PREPARATION AND CHARACTERISATION OF IMMUNOADSORBENTS FOR HIGH PERFORMANCE LIQUID AFFINITY CHROMATOGRAPHY (HPLAC)

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ABSTRACT : *The efficacy of activation methods and coupling were studied in the context of performance in batch and fixed bed binding experiments utilising cell culture fluids or blood plasma as feedstock. Conclusions were drawn regarding selection of solid phase according to pore size, rigidity, pH stability, chemistry of derivation and activation, and gross concentration of immobilised ligand required for optimal performance in analytical and preparative fractionation of complex feedstreams.*

KEY WORDS : *Immunoadsorbent, High Performance Liquid Affinity Chromatography (HPLAC).*

INTRODUCTION :

The application of high performance liquid affinity chromatography (HPLAC) is well established both in analytical as well as preparative methods in biotechnology [1-5].

Bioaffinity interaction may be regarded as specific (monoclonal antibody- antigen), generic (lectin- glycoprotein) or pseudo- affinity (triazine dye albumin or dehydrogenase), but the majoroity of so called chromatographic applications exploit bulk adsorption- desorption characteristics rather than true chromatographic partition between solid and mobile phases.

Solid phases applicable to the assembly of affinity HPLC adsorbents for macromolecular

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analysis or recovery require unique properties in terms of porosity, surface area and ease of biochemical derivation. Such characteristics are not mandatory in bioaffinity purification systems, as evidenced by the wide application of agarose, acrylamide and dextrans in LC processes [6].

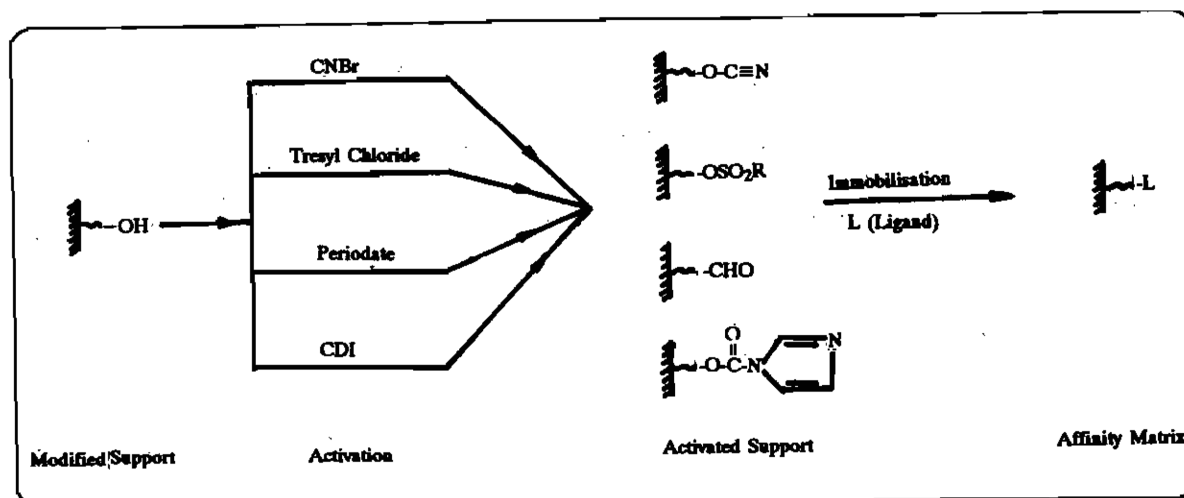
In the present study, various types of rigid materials were used, including *Lichrosorb* Si-60 (10μm particle diameter(dp),60Å pore size (ps)), hydrophilic macroporous polymer supports (HMPS) (10,20 μm dp,1000Å ps)and non-porous polymers presently available for HPLC. Nevertheless, the application of silica is limited due to presence of negatively charged silanol groups. These promote both non specific adsorption and the denaturation of proteins directly adsorbed. The alkaline solubility of silica above pH values of 8.0 is also another disadvantage of these supports. Chemical modification of silica surfaces results in a hydrophilic character suitable for the activation and immobilisation of affinity ligands. These bioselective agents are characterised by low or high molecular weights and are applicable to purification processes (e.g. purification of MCAB in culture supernatant or low level of other biochemicals in a crude mixture). Chemical modifications can be achieved under controlled reaction conditions. As an alternative to silica; examination has been made of materials such as HMPS having rigid characteristics and hydrophilic surfaces suitable for working at high pressures. The major advantages of these supports over silica materials include stability whithin a wide range of pH 1-13, and hydrophilic surfaces suitable for both direct activation and processes of ligand immobilisation.

However, the controlled assembly and operation of affinity HPLC materials will benefit applications requiring rapid cycle times, automated processing and sensitive detection of product contaminants. In addition, particular advantage is demonstrated in the utilisation of miniaturised preparative affinity systems for the rapid off- line bioquantitation of specific analytes present in the complex mixtures associated with productive fermentations, protein purifications and effluent treatments [7].

The establishment of affinity HPLC methods for macromolecular fractionation involves important additional considerations. The pore size of raw materials, the hydrophilic derivation of surfaces and the molecular dimensions of affinity ligands and products strongly influence the performance of matrices.

The perparation of biospecific adsorbents for affinity chromotography requires efficient activation and coupling methods for attachement of ligands to suitable matrices. Although a variety of activation procedures and coupling strategies is now available, problems such as reagent toxicity, activation pH, side reactions, non specific adsorption and ligand leakage and subsequent coupling, limits applicability. The current work has used tresyl chloride,periodate,1,1′ carbonyldiimidazole (CDI) and cyanogen bromide for various types of silica and polymer supports. Comparsion has been made between silica and polymer solid phases which have been appropriately derivatised and activated (Scheme 1).

Matrices for analytical HPLAC require supports having pore sizes which allow macromolecules(e.g.IgG,150KD; 70-90Å diameter) to freely penetrate the adsorbent particles. Such matrices include Lichrosorb Si-4000 and HMPS having 4000Å and 1000Å pore diameter respectively. Materials with small pore sizes (e.g. nominally 60Å) may also be used where such macromolecules are totally excluded from penetrating particles. Furthermore, application of small, nonporous supports (3-8μm dp) may be recommended in rapid analysis of biochemicals for reasons of unrestricted mass transfer and minimal dispersion. In contrast, supports possessing intermediate particle diameters and pore size (10-30μm dp and 200-1000Å ps) may be preferred for preparative HPLAC [8]. Since these matrices are charactrised by large surface areas and have demonstrated low restricted diffusion and diminished resistance to fluid flow as compared to supports having small particles (10μm).



Scheme 1

EXPERIMENTAL :

Materials

Spherisorb VLS was the gift of phase separation, Clwyd, U.K.; Lichrosorb Si-60 and Si-4000 (10μm dp, 4000Å ps) were obtained from *Merck*, Darmstadt, Germany. Both macroporous hydrophilic polymer supports (MHPS) (10, 20μm dp, 1000Å ps) and nonporous (8μm) supports, were donated by *Polymer Laboratories*, Church Stretton U.K.; 3- Glycidoxypropyltrimethoxysilane was obtained from *Aldrich Chemicals*, U.K.; Tresyl- Chloride from *Fluorochem Ltd.* (Glossop, U.K) and 1,1- carbonyldiimidozole (CDI) from *Pierce*, U.K. Human immunoglobulin (HuIgG) was purified from outdated blood using ion exchange chromatography [9]. All solvents and other chemicals used were analytical grade from commercial sources and were used without further purification.

Preparation of 3- Glycidoxypropyl silica under Aqueous and Dry Conditions

The bonding procedure in this study was adapted from *Ohloson* et al [1]. 5g silica was suspended in 200mL aqueous solution of 3-glycidoxypropyltrimethoxysilane (1% V/V, pH 5.5.). The slurry was degassed under vacuum and then heated and maintained at 90°C for 2 hours with occasional agitation. The mixture was adjusted and maintained at pH 3.0 and heated for 1 hour to convert the oxirane groups to glycol groups.

The procedure used for coating under dry conditions was a modification of the method by *Larrson* et al. [10]. Silica (20g) was dried at 190°C for 72 hours, and placed in a three necked reaction flask for a further-48 hours at 190°C. The reaction flask was cooled and sodium dried toluene (300mL) was added, followed by 20mL of 3- glycidoxypropyltrimethoxysilane and 0.5mL of KOH- dried triethylamine. The suspension was gently stirred on a magnetic stirrer and refluxed for 24 hours. Anhydrous conditions were sustained under a slow stream of nitrogen. The epoxy silica was washed on a glass filter with 5 volumes of toluene, acetone and ether, and dried under vacuum. The epoxy group content was estimated at approximately 400μmol/g.

The tresyl chloride activation method was employed as follows: 10g diol silica was washed in 5 volumes of dry actone and suspended in a mixture of dry acetone (15mL) and dry pyridine (1.1mL). The well mixed suspension was cooled to 0°C and tresyl activation was initiated by dropwise addition of 0.5mL tresyl chloride. The reaction was terminated after 20 minutes by washing the activated gel on a glass filter with four successive 25mL volumes of dry acetone, followed by 25:75, 50:50 and 75:25 mixtures (V/V) of 5mM HCl in acetone and subsequently

by 5mM HCl. The gel was washed with 10 volumes of coupling buffer (0.1M $NaHCO_3$, pH 8.0) containing 0.5M NaCl and transferred to a vessel containing a solution of the ligand (e.g. 15mg/mL HuIgG) in 2 volumes of phosphate buffer saline (PBS). Coupling was continued overnight at 4°C and the suspension filtered and successively washed with 5-10 volumes of coupling buffer, saline buffer (0.1M acetate buffer containing 1.0M NaCl, pH 4.0), PBS pH 7.5, 3M KSCN and finally PBS. In order to block the remaining activated groups, 10 volumes of ethanolamine (1M, pH 8.0) or 100mM glycine in PBS pH 7.5 was stirred with solid phases for 1 hour, then filtered and washed with PBS, and stored at 4°C in PBS containing 0.02% NaN_3 [11].

Activation of diol silica with 1,1 carbonyl-diimidazole activation (CDI) and the coupling of protein ligands were carried out by a modification of the method by *Bethall* et al [12].

The modified method of *March* et al [13] was used for cyanogen bromide activation of hydrophilic macroporous polymer supports (HMPS).

Aldehyde-silica was prepared in the present work by a modified method of *Wikstrom* et al. (reviewed in 4). Epoxy silica (6g) was suspended in 100mL of acetic acid: water (90:10), 5g of NaOH was added and the mixture stirred for 2 hours at room temperature. The aldehyde- silica thus formed was washed with water (5×10mL), acetone, and ether and finally dried in vacuum.

Purification of Protein Ligand

Human immunoglobulin (Hu.IgG) was purified from outdated blood using *Zeta-Prep* QAE anion oxchanger (LKB- company) as follows. The cartridge was equilibrated with 250mL of 0.2M Tris- HCl pH 7.5, and then 1.5L of 10mM Tris-HCl pH 7.5 was added at a flow rate of 25mL/min until the pH and conductivity of the effluent matched the inlet. Serum (50-60mL) was filtered through glass wool, diluted 10 fold with 10mM Tris-HCl pH 7.5 and passed through Zeta-Prep at a flow rate of 10mL/min. Fractions were collected as protein appeared in the breakthrough point. The purity of the product was assessed by SDS-PAGE method [9].

Characteristics of Immunoadsorbents

Batch Binding Performance

HuIgG-silica(0.27g dry) and HuIgG- polymer (0.2g dry) were dispensed by packing equal volumes in a mini-HPLC column and then transferred to 10mL of animal cell culture. The supernatants containing various MCAB concentrations (0.1-0.7mg/mL) along with some protein impurities (2.5mg/mL including BSA, transferrin, insulin and protease) were suspended with solid phases in screw cap universal tubes (25mL). Suspensions were equilibrated by mixing on a rocking reaction platform at 4°C for 16 hours, centrifuged at 16000×g for 15 minutes and supernatants taken for ELISA (Enzyme linked immunosorbent assay)determination.The amount of MCAB bound to immunoadsorbents at equilibrium (q^*) was calculated from the total MCAB activity present at zero-time (C_o) substracted from that in solution at equilibrium (C^*). Control experiments were carried out to validate the stability of MCAB activity under the conditions and time-scale of the experiment. Values of k_d and q_m were determined respectively, where C^* is the intercept and k_d is the slope according to following equation [14]:

$$\frac{C^*}{q^*} = \frac{C^*}{q_m} + \frac{k_d}{q_m}$$

Measurement of Dynamic Capacity Using a Mini-HPLC Column

Immunoadsorbents (HuIgG - silica and polymer) were packed into a mini-HPLC column (10×6mm) washed with 5 column volumes of PBS, 3M KSCN in PBS and PBS respectively. The packed column was loaded with 50mL of MCAB (0.4-0.7mg/mL, ELISA activity) at flow rates of 0.4-0.6mL/min at 4°C. The column was washed with PBS at 0.8mL/min, until the outlet absorbance- monitored by continuous spectrophotometry at 280nm reached that of the inlet.

Specifically bound MCAB was eluted from the column with 3M KSCN in PBS until the A_{280} of the eluent equalled that of pure 3M KSCN solution.

Similar binding experiments were performed with activated matrices blocked with ethanolamine to determine any non specific binding.

Comparison of Batch and Dynamic Adsorption Capacities

In order to compare the dynamic and batch capacities of HPLAC materials, matrices were contacted with MCAB by equilibrium batch binding using monoclonal antibodies far in excess of the available antigen ligands. Materials so saturated were packed into mini-column (10×6mm) and washed with PBS, then eluted with 3M KSCN as above. A comparison of the two elution profiles from dynamic and batch loadings was used to indicate the variation of capacity resulting from different adsorption conditions (Figs. 1, 2).

RESULTS AND DISCUSSION :

Activation Procedures

The results obtained from the activation and subsequent coupling of antigen HuIgG to: Lichrosorb Si-4000, Lichrosorb Si-60, Spherisorb VLS and hydrophilic macroporous polymer using identical tresyl chloride, periodate and CDI procedures are presented in Table 1. The results were obtained using challenges of antigen 9.4-61mg/g) within the concentration range of 0.3-25mg/mL. Quantitation by UV absorption indicate that all of the materials react with

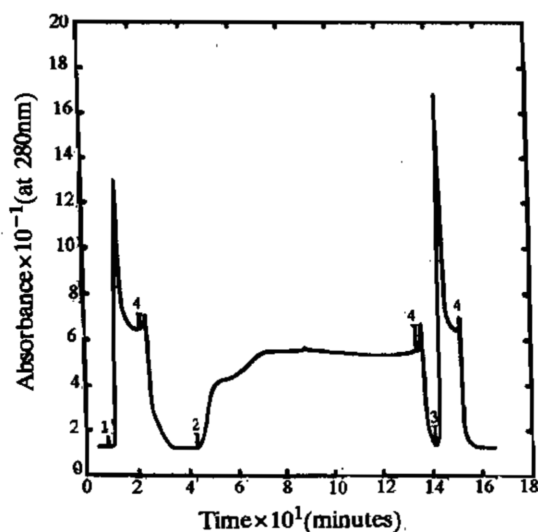


Fig. 1: Diagram comparing the elution profiles of MCAB bound to HuIgG- Lichrosorb Si-4000 (ligand density= 8.5mg/g) under both batch and fixed bed (HPLC) conditions.
(1) Elution of saturated matrix from batch binding;
(2) Load of column with pure MCAB to equilibrium;
(3) Subsequent elution;
(4) Washing with PBS.
Dimension of the mini- HPLAC column= 10×6mm
Loading flow rate= 0.4mL/min, washing and elution flow rate= 0.8mL/min.
Eluent= 3M KSCN in PBS.

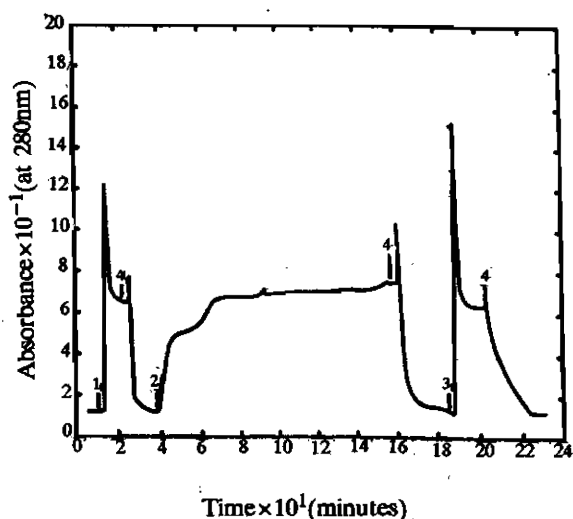


Fig. 2: Diagram comparing the elution profiles of MCAB bound to HuIgG- Lichrosorb Si-4000 (ligand density= 6mg/g) under both batch and fixed bed (HPLC) conditions.
(1) Elution of saturated matrix from batch binding;
(2) Load of column with pure MCAB to equilibrium;
(3) Subsequent elution;
(4) Washing with PBS.
Dimension of the mini- HPLAC column= 10×6mm
Loading flow rate= 0.4mL/min, washing and elution flow rate= 0.8mL/min.
Eluent= 3M KSCN in PBS.

Table 1 : Results of ligand immobilisation for various supports

Activation method	Matrix ps dp (Å)(μm)	Total challenge HuIgG mg	mg/mL	wt of matrix (g dry)	bound ligand (mg/g dry)	coupling efficiency (%)
Periodate	Sph.280,20	24	1.3	1.5	14.5	90.6
		75	25	2	37.0	98.7
	Li.4000,10	34	1.7	1.7	20	100
		15	0.75	1.6	8	85
	HMPS 1000,10	36.6	1.26	1.77	5.5	25.6
	Li.60,10	13.5	0.4	0.74	6.4	35
		45	1.2	0.74	20.2	33
Tresyl Chloride	Sph.280,5	15.7	1	0.75	10	47
	Sph. 280,20	24	1.3	1.5	14	87.5
		100	10	4	10.2	41
	Li.60,10	13	0.4	0.75	4	23
	Li.4000,10	15	0.29	1.5	4	40
		25	0.68	1.5	10.4	62
		13.6	0.62	0.65	85	40
	HMPS 1000,10	29.6	1.0	2.4	10	80
CDI	non-porous polymer-8	19.2	0.914	2.5	1.3	17
	Sph.280,20	74	1.4	5	10	67
	HMPS 1000/20	32	1.8	1.8	18	100
CNBr	HMPS 1000/10	35.7	2	1.77	8.9	44

Sph = Spherisorb VLS, Li= Lichrosorb

HMPS = Hydrophilic macroporous polymer support

$$\%efficiency = \frac{[\text{ligand (mg/g dry)}][(\text{wt. of matrix (g dry)} \times 100)]}{[(\text{total challenge})(\text{mg of HuIgG})]}$$

ps = pore size (Å)

dp = particle diameter (μm)

different percentages of antigen (20-100%). The difference between reactivity of various activated matrices may be due to the following parameters (Table 1).

(a) concentration and total mass of challenge

(b) difference between activation methods

(c) dimensions of matrices (dp and ps).

Activation methods such as tresyl chloride, periodate and 1,1′ carbonyldiimidazole (CDI) are applicable to modified silica and polymer supports. Periodate activation is recommended for matrices such as diol silica, because of high levels of vicinal hydroxyl groups for oxidation to aldehyde groups. This activation method has proven less successful for the polymer matrices (HMPS) containing few vicinal hydroxyl groups. Tresyl chloride activation is generally applicable for most supports, but there are practical problems associated with the method. The reagent is expensive, mixing condition and temperature require careful control.

CDI activation has been successful for all HPLC supports used in this study, and is probably applicable to other commercially available matrices. The method yields a material characterized by high efficiency, productivity, low non-

Table 2 : Results of batch and fixed bed binding capacity and subsquent recovery and productivity for various affinity matrices

Matrix	Activation	Ligand density (mg/g)	qm Batch isoth. (mg/g)	qm* Fixed bed (mg/g)	qm Single batch	k_d (M)	Recovery (%)	Productivity binding (%)
Sph.(20μm, 280Å)	T.Ch.	14.2	15.2	13.0	-	8×10^{-9}	86	91.5
Sph.(20μm, 280Å)	P.	14.0	16.0	8.3	-	5×10^{-8}	87	59.3
Sph.(20μm, 280Å)	CDI	10.0	6.6	13.0	15.0	-	46	130
Li.(10μm, 4000Å)	T.Ch.	8.0	2.25	9.2	6.6	6×10^{-7}	66	115
Li.(10μm, 60Å)	P.	20.6	11.2	28.5	3.0	6×10^{-8}	45	138.3
Li.(10μm, 60Å)	P.	6.4	1.1	19.6	2.5	3×10^{-7}	56	306.2
Li.(10μm, 60Å)	P.	20.2	-	33.5	3.0	2×10^{-8}	66	166
HMPS(20μm, 1000Å)	CDI	19.0	-	52.5	25.5	-	53	276.3
HMPS(20μm, 1000Å)	CNBr	8.8	2.3	11.5	-	5×10^{-7}	44	130.7
HMPS(20μm, 1000Å)	P.	5.5	1.52	8.5	-	5×10^{-8}	50	154.5
HMPS(20μm, 1000Å)	T.Ch.	10.0	4.3	6.48	-	6×10^{-7}	87	64.8
+HMPS(20μm, 1000Å)	CDI	5.8	34.0	30.0	-	4×10^{-7}	-	517.2
HMPS(20μm, 0Å)	CDI	1.3	-	3.0	4.2	-	-	230.8

sph.= Spherisorb VLS; Li.= Lichrosorb; HMPS= Hydrophilic macroporous polymer support; T.Ch.= Tresyl chloride; P.= Periodate; CDI= Carbonyldiimidazole; CNBr= Cyanogen bromide. Recovery based on A_{280} for fixed bed adsorption (HPLAC minicolumn). $\text{Productivity}_{(bind)}$= Fixed bed binding capacity × 100/ligand. density.* refers to equilibrium binding capacity. + Ligand= protein A.

specific binding, and stability towards a wide range of pH(2.5-10). The method is simple, and the chemical reactions is rapid, allowing termination within 15-20 minutes.

Immunoadsorbents :

Batch binding capacities of HuIgG Lichrosorb Si-4000 were five times greater than those of HuIgG Lichrosorb Si-60 (Table 2) for anti-HuIgG MCAB as product.The low batch binding capacity for HuIgG- Lichrosorb Si-60 is assumed to be due to restricted diffusion of macromolecular HuIgG within the pores of HPLC supports under batch test.

Adsorption isotherms of most fabricated immunoadsorbents(ligand: HuIgG or protein A) are favourably non- linear, but this positive observation masks a complex binding mechanisms (Figs. 3,4). The chemical structure and

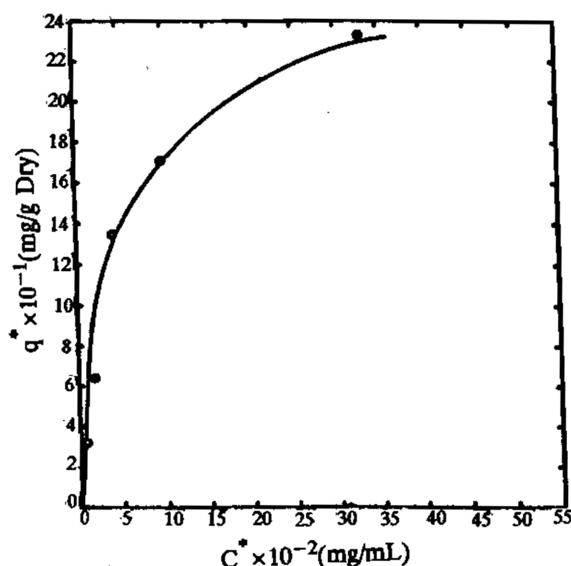


Fig. 3: Adsorption isotherm for the binding of TB/C3 MCAB to HuIgG- HMPS(20μm dp) according to the modified method of Chase[8].

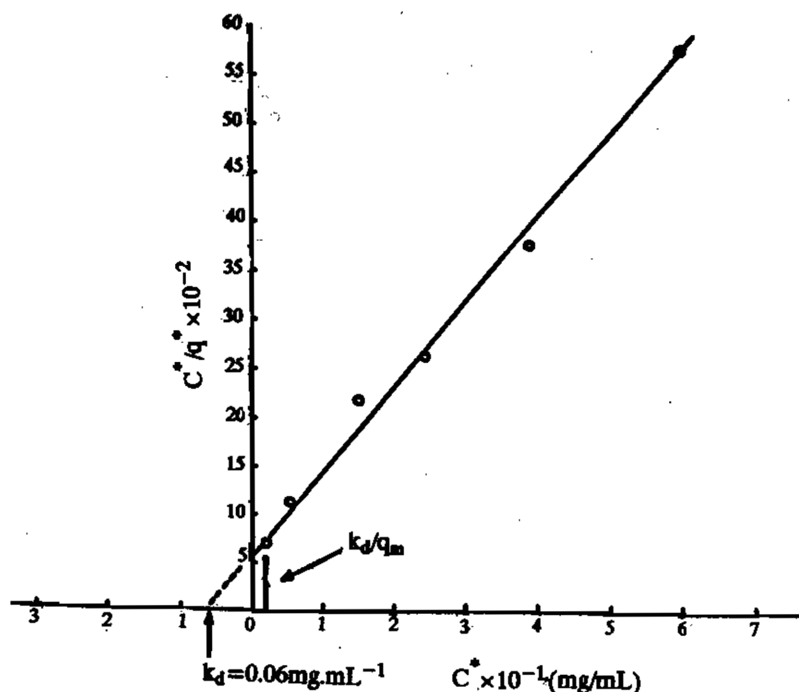


***Fig. 4:** Graphical plot for experimental values of C^*/q^* against C^*, for measurement of $1/q^*$ (slope) and k_d/q_m(intercept where $C^*/q^*=0$)*

geometry of adsorbent particles strongly influence determination methods and final values for kinetic factors characteristic of binding interaction. In the case of small porous particles, the rate determining step can not be modelled by a single surface adsorption, since observed behaviour results from a combination of determining factors such as film mass transfer and pore diffusion. Further work is essential for the complete understanding of the nature of adsorption processes upon and within such materials. Thus any contemporary model should be based upon determined kinetic parameters (q_m, k_d, k_1, k_2, etc) and quantites describing diffusion and dispersion of macromolecules within a porous environment.

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