome(s) P450 by forming high-

affinity ligand complex thus inhibiting the drug metabolizing enzymes *in vitro* (18).

Structural formulas

3. Experimental Section

Compounds - Testosterone was obtained from Schering, Berlin, Germany; Stanozolol was a gift from Sterling Winthrop Group Ltd, Guilford, UK; Epitestosterone was kindly supplied by Prof. Dr. M. Donike, Institut für Biochemie, Deutsche Sporthochschule, Köln, Germany. The compounds were used without further purification. HSA was obtained from the Institut of Immunology, Zagreb, Croatia. All other chemicals were p.a. grade, and deionized water was used throughout.

Determination of Binding to HSA by Gel Filtration - Binding experiment were performed according to the method described elsewhere (18) using columns (30x9 cm, Pharmacia, Uppsala, Sweden) filled with Sephadex G-25 Fine (Pharmacia, Uppsala, Sweden) and equilibrated with 0.067 mol L^{-1} phosphate buffer (pH 7.4). The sample (20 mL) was applied to the column, and a constant flow rate (24 mL/h) was maintained with a peristaltic pump (P-3, Pharmacia, Uppsala, Sweden). Collected samples were monitored by UV absorption measurements and HPLC analysis; fractions containing protein were monitored at 280 nm, whereas protein free fractions were monitored at 248 nm. Calibration curves were tested daily and compared statistically.

The concentration of HSA in the experiments was kept constant at 1.45×10^{-5} mol L^{-1} the final concentration of compounds applied to the column, with and without the HSA, was in the range from $0.8\text{-}7.0 \times 10^{-5}$ mol L^{-1}. Stock solution of testosterone, epitestosterone and metandienone were prepared in methanol and added to HSA or buffer solution in uL amounts.

Displacement experiments were performed with stanozolol as displacing agent. Stanozolol was dissolved in absolute ethanol and displacement experiments were performed at two concentrations of stanozolol (0.8×10^{-5} mol L^{-1} and 1.45×10^{-5} mol L^{-1}). The concentrations of testosterone, epitestosterone, metandienone and HSA were the same as in binding experiments.

Calculation of results - The results are presented and calculated according to Scatchard (19-23). Data for the Scatchard plots were fitted and calculated with a computer program for linear and non-linear (limiting slope) regression (23). A significance test was used for evaluation of the experimental data and fitting to the appropriate model of binding.

4. Results and discussion

The Scatchard plots calculated for testosterone, epitestosterone and metandienone fitted and calculated by both the linear and non-linear regression are presented in Figures 1-3. The experimental data for testosterone and epitestosterone could be best approximated by two straight lines with linear regression fit, suggesting for both compounds two independent and nonequivalent classes of binding sites. For metandienone, the results could be best approximated by a single straight line indicating a single set of binding site for the compound of similar affinity. The binding parameters and the results of statistical evaluation are presented in Tables 2 and 3. The high affinity binding constant for testosterone was significantly higher ($p<0.05$) when compared with the binding constant for epitestosterone. Because of high SD values obtained for the low affinity binding constant for epitestosterone (Table 2) the values could not be compared with those obatined for testosterone The high affinity binding constant for testosterone obtained (K_1 /n=9.7 +/-2.2 x 10^5 mol L^{-1}) is for one order of magnitude higher when compared with the value of the primary bonding connstant reported and obtained by different experimental method (K_1 =2.0+/-0.2 x 10^4 mol L^{-1}, ref.15). However, the value obtained for the low affinity binding constant (K_2 /n=0.22+/-0.03 x10^5 mol L^{-1}) is in good agreement with the affinity reported for the primary binding constant. Our results show that there are two primary binding sites for testosterone and epitestosterone, and that the value for the high affinity binding constant is statistically different when compared to the value obtained for epitestosterone.

Figs. 1 - 3, and Tables 2 and 3

The number of binding sites (n) for the high affinity binding site was as low as 0.2 for both isomers indicating that only a limited number of HSA molecules are occupied at the lower range of concentration of testosterone and epitestosterone. The number of binding sites at the higher range of concentration for the low affinity binding site was 2 and 3 for testosterone and epitestosterone, respectively. These values agrees very well with the reported values for testosterone and pregnenolone (n=1 and n=2, respectively) obtained by equilibrium dialysis (15).

The plot of the percent of binding versus total concentration of the compound for testosterone in comparison with epitestosterone and metandienone are presented in Figs. 4 and 5. The experimental data indicate non-linear binding with increasing concentration of the compounds tested. Also these plots, as well as the results on the percent of binding presented in Table 4, show differences in the extent of binding to HSA. Particularly obvious difference was obtained for the values of testosterone and epitestosterone (40.5% and 24%, respectively). The results on the binding of

metandienone in the presence of stanozolol could not be interpreted because of inconsistences of the values obtained repeatedly.

Figs. 4 and 5

The binding parameters of testosterone in the presence of stanozolol are presented in Table 5. Statistical evaluation of the data indicate that the affinity of binding, as well as number of binding sites obtained in the presence of two concentrations of stanozolol are statistically equivalent. The Scatchard plots and the plots of the percent of binding versus total concentration of testosterone, without and in the presence of stanozolol, are presented in Figs. 6-8. These data indicate that

Figs. 6-8 and Table 5

stanozolol, at the experimental conditions applied, does not interfere with the binding of testosterone. Different results were obtained when binding of etomoxir isomers was investigated in the presence of stanozolol (18). Statistically significant decrease of the binding affinity was observed for both isomers in addition to displacement of the (+)-isomer from its high affinity binding site. The effects were explained by both the competitive displacement and by displacement caused by conformational changes of the protein molecule. Recent report (16) confirmed the ability of both testosterone and stanozolol to induce conformational changes in the human serum albumin molecule. The changes were dependent on drug to protein ratio, and different patterns were observed for either of the compound. Testosterone induced conformational changes resulted from binding to the high affinity site without change of conformation when the low affinity binding site were occupied. In difference, stanozolol induced conformational changes were not directly related to the total drug concentration indicating more complex drug-protein interaction (16). These observations supports the results from our investigations showing diverse effects of stanozolol on the binding of testosterone, metandienone and isomers of etomoxir.

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Table 2. Estimated binding parameters for Testosterone, Epitestosterone and Metandienone - linear regresion

Compound Range of conc. ($x10^{-5}$), mol L^{-1}	K/n($x10^{5}$) L mol^{-1}	n mol/mol	SD Around Straight Line	r/c_f
Testosterone				
0.8 - 1.12	9.8 ± 2.2*	0.2 ± 0.06	0.029	0.005
1.6 - 7.0	0.22 ± 0.03	1.8 ± 0.23	0.017	0.013
Epitestosterone				
0.64 - 1.12	3.7 ± 0.47	0.2 ± 0.03	0.027	0.009
1.84 - 7.0	0.05 ± 0.08**	2.8 ± 4.49	0.013	0.011
Metandienone				
0.96 - 7.0	0.18 ± 0.038	1.1 ± 0.25	0.02	0.015

* Statistically significant difference ($p<0.05$)
**Results not comparable because of high SD value
HSA concentration, 1.45×10^{-5} mol L^{-1}

Table 3. Estimated binding parameters for Testosterone, Epitestosterone - non-linear regresion

Compound Range of conc. ($x10^{-5}$), mol L^{-1}	K/n($x10^{5}$) L mol^{-1}	n mol/mol	SD Around Curve
Testosterone			
0.8 - 1.12	11.53	0.2	0.11
1.6 - 7.0	0.2	1.7	
Epitestosterone			
0.64 - 1.12	4.64	0.1	0.06
1.84 - 7.0	0.05	2.7	

HSA concentration, 1.45×10^{-5} mol L^{-1}

Table 4. Percentage of binding of testosterone, epitestosterone and metandienone*.

Compound**	Percentage of binding (%)
Testosterone	40.5
Testosterone + Stanozolol[1]	37
Testosterone + Stanozolol[2]	33
Epitestosterone	24
Metandienone	23
Metandienone + Stanozolol	not consistent

* HSA concentration 1.45×10^{-5} mol L^{-1}
** Steroid concentration 1.04×10^{-5} mol L^{-1}

[1] Stanozolol concentration 0.8×10^{-5} mol L^{-1}
[2] Stanozolol concentration 1.45×10^{-5} mol L^{-1}

Table 5. Estimated binding parameters for Testosterone in the presence of Stanozolol

Testosterone Range of conc. ($\times 10^{-5}$), mol L^{-1}	$K/n(\times 10^{5})$ L mol^{-1}	n mol/mol	SD Around Straight Line	r/c_f
	A. Stanozolol (0.8×10^{-5} mol L^{-1})			
0.8 - 1.04	3.7 ± 3.3	0.3 ± 0.27	0.054	0.018
1.6 - 7.0	0.17 ± 0.04	1.9 ± 0.4	0.023	0.017
	B. Stanozolol (1.45×10^{-5} mol L^{-1})			
0.8 - 1.04	2.7 ± 0.9	0.3 ± 0.1	0.016	0.01
1.6 - 7.0	0.14 ± 0.04	1.9 ± 0.51	0.02	0.015

HSA concentration, 1.45×10^{-5} mol L^{-1}

Figure captions:

Fig. 1. Scatchard plot for the binding of testosterone to HSA.
A. Linear regression; B. Limiting slope method
All points are experimental, whereas the solid lines were computed from the binding parameters. Results of statistical evaluation and concentration of the compound and HSA are given in Tables 2 and 3.

Fig. 2. Scatchard plot for binding of epitestosterone to HSA.
A. Linear regression; B. Limiting slope method
For description see Fig. 1, and Tables 2 and 3.

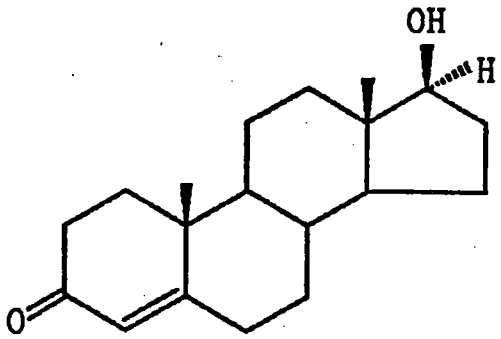
Fig. 3. Scatchard plot (linear regression) for binding of metandienone to HSA. For description see Fig. 1 and Table 2.

Fig. 4. Direct plot of concentration dependent on the percentage of bound testosterone (y1), and epitestosterone (y2).
For concentrations see Table 2.

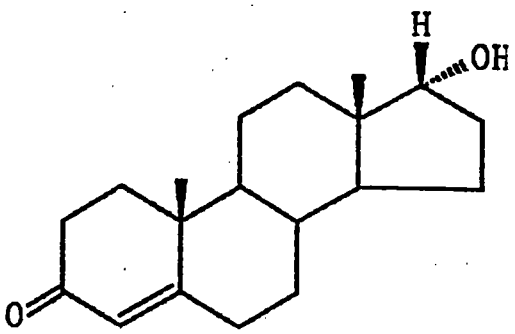
Fig. 5. Direct plot of concentration dependent on the percentage of bound testosterone (y1) and metandienone (y2).
For concentrations see Table 2.

Fig. 6. Scatchard plot (linear regression) for binding of testosterone to HSA in the presence of stanozolol. For description see Fig. 1 and Table 5.
A. Concentration of stanozolol: 0.8×10^{-5} mol L^{-1}.
B. Concentration of stanozolol: 1.45×10^{-5} mol L^{-1}.
For concentrations of testosterone and HSA see Table 5.

Fig. 7. Direct plot of concentration dependent on the percentage of bound testosterone alone (y1), and in the presence of stanozolol (y2).
A. Concentration of stanozolol: 0.8×10^{-5} mol L^{-1}.
B. Concentration of stanozolol: 1.45×10^{-5} mol L^{-1}.
For concentrations of testosterone and HSA see Table 5.



TESTOSTERONE



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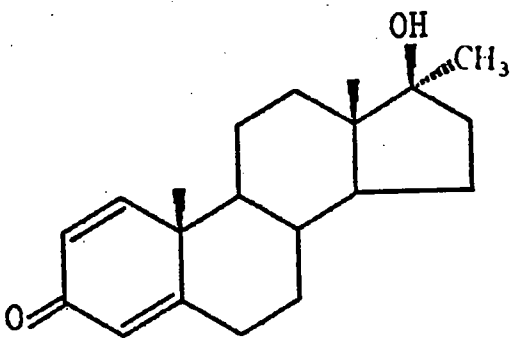

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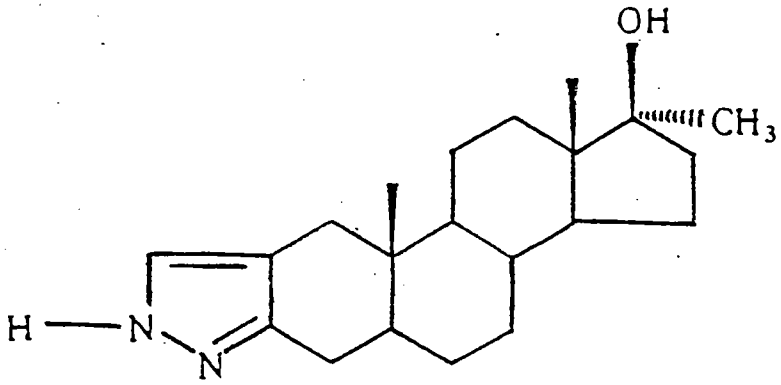

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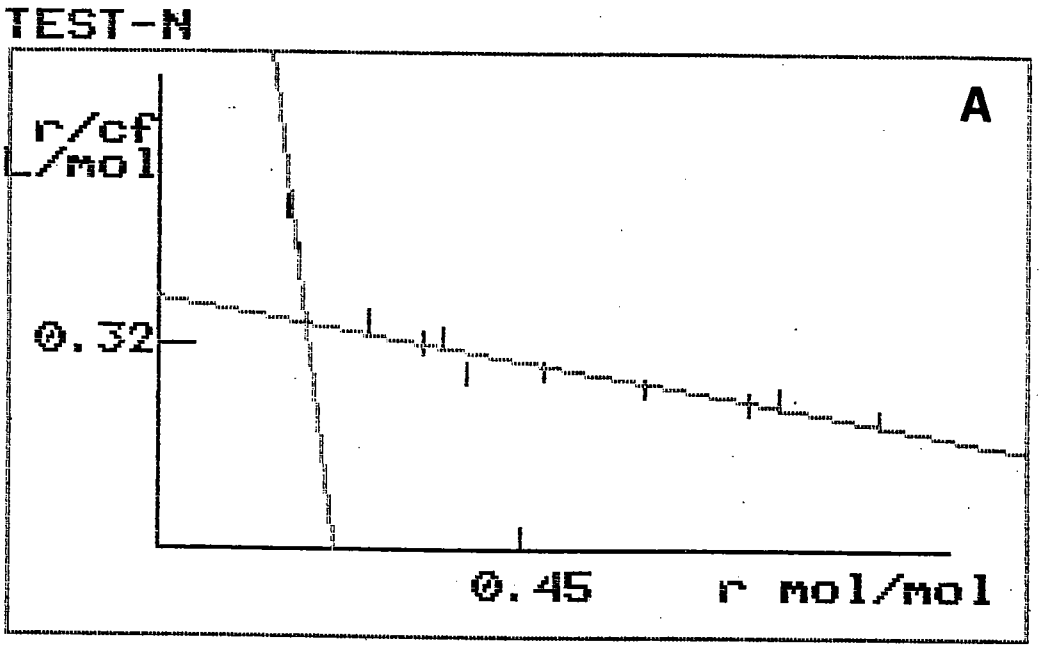
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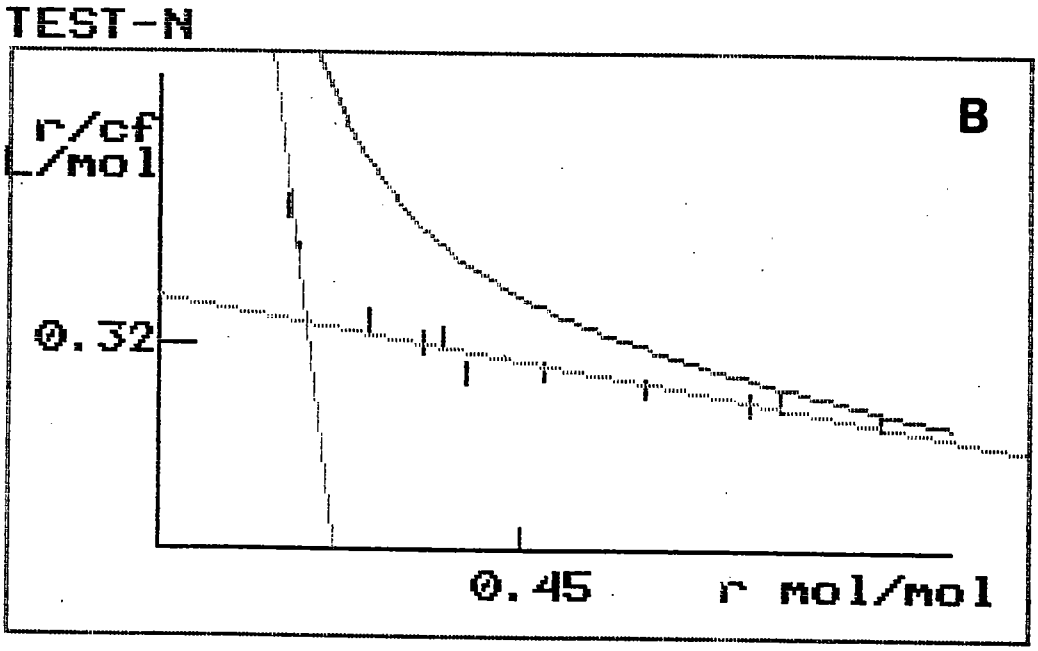
r mol/mol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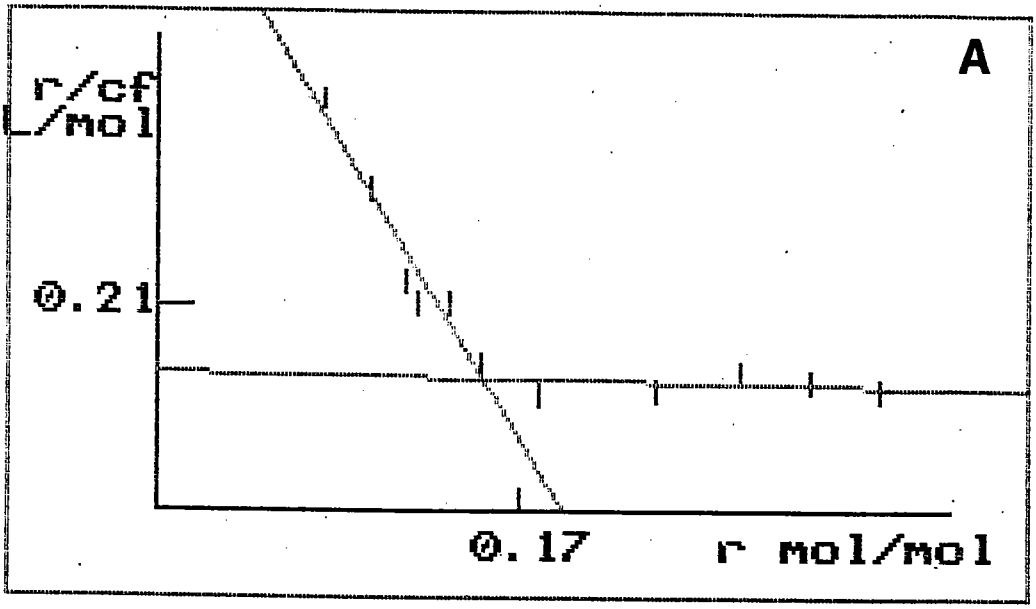

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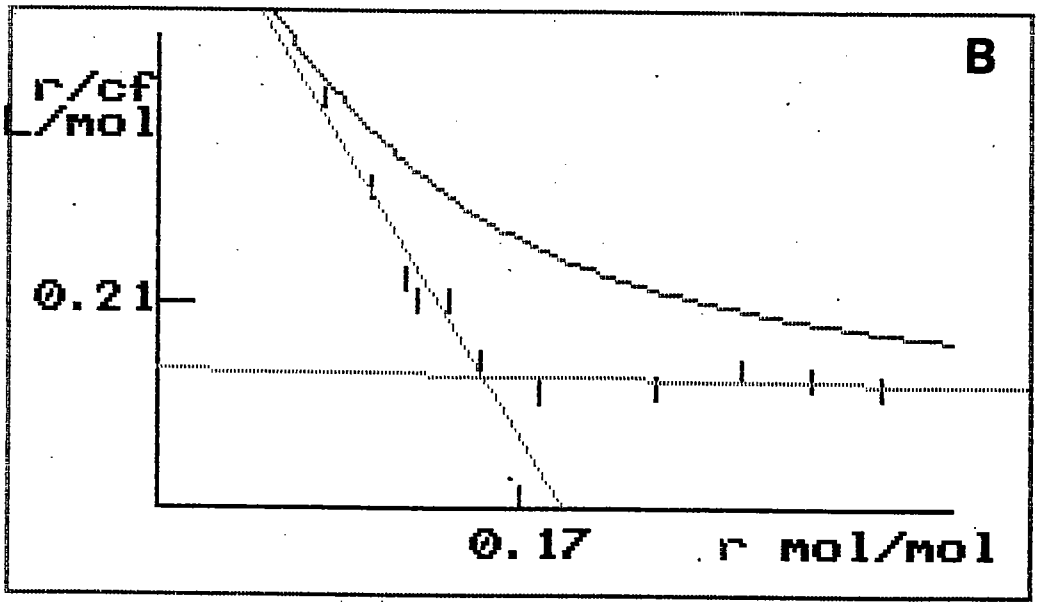

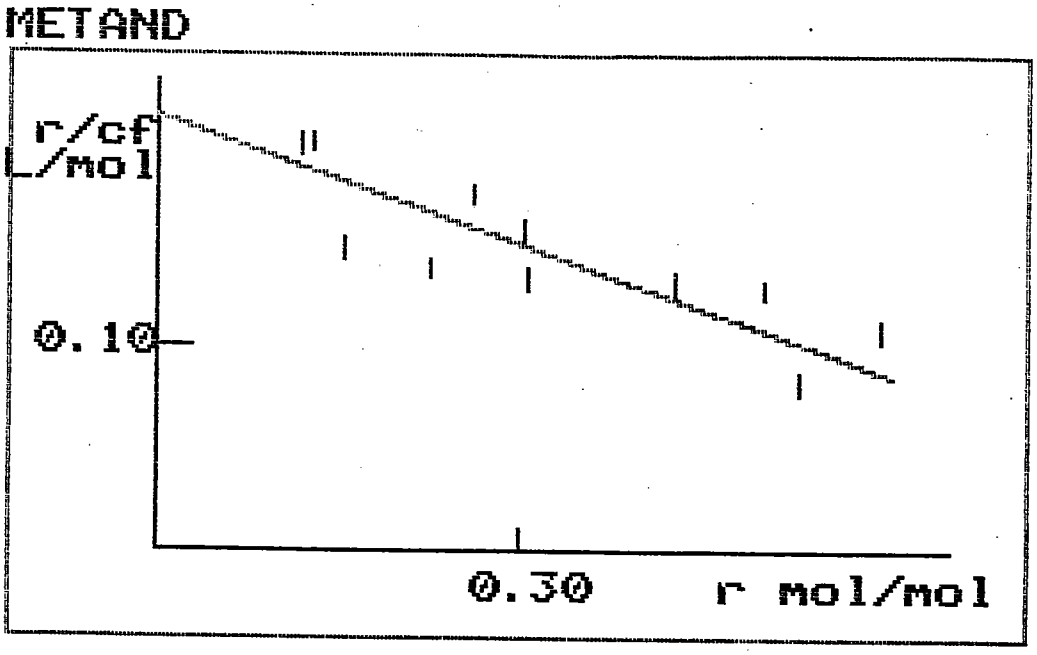
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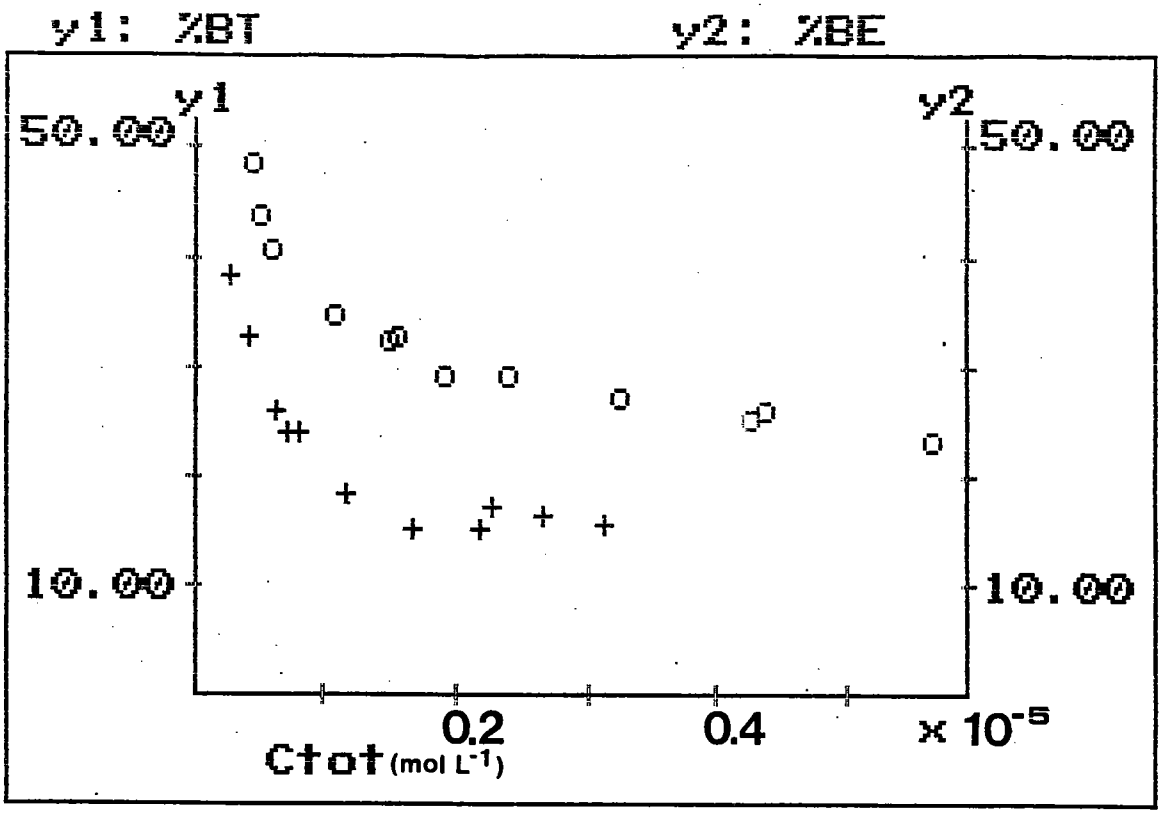
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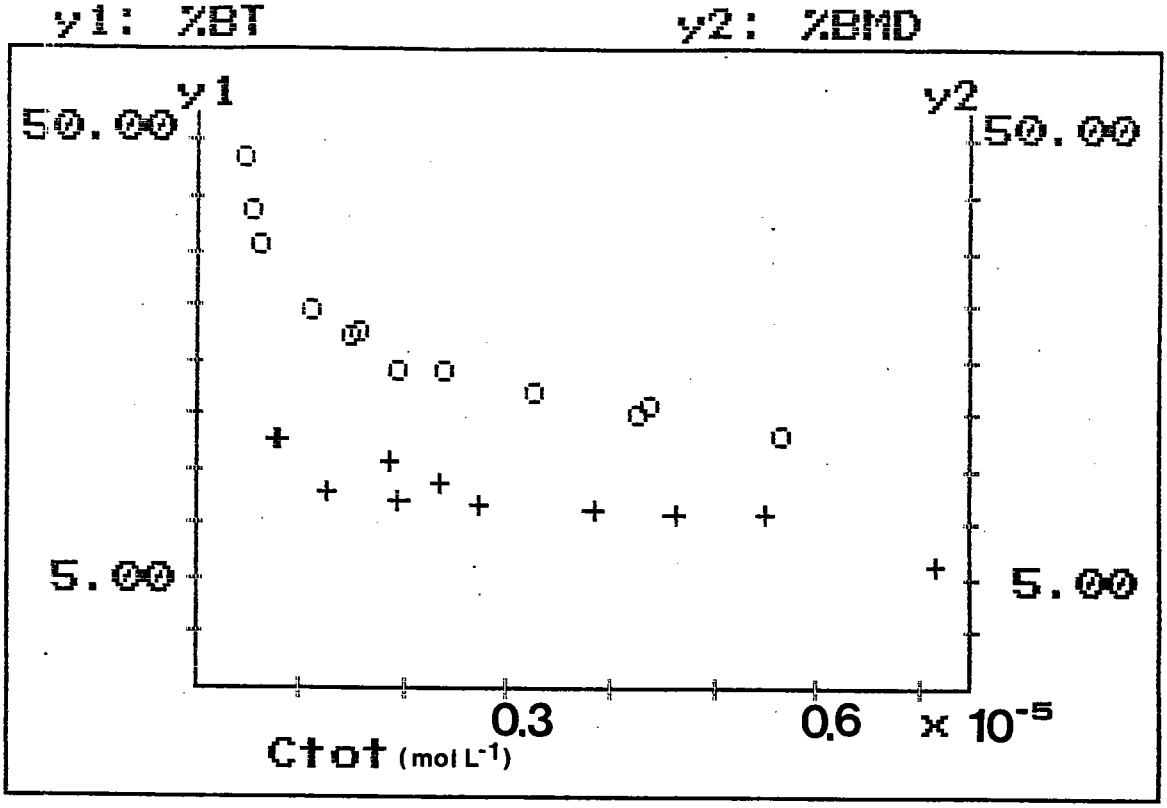
y2: %BE
y1
y2
50.00
50.00
10.00
10.00
0.2
0.4
x 10-5
Ctot (mol L-1)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y1
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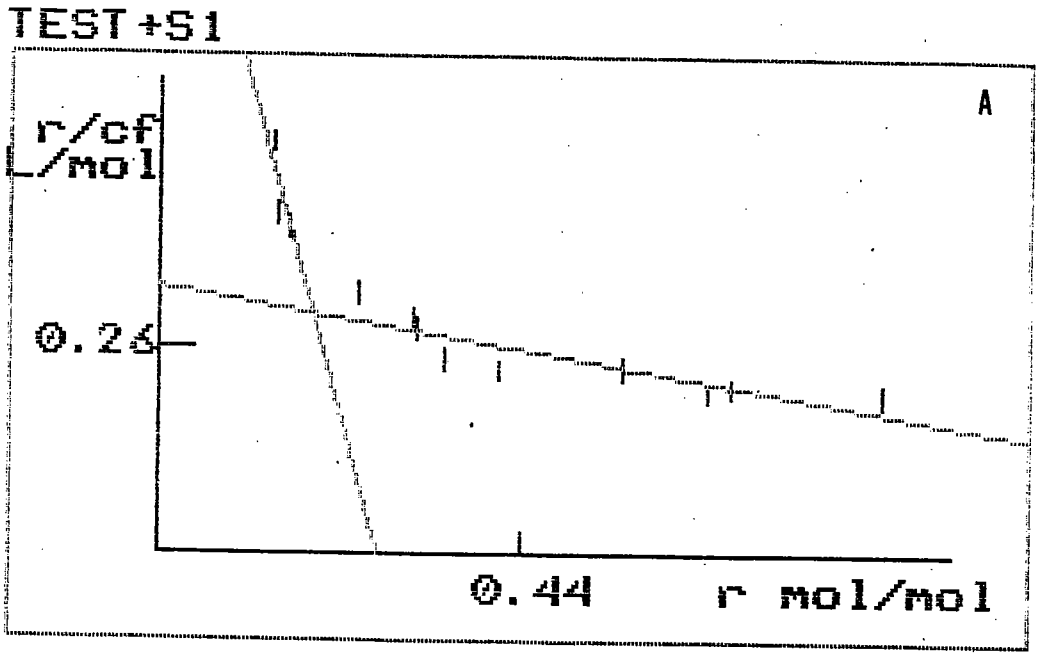
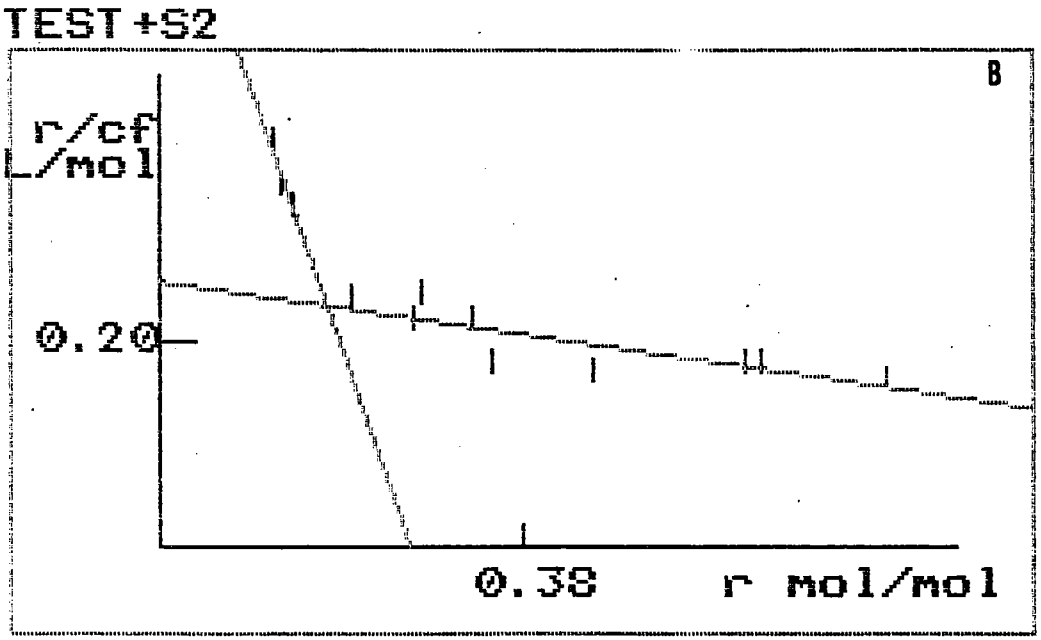
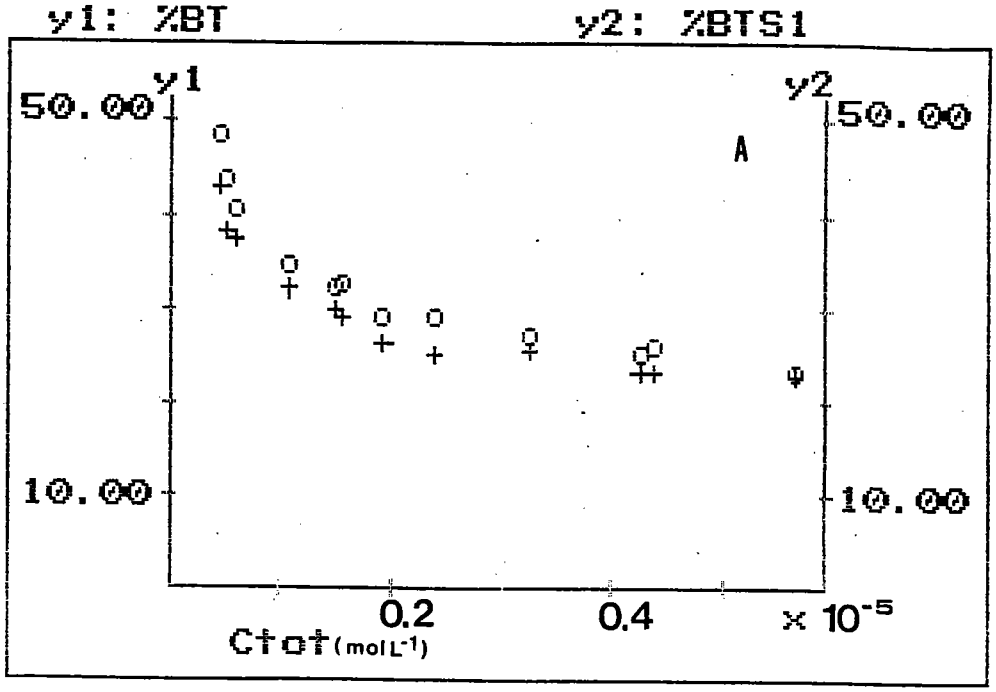
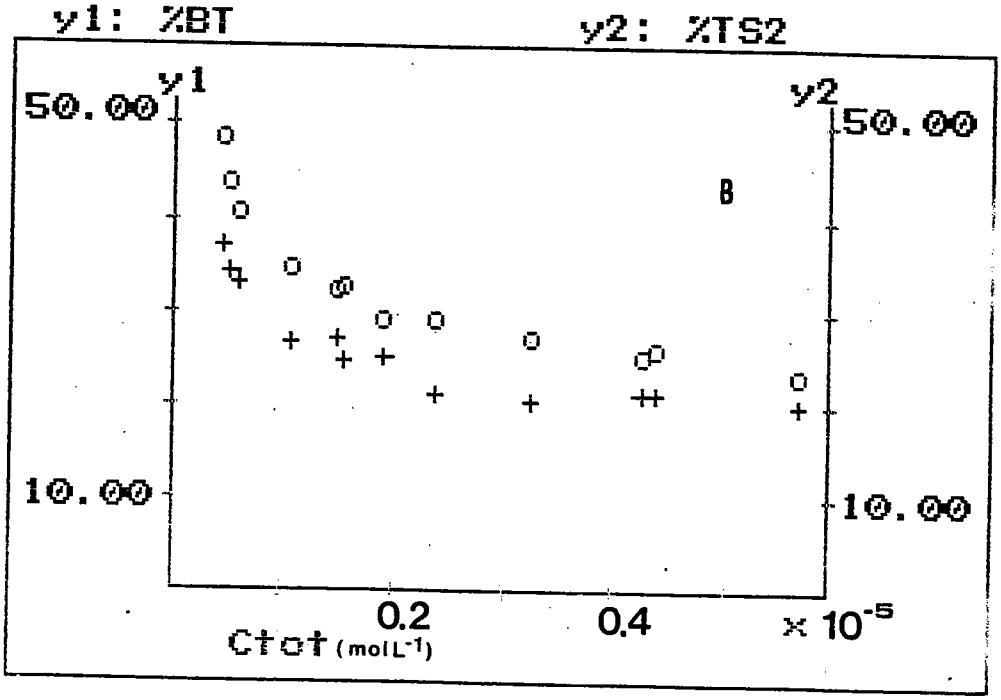
x 10⁻⁵
Ctot (mol L⁻¹)


TEST+S1
A
r/cf
L/mol
0.26
0.44
r mol/mol


TEST+S2
B
r/cf
L/mol
0.20
0.38
r mol/mol


y1: %BT
y2: %BTS1
y1
y2
50.00
50.00
10.00
10.00
A
0.2
0.4
× 10⁻⁵
Ctot (mol L⁻¹)


y1: %BT
y2: %TS2
y1
y2
50.00
50.00
10.00
10.00
B
0.2
0.4
× 10⁻⁵
Ctot (mol L⁻¹)