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Automated flow-injection serial dynamic dialysis technique in the study of drug binding with cyclodextrins

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Abstract

A flow-injection dynamic dialysis technique is presented for the determination of binding parameters of drugs to cyclodextrins (CDs). The automated system consists of a flow-injection unit, the sample loop of which is the receiving compartment of a dialyser unit, and a home made timing module for operation control through two flow switching solenoid valves. The procedure of binding studies is rapid and yields reproducible results. Binding parameters of CD–micromolecule complexes were calculated using the Scatchard model. Typical examples of the binding of *p*-nitrophenol with α-CD ($K_{as} = 1.58 \times 10^3$ M^{-1} at pH 7.4 and 2.06×10^3 M^{-1} at pH 9.0), salicylic acid with β-CD ($K_{as} = 3.8 \times 10^2$ M^{-1} at pH 1.5 and 51 M^{-1} at pH 7.4) and ibuprofen with β-CD ($K_{as} = 2.2 \times 10^2$ M^{-1} at pH 2.5) are presented and the binding constants obtained are compared to literature values. 1:1 stoichiometry was found in all cases and within run precision ranged from 2 to 14% R.S.D. The between run precision for the binding of *p*-nitrophenol to α-CD was 2% ($n = 3$).

Key words: Flow injection; Automated serial dynamic dialysis technique; Cyclodextrins; Drug binding to cyclodextrins; Ibuprofen; *p*-Nitrophenol; Salicylic acid; Scatchard model

1. Introduction

Cyclodextrins (CDs) are torroidally shaped, cyclic oligosaccharides, consisting of six, seven, or eight glucopyranose units (α-, β- and γ-cyclodextrin, respectively), which are linked with α-1,4-glycosidic bonds. CDs form inclusion complexes of the "host-guest" type with a wide variety of ionic and molecular species, the main requirement being that the guest fits entirely, or partly, in the CD cavity [1,2]. The intermolecular interactions involved in the formation of these complexes are non-covalent, consisting mainly of Van der Waals forces, hydrophobic interactions and, when possible, hydrogen bonding [1,2].

The enclosure of a molecule in the CD cavity causes considerable modification of its physical, chemical and biological properties. In addition, upon dissociation of the complex the molecule regains its former properties. These characteristics, and their insignificant per os toxicity, have resulted in a large number of applications for CDs in pharmaceutical technology [2–4], analyti-

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cal chemistry [5], chemical technology [6] and various industrial uses [7].

Increasingly important is the capability of CDs to form complexes with drug molecules, as various properties of the guest drug molecule are altered upon complexation. These properties can thus be modified and the biopharmaceutical and pharmacokinetic behaviour of a drug can be improved. The solubility and dissolution rate are usually increased, the absorption rate is improved and the bioavailability of the drug is enhanced; also, stability is usually increased and the use of CDs in drug formulation has been extensively reported [2–4].

The binding of CDs to various molecules has been studied by various techniques, including phase solubility, spectroscopy (spectrophotometry, circular dichroism, fluorimetry, NMR), chromatography, and electrochemistry (potentiometry) [1,2]. In the so-called "direct methods, the measurement of the analytical signal is performed in the micromolecule–CD mixture under study, while the "indirect methods" require a separation step of the complexes from the free micromolecules. Equilibrium and dynamic dialysis methods are the most common methods in the latter group.

In the present paper, a technique based on the principle of dynamic dialysis in conjunction with the flow-injection (FI) concept is introduced for studying binding parameters of CD complexes. Dynamic dialysis methods [8–10] have been used for in vitro studies of drug–protein interactions, offering the advantages of relatively increased speed (in comparison with the time-consuming equilibrium dialysis methods) and measurement of drug–protein binding over a range of drug concentrations in a single experiment. These methods are based on the principle that the macromolecule–drug complex reaches rapid equilibrium with free macromolecule and diffusible drug in a macromolecule compartment, which is separated from a sink compartment by a semipermeable (dialysis) membrane. The rate of diffusion across the membrane is directly proportional to the free drug concentration in the macromolecule compartment. Sampling of dialysed drug is accomplished by periodical removal of a volume from the sink compartment which is replaced with fresh buffer solution in order to maintain sink conditions. Measurement is usually accomplished using UV-spectrophotometry.

The coupling of dynamic dialysis with the flow-injection concept results in an advantageous

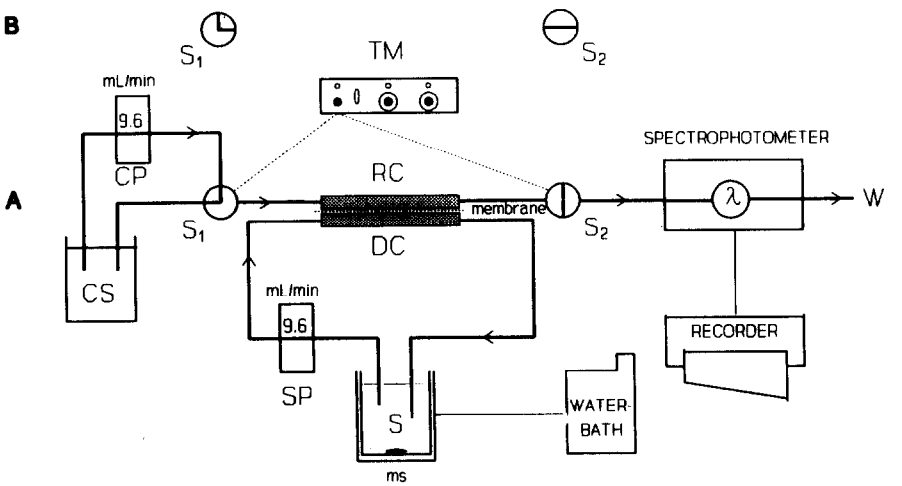

Fig. 1. Schematic diagram of the FISDD system used. CS, carrier solution; CP, carrier pump; RC, receiving compartment; DC, donor compartment; SP, sample pump; S, sample (cyclodextrin–micromolecule) solution; W, waste; S_1, S_2 three- and two-way solenoid valves, respectively; TM, timing module; ms, magnetic stirrer. B, sampling-washing and A, dialysis position of solenoid valves, respectively.

automated fast technique for binding studies. By inserting a dialyser unit in an FI system, the receiving compartment of the former functioning as a sample loop, the binding experiment can be accomplished automatically through a successive series of dynamic dialysis runs. This concept, called flow-injection serial dynamic dialysis (FISDD), has been exploited in protein binding studies [11] and dissolution studies of drug formulations in milk [12], by use of an automated FI analyzer.

In this paper a very simple timing module is used for operation control through two flow switching solenoid valves, and the technique is applied to the recently interesting topic of cyclodextrin–micromolecule binding studies. The performance of the system and the technique were evaluated in the study of the binding of *p*-nitrophenol with α-CD, and salicylic acid and ibuprofen with β-CD. The Scatchard model for one class of binding sites (the CD cavity) was used in order to calculate the binding parameters. This model, frequently used in binding studies of drugs to proteins, has been previously used in the determination of binding constants of drugs to CDs [13].

2. Experimental

2.1. Apparatus

The home-made FISDD apparatus used, shown in Fig. 1, was a simplified version of the system devised previously in our laboratory [11]. In this study, the sample injection rotary valve and the microcomputer were replaced by a home-made timing module, a three-way (S_1) and an on-off (S_2) flow switching 6V DC solenoid valves (Angar).

The dialyser unit, constructed from plexiglas, had a dialysis surface of 670 mm^2 with two identical chambers of 1.0 ml volume each. The two chambers were separated by a semipermeable membrane with 20 μm pores (Cuprophan, Technicon Chemicals), which was hydrated for a short time before assembling the unit. The donor compartment was connected to the sample pump (Masterflex, Cole Palmer), which circulated the cyclodextrin–micromolecule solutions from a thermostatted (25 $\pm$ 0.5°C) cell of 100 ml volume. The inlet of the receiving compartment was connected to the carrier pump via the three-way solenoid valve and the outlet to the UV–visible

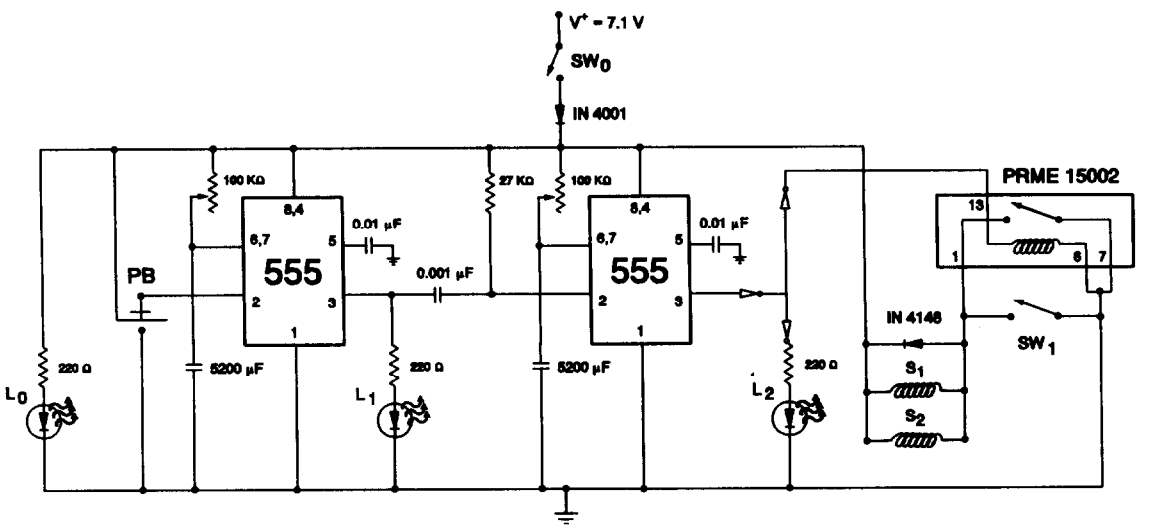

Fig. 2. Circuit diagram of the timing module. SW_o, voltage supply switch; S_1 and S_2 solenoid valves; SW_1, manually operated switch turning S_1 and S_2 to the sampling-washing position overriding timer control; PB, push button triggering timing sequence; L_o, voltage supply indication LED; L_1 and L_2 dialysis and sampling-washing time indication LEDs, respectively.

spectrophotometer (UV-120-02, Shimadzu) equipped with a 400-μl flow cell (Hellma) via the on-off solenoid valve. The absorbance peaks were recorded on a chart recorder (Model 7334, Knauer).

The circuit of the home made timing module (Fig. 2) consists of two cascaded 555 (National Semiconductor) integrated circuit (IC) timers that are configured in the monostable (one-shot) mode and a solid state relay (Clare, PRME 15002) controlled by the second 555 IC. This relay controls the solenoid valves by opening and closing their common power supply. Light emitting diodes (LEDs) connected to the timer's output indicate timing sequence to the operator. The timing element in the 555 ICs is an RC circuit and the time measured is 1.1RC (s). Fixed 5200 ($\pm$10%) μF capacitors were used and the time was set by two 100 ($\pm$5%) kΩ ten-turn potentiometers (Sectrol mod-534) that are linear within $\pm$0.25%. Triggering of the timing sequence was achieved by pressing a push button.

Binding experiments were carried out at constant temperature, flow-rates, and dialysis and sampling times. Data (concentration of free micromolecule calculated from a calibration curve) were treated using the Statgraphics statistical graphics system on an IBM PS/2 computer.

2.2. Reagents

p-Nitrophenol binding study

Concentrated stock solutions of 0.100 M *p*-nitrophenol (Merck) and 5.00×10^{-4} M α-CD (Fluka) were prepared in (a) phosphate buffer 0.010 M, pH 7.4 or (b) borate buffer 0.010 M pH 9.2.

Salicylic acid binding study

Concentrated stock solutions of 0.0100 M sodium salicylate (Ferak) and 1.00×10^{-3} M β-CD (Sigma) were prepared in (a) water and the pH was adjusted to 1.5 by H_2SO_4 2 M (final H_2SO_4 concentration about 0.1 M) or (b) phosphate buffer 0.10 M, pH 7.4.

Ibuprofen binding study

Concentrated stock solutions of 0.010 M ibuprofen (kindly donated by a local manufacturer) and 3.00×10^{-4} M β-CD were prepared in 50% (v/v) methanol in water and adjusted to a phenomenal pH 2.5 with concentrated HCl.

The carrier solution in all cases was the same buffer used in the binding experiment.

The reagent solutions used (0.10 M $NaH_2PO_4 \cdot H_2O$, 2.0 M H_2SO_4 and 50% (v/v) methanol) were prepared from analytical grade reagents. Deionized water was used throughout.

2.3. System set up and operation

In the FISDD system the receiving compartment of the dialyser unit is the "sample loop" of the FI analyzer. Carrier solution is enclosed in the "sample loop" to receive the dialysable micromolecule (drug) of the donor compartment (cyclodextrin compartment) through which the CD–micromolecule (drug) solution is continuously circulated. Periodically this "sample zone" is transferred to a UV–visible spectrophotometer for measurement, and fresh carrier solution is enclosed in the "sample loop". The change of the total concentration of the micromolecule in the donor compartment was achieved by successive manual additions of small volumes of a concentrated standard micromolecule solution.

The dialysis time (3 min for *p*-nitrophenol, and 2 min for salicylic acid and ibuprofen) and sampling time (25 s for all experiments) are set on the timing module and the appropriate wavelength is set on the spectrophotometer (399 nm for *p*-nitrophenol, 237 nm for salicylic acid in pH 1.5, or 296 nm in pH 7.4, and 220 nm for ibuprofen). Then 50.0 ml of the appropriate buffer (for the construction of the calibration curve) or buffered CD solution (for the binding experiment) are pipetted into the beaker, and the carrier and sample pumps are started at the appropriate flow-rate (9.6 ml/min in all experiments). The valves are set, by a manually operated switch overriding timer control, to the sampling-washing position (Fig. 1B), diverting the carrier flow through the receiving compartment, and 100% transmittance and recorder baseline are set. The first portion of the standard stock solution of the micromolecule (drug) under test is added and the timing module is activated after a 2 min mixing/

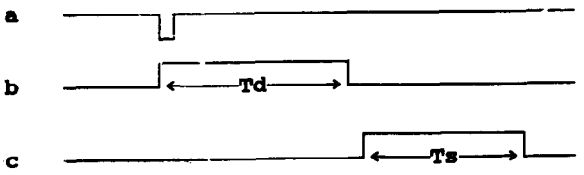

Fig. 3. Timing sequence of the timing module. a, triggering pulse; b and c, output of first and second 555 IC, respectively. Td and Ts, dialysis and sampling-washing time, respectively.

incubation time. During the preset dialysis period, the solution in the beaker is recycled through the donor compartment. Carrier solution is trapped in the receiving compartment and the carrier flow is recycled through the S_1 valve (as shown in Fig. 1A). Then, the timing module initiates the sampling-washing period, switching the S_1 and S_2 valves to the positions shown in Fig. 1B, so that carrier solution flows through the receiving compartment transferring the dialysate to the spectrophotometer, where an absorbance peak corresponding to the dialysed drug concentration is recorded. The next portion of the standard stock solution is added and the cycle is repeated. The timing sequence is shown in Fig. 3.

By using the procedure of successive additions in the selected buffer, calibration curves (absorbance peaks vs. free micromolecule concentration (D_F)) were obtained in a concentration range of $1.0–10 \times 10^{-4}$ M for *p*-nitrophenol and $1.0–30 \times 10^{-4}$ M for salicylic acid and ibuprofen. From the absorbance peaks obtained in the binding experiments the concentration of the free (unbound) micromolecule can be obtained from the constructed calibration curves.

In order to obtain the dialysis–time profiles, a standard solution of the drug under study in the appropriate buffer is placed in the sample beaker, and a series of dialysis-measurement experiments are conducted varying the dialysis time within a range of 1–10 min. The data obtained from this procedure were used to calculate the experimental dialysis rate constants, and thus to optimize the dialysis time chosen.

3. Results and discussion

3.1. System evaluation

Before using the timing module a calibration curve of the potentiometer turns (settings) versus time was constructed for each 555 IC. The time range found was 0.65 s to 10 min with a resolution of 0.65 s. The 555 ICs calibration curves were stable within 2% for six months of operation. These two calibration curves were used in order to set the dialysis and sampling-washing time. The flow-rate used (9.6 ml/min) was selected on the basis of fast donor recycling and low sampling-washing time. The second solenoid (S_2) was included to prevent a small flow of the solution in the receiving compartment to the waste, due to the force of the hydrodynamic pressure gradient during the dialysis period.

Since α- and β-CD are not large macromolecules (MW 973 and 1135, respectively), experiments had to be conducted to assure that they do not diffuse through the membrane under the experimental conditions used during the binding experiments. Buffered solutions of CDs were dialysed for a time period equal to that used in binding experiments. Then the dialysates were collected in test tubes containing phenolphthalein (for β-CD) or methyl-orange (for α-CD) solution, and the absorbances were measured and compared to those of blank experiments (the dialysate was substituted with an equal volume of buffer). No difference in the absorbance was detected, therefore CDs did not diffuse through the membrane under the experimental conditions used. This test is based on the resulting decoloration of phenolphthalein or methyl orange solutions upon addition of β- or α-CD, respectively, due to the formation of the corresponding inclusion complexes [2].

3.2. Determination of dialysis rate constants

The selection of the dialysis time for the micromolecules studied was based on the dialysis rate constants. The experimental dialysis rate constant (k_d) can be calculated according to Eq. 1 [11]

$$\ln[(D_f - D_i)/D_f] = -k_d t \quad (1)$$

where D_f is the concentration of the free (unbound) micromolecule in the donor compartment and D_i is the micromolecule concentration in the receiving compartment after dialysis for a period of time t. D_i is calculated from absorbance measurements using experimentally determined molecular absorptivities. D_f remains practically constant during the experiment, due to the large volume of the solution in the cell (50 ml), as compared to the volume trapped in the receiving compartment (1.0 ml), and can be considered as equal to D_{fo}, which is the initial free micromolecule concentration in the donor compartment. Then the dialysis time required to attain a specified percent of dialysis is calculated according to Eq. 2 [11]

$$t = -\ln[1 - (\%D_i/100)]/k_d \quad (2)$$

The calculated experimental dialysis rate constants for *p*-nitrophenol, salicylic acid and ibuprofen, along with the percent of dialysed micromolecule during the dialysis time chosen and their pK_a values are shown in Table 1. The good within run precision and the correlation coefficients found verify the validity of Eq. 1.

Due to the hydrophobic character of the cuprophan membrane, the non-ionised form of *p*-nitrophenol and salicylic acid diffuse at a higher rate than their corresponding anions (Table 1). As ibuprofen has the highest molecular weight, its k_d value was found to be the lowest one.

3.3 Determination of binding constants

Typical absorbance peaks recorded during the binding study of ibuprofen are shown in Fig. 4. Dashed-line peaks obtained from solutions of pure ibuprofen in various concentrations are shown superimposed to the corresponding (solid-line) ones obtained in the presence of a constant β-CD concentration. It is very clear that binding lowers the amount of ibuprofen dialysed by decreasing the free ibuprofen concentration.

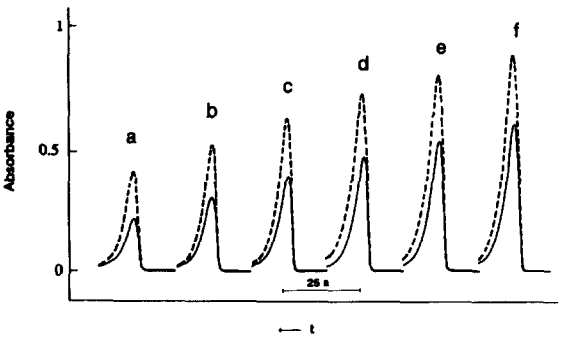

Fig. 4. FIA peaks for a typical binding study of ibuprofen. Dashed line, calibration curve; Solid line, same experiment in the presence of 3.00×10^{-3} M β-CD. Dialysis time 2 min. Total (free plus bound) drug concentration in donor compartment: (a) 1.07, (b) 1.47, (c) 1.86, (d) 2.25, (e) 2.64 and (f) 3.03×10^{-3} M.

The binding parameters were estimated by the use of the well-known Scatchard equation (Eq. 3.), for one class of binding sites

$$(r/D_f) = nK_{as} - K_{as}r \quad (3)$$

where $r = D_b/CD_t$, D_b being the concentration of the bound micromolecule (drug) ($D_b = D_t - D_f$) and CD_t the total cyclodextrin concentration, n the number of cyclodextrin molecules complexed with each micromolecule and K_{as} the association constant of the complex. The parameters n and K_{as} were calculated by linear regres-

Table 1
Calculation of experimental dialysis rate constants at 25°C

Compound	C ($M\times10^4$)	pK_a	pH	$k_d \pm$ S.D. (min^{-1})	r [a]	dialysed % [b]
p-Nitrophenol	1	7.10	7.4	0.075 ± 0.003	0.996	20.1
	1		9.2	0.056 ± 0.002	0.995	15.5
Salicylic acid	4	2.97	1.5	0.093 ± 0.006	0.993	17.0
	1		7.4	0.058 ± 0.003	0.99	10.9
Ibuprofen	2	5.20	2.5	0.041 ± 0.002	0.996	7.9

[a] Correlation coefficient for the fitting of experimental data to Eq. 1.
[b] Calculated using Eq. 2 and the dialysis times listed in system setup and operation.

Table 2
Binding data for the studied compounds to cyclodextrins [a]

Compound	C_{CD} ($M \times 10^4$)	pH	n	K_{as} (M^{-1}x10)
p-Nitrophenol	5.00	7.4	0.77 ± 0.01 [b]	158 ± 3 [b]
	10.0	9.0	0.82 ± 0.07 [b]	206 ± 16 [b]
Salicylic acid	10.0	1.5	1.07 ± 0.03 [b]	38 ± 1 [b]
Salicylate	30.0	7.4	1.3 ± 0.2 [b]	5.1 ± 0.7 [b]
Ibuprofen	30.0	2.5	0.96 ± 0.09 [b]	22 ± 2 [b]

[a] α-CD for *p*-nitrophenol, β-CD for the others; temperature, 25°C.
[b] Within run standard deviation.

sion of r/D_f versus r values. Examples of Scatchard plots obtained for the three model molecules studied are shown in Fig. 5. Binding data calculated from Eq. 3 is presented in Table 2 and a comparison to literature values is shown in Table 3. As expected, $n = 1$, i.e. the cavity of

Table 3
Comparison of determined binding constants with literature values at 25°C

K_{as} (M^{-1})	pH	Medium, method	Ref.
p-Nitrophenol–α-CD			
2230 [a]	11.0	0.5 M phosphate, spectrophotometry	[14]
2200		pH–Potentiometry	[15]
2500	11.0	Phosphate, spectrophotometry	[16]
2700	11.0	Phosphate, NMR	[16]
1590	11.0	Phosphate, optical rotation	[16]
1890	11.0	0.1 M Phosphate, spectrophotometry	[24]
2408		pH–Potentiometry	[19]
3550	10.0	0.1 M Na_2CO_3, gel filtration	[25]
1770	9.0	0.01 M Borate, ISE–potentiometry	[13]
1579	7.4	0.01 M Phosphate	This study
2060	9.0	0.01 M Borate	This study
Salicylic acid–β-CD			
700	2.5	Water [b], phase-solubility	[26]
378	1.5	0.1 M H_2SO_4	This study
51	7.4	0.1 M Phosphate	This study
Ibuprofen–β-CD			
10800 [c]	7.0	1/15 M Phosphate, spectrophotometry	[27]
10000 [c]	5.0	Water [b], phase-solubility	[26]
410	2.0	Phase-solubility	[23]
221	2.5	Methanol 50% (v/v) [b]	This study

[a] Original measurement for 20°C; calculated for 25°C by Connors and Lipari [15].
[b] Adjusted to pH value by addition of HCl solution.
[c] Complexation stoichiometry 2:3 (drug to CD) was detected.

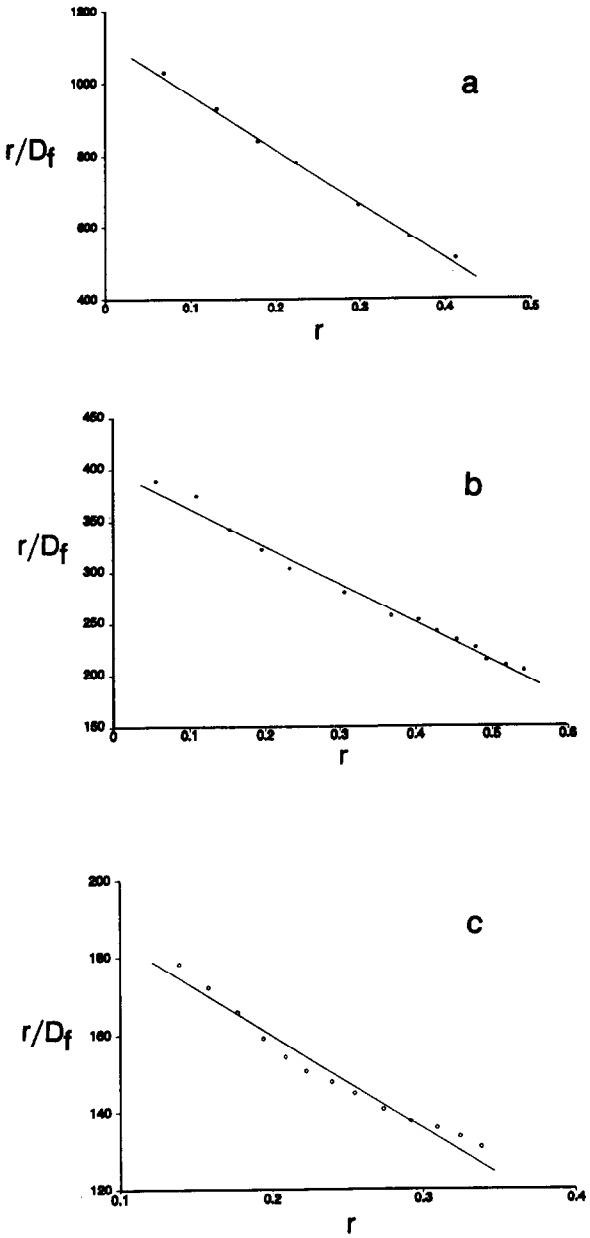

Fig. 5. Scatchard plots for binding experiments of (a) *p*-nitrophenol, (b) salicylic acid and (c) ibuprofen.

the cyclodextrin. Within run precision ranged from 2 to 14% R.S.D., while the between run precision for the binding of *p*-nitrophenol to α-CD was 2% ($n = 3$).

The reactions of micromolecules with CDs in solution are fast enough and completed in a few seconds, as was revealed by in situ monitoring with ion-selective electrodes [13]. The 2 min mixing/incubation time after each successive addition of the micromolecule solution, in conjunction with the 2–3 min dialysis time, ensures that equilibrium is reached.

While the estimated value of K_{as} for *p*-nitrophenol at pH 9.0 is within the range of previously reported estimates (Table 3), at pH 7.4 the K_{as} value is lower. This can be explained on the basis of the degree of ionization of *p*-nitrophenol at the two pH values. At pH 7.4 *p*-nitrophenol is only partially (66%) ionized. It is well-established that *p*-nitrophenolate is bound much stronger than *p*-nitrophenol to α-CD, as proven by a negative change of its pK_a upon complex formation with α-CD ($\Delta pK_a = -0.94$) [14–19].

In contrast to phenols, carboxylic acids are bound to CDs much stronger than their conjugate bases, resulting in a positive change of their pK_a values upon complex formation [15–17,20,21]. This is clearly shown by the two estimates of K_{as} as obtained for salicylic acid at pH 1.5 and 7.4. Although the K_{as} found (378 M^{-1}) at pH 1.5 is in the same order of magnitude with the literature value (700 M^{-1}) at pH 2.5, the difference shows the effect of pH and the method used on the K_{as} values.

Methanolic medium was used to solubilize ibuprofen, which is sparingly soluble in water at pH 2.5. Ethanolic medium was also tried as a solvent, but the dialysis rate was found to be negligible compared to that of the methanolic one. Ethanol was also found to affect complex formation to such a degree, that no useful binding data could be obtained. Various methanol concentrations in the range of 10 to 60% (v/v) were tried and the 50% (v/v) concentration was found to be adequate. The effect of organic solvents on inclusion complex formation with CDs has been studied [22] and it has been shown that methanol is bound much weaker than ethanol in the CD cavity due to its smaller size [2]. However the weak binding of methanol may account for the somehow lower K_{as} of ibuprofen compared to that found by Orienti et al [23].

4. Conclusion

The serial dynamic dialysis technique offers the advantage of separation of free from bound drug and diminishes the long times usually required in static experiments (equilibrium dialysis, phase-solubility studies). The combination with the flow-injection technique results in automated measurements utilizing an exceptionally simple and low-cost system based on two solenoid valves and a home made timing module. The detection scheme used in this study was a direct UV measurement. To improve sensitivity, or when studying drugs that do not absorb in the UV–visible range, the carrier stream leaving the receiving compartment could be merged with appropriate reagent stream(s), allowed to react for a limited time period in a reaction coil and afterwards measured in the appropriate wavelength. Drug ion-selective electrodes, fluorimetry and other detection principles could also be adapted to this system without any modifications. It was also shown that the FISDD technique can be used in the binding studies of cyclodextrins with micromolecules.

5. Acknowledgements

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