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A method for the quantitative determination of the total concentration of radiopaque agents in plasma

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The total concentration of radiopaque agents in plasma can be determined spectrophotometrically after elimination of interfering factors using a double column filled with cross-linked dextran-gel Sephadex G-25c and Se-Sephadex 25c. The method gives a linear relationship between the total concentration in the sample and the maximum concentration in the eluate and is sensitive to about 50 μg/ml of radiopaque agent.

IN investigations of the excretion pattern of radiopaque agents it is necessary to know the fraction of the total plasma concentration of the agent bound to plasma protein. This may be obtained using dialysis or ultrafiltration, from the free concentration in equilibrium and the quantity of agent originally applied to the system (Goldbaum & Smith, 1954; McMenamy & Oncley, 1958; Davison & Smith, 1961) but this is possible only when the drug or agent is not adsorbed on a membrane. We found radiopaque agents to be adsorbed (8–18 mg from concentrations of 1·2 mg/ml) onto Visking tubing (1·4 cm × 8 cm) and collodion (5 cm diam. × 6 cm) and the adsorption to depend on the concentration of the agent and to vary with the individual membrane used. Thus to determine the plasma protein binding of the radiopaque substance (Langecker, Harwart & Junkmann, 1953; Lasser, Farr, Fujimagari & Tripp, 1962), we had to measure the total concentration in the plasma or protein phase and the free concentration in the protein-free dialysate, at equilibrium. Therefore for *in vivo* experiments, and also for the determination of protein binding in *in vitro* experiments, we needed a method to measure the total concentration of radiopaque agent in the plasma or protein phase.

The agents can be determined spectrophotometrically but it is first necessary to separate them from plasma proteins and protein fragments, especially creatinine, which interfere with spectrophotometric determinations. It seemed probable that as the dextran gel Sephadex in a column had been shown to separate molecules of different sizes (Porath & Flodin, 1959; Porath, 1960; Barlow, Firemark & Roth, 1962), this approach promised a solution to the problem of eliminating interfering material. If, and how far, a dissociation of the protein-bound radiopaque agents can be obtained by this method, must be investigated (Barlow & others, 1962; Jacobsson & Widström, 1962; Lissitzky, Bismuth & Rolland, 1962).

For a column filled with Sephadex acting as a sieve for molecules of different size

$$K_D = \frac{V_e - V_o}{V_i}$$ (Barlow & others, 1962)

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where the parameters of the column are:

V_o = the water outside the gel grains, V_i = the water within the gel grains, V_e = the volume of eluate required to deliver the peak concentration of the experimental substance, K_D = the distribution coefficient; this indicates the fraction of the inner volume accessible to a particular molecular species.

For low molecular weight compounds eluted on a neutral Sephadex column, $V_e = V_i + V_o$, so $K_D = 1$; deviations from the $K_D = 1$ value are due to adsorption onto the gel comparable with the adsorption which takes place by column chromatography on a column filled with cellulose. The gel is also available with ion-exchange properties and here, for low molecular weight compounds, deviations from the $K_D = 1$ value depend on the pK_a value of the compound and the pH of the eluent. Thus the ion-exchange gel not only differentiates on the basis of the dimensions of the molecules but also on basic or acidic properties (Porath & Lindner, 1961; Carnegie, 1961; Scheffer, Kikuth & Lorenz, 1965).

If a mixture from which acids or bases have to be separated contains proteins, the choice of the pH of the eluent is limited because the proteins may precipitate. For this reason these must first be removed in neutral conditions, therefore a neutral Sephadex column is required. If the mixture then contains only one acidic compound to be measured, separation can be effected by a cation-exchanger, and for this purpose a Sephadex cation exchanger can be used.

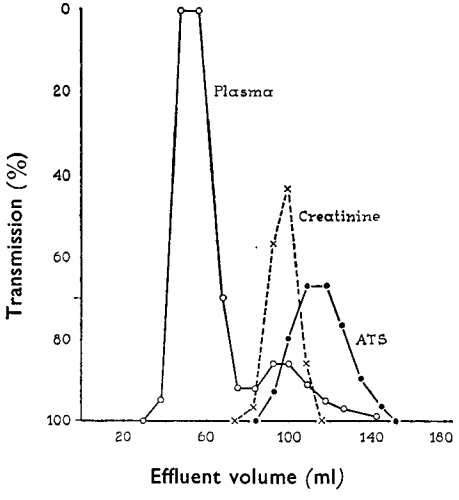

FIG. 1. The % transmission at 237 mμ as a function of the effluent volume. The curves represent the transmission pattern of 8 ml fractions of the eluate, obtained with 1 ml plasma, 1 ml creatinine (0·1 mg/ml in distilled water) and 1 ml acetrizoate sodium (ATS) (0·1 mg/ml in distilled water) respectively, eluted on a 40cm Sephadex G-25c column with Tyrode pH 7·6.

Note: The mean zone of plasma proteins does not overlap the zone of small molecules; for the separation of the small molecules a second step is necessary.

ELUTION ON NEUTRAL SEPHADEX (G-25C)

In experiments in which the elution of plasma proteins, creatinine and various radiopaque agents was examined singly (Fig. 1), we found (*a*) no permanent adsorption of the radiopaque agents onto the Sephadex (of the quantity of agent applied to the column 99·5–99·9% was recovered in the eluate), (*b*) the zone containing the plasma proteins did not overlap that containing the radiopaque agents, (*c*) the zone containing the agents and that containing creatinine as well as the zone obtained from the smaller molecules in plasma clearly overlapped. Therefore to determine radiopaque agents in plasma a second separation step was required. In experiments with combinations of plasma and radiopaque agents we found that there was no mutual influence as far as the elution zones are concerned; the minima in the light transmission in these zones remain unchanged. So a total dissociation of the radiopaque-protein complex is obtained. From the latter experiments the values for V_o, V_i and V_e, and consequently K_D can be calculated (see Table 1).

TABLE 1. EXPERIMENTAL DATA FOR THE RADIOPAQUE AGENTS ELUTED ON A SEPHADEX G-25C COLUMN

Sample placed	Start of the peak	Minimum of the peak		End of the protein peak		Vo	Vi*	Ve	KD
	EV in ml	EV in ml	% Trans-mission	EV in ml	% Trans-mission				
Blank plasma		40	1	60	96	40			
Creatinine, 0·4 mg/ml in H_2O		90	55				50	90	1·0*
Acetrizoate-Na, 0·4 mg/ml in H_2O	70	100	75					100	1·2
Acetrizoate-Na, 0·4 mg/ml in plasma	72	102	74	60	96			102	1·24
Iodohippurate-Na, 0·4 mg/ml in H_2O ..	82	112	84					112	1·44
Iodohippurate-Na, 0·4 mg/ml in plasma ..	83	110	84	65	97			110	1·40
Iodopyracet-Na, 0·4 mg/ml in H_2O ..	102	135	87					135	1·9
Iodopyracet-Na, 0·4 mg/ml in plasma ..	106	138	87	65	97			138	1·96
Diatrizoate-Na, 0·4 mg/ml in H_2O ..	65	88	68					88	0·96
Diatrizoate-Na, 0·4 mg/ml in plasma ..	64	90	66	60	96			90	1·0

EV = Effluent volume; 0·2 ml sample was placed in each case. Each determination was done in quadruplicate.

* The Vi values are calculated assuming that the KD value of creatinine is 1·0. As far as the radiopaque solutions in plasma are concerned the data of the peak minimum have reference to the radiopaque agent.

COMBINATION WITH A SE-SEPHADEX 25C COLUMN

As expected at lower pH values, there was a stronger retardation of the passage of basic and amphoteric compounds, while for the acids the tendency to pass unhindered was increased (Table 2). Therefore the column of neutral Sephadex was used with a column of the cation-exchange gel, Se-Sephadex 25c.

Materials and methods

Neutral Sephadex, Sephadex G-25c (approximate exclusion limit 5000 M.W.) and the cation exchanging Sephadex, Se-Sephadex-25c

(Pharmacia, Uppsala, Sweden) were used. Columns of 2 cm in diameter and 40 cm long for the neutral and 25 cm long for the cation exchanger Sephadex were combined, each having a sintered glass filter at the end (Fig. 2). After carefully washing the Sephadex in distilled water, followed by three washings in the elution fluid to be used, the column was filled as described by Porath (1960). Precipitation of plasma proteins was avoided by using a 0·08 M phosphate buffer. At the fixed pH 7·6, Tyrode 0·08 M can also be used.

TABLE 2. EXPERIMENTAL DATA OF COMPOUNDS ELUTED ON A SE-SEPHADEX-25C COLUMN, 25 CM OF LENGTH

Sample placed	Eluted with 0·08 M phosphate buffer		
	pH 7·4 Ve in ml	pH 6·0 Ve in ml	pH 5·0 Ve in ml
0·6 ml Plasma	30	30	30
0·6 ml Acetrizoate-Na, 0·1 mg/ml in H_2O	44	44	40
0·6 mg Creatinine, 0·1 mg/ml in H_2O	69	94	180

Each determination was done in triplicate.

In the experimental set-up (Fig. 2) the combined column is pretreated with 60 ml phosphate buffer pH 6·4. A 0·6 ml sample is placed on the top of the neutral (N) column with an Agla-micrometer supplied with a ball joint KVII. KII is turned to the position in which the N column is eluted with phosphate buffer pH 5·0. KI is turned into the position

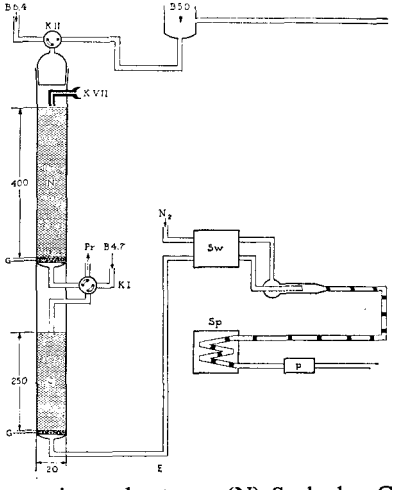

FIG. 2. Diagram of experimental set up: (N) Sephadex G-25c column; (C) Se-Sephadex 25c column; (G) glass filter; (KI) and (KII) multiway taps. (B 6·4) inlet phosphate buffer, pH 6·4, for pretreatment column (N), (B 5·0) inlet elution fluid, phosphate buffer pH 5·0, (KVII) socket joint for injection sample. The fixed height of the supply vessel, and the capillary tube of the inlet, makes it possible to place the sample on the column, while the column is eluting. (Pr) outlet protein fraction, (B 4·7) inlet phosphate buffer, pH 4·7, for pretreatment column (C); (SW) magnetic valve for inlet nitrogen from (N_2); (Sp) glass-spiral; (p) peristaltic pump used during the elution to give gradual filling (1·5 ml/min) of the glass-spiral with elution fluid. After filling, the spiral is connected to the flow cell of a spectrophotometer for recording.

shown in Fig. 2. While the protein fraction is removed in this way from the end of the N column (Pr in Fig. 2), the cation (C) column is pretreated with phosphate buffer pH 4·7. As soon as the desired effluent volume from the N column is removed—this volume depends on the K_D-value of the radiopaque agents (Table 1)—KI is turned to the position in which the N and C column are connected. Instead of taking separate samples, the per cent transmission in the elution fluid coming from the C column is measured continuously. The eluate is divided in small portions by nitrogen bubbles and stored in a glass spiral 2 mm diameter and 10 m long with a total content of 130 ml. When the fluid is passed through the column and stored in the spiral it can be passed at any suitable time and at any speed required through a flow cell placed in the Beckman DB spectrophotometer for the measurement of the per cent transmission. Before passing through the flow cell the nitrogen bubbles, which served to prevent convection and mixing of the fluid, are removed. The light transmission is recorded.

Results

The heights of the transmission peaks obtained with various samples of identical size and containing identical concentrations of the radiopaque agent were remarkably consistent. Table 3 gives an example of a comparison of the transmissions measured with 4 radiopaque agents dissolved in water and in plasma. Calibration curves for 4 radiopaque agents in water were prepared (see Fig. 3). With plasma samples identical curves

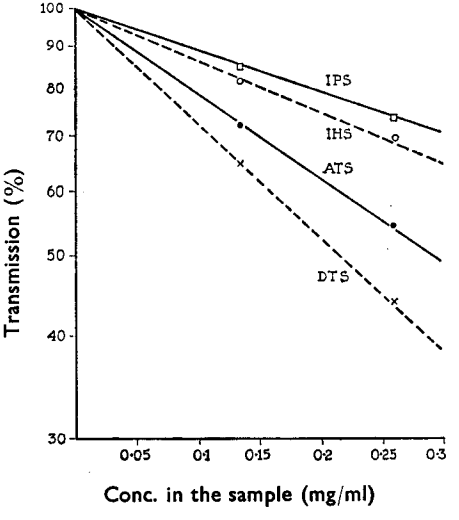

FIG. 3. The minimum in the transmission at 237 or 230 mμ measured for various radiopaque agents in the effluent of the double column system, expressed in percentage on a logarithmic scale as a function of the concentration of the radiopaque agent in the sample. (IPS) iodopyracet-sodium; (IHS) iodohippurate-sodium; (ATS) acetrizoate-sodium; (DTS) diatrizoate-sodium. Note the linearity in the relationship which indicates a practically 100% recovery of the drug and makes it possible to use the curves for calibration.

were obtained (see Table 3). By means of these calibration curves the concentration of the radiopaque agents in the unknown samples could be determined.

Calculation of a known concentration of 0·200 mg/ml with the aid of the calibration curves in Fig. 3, gives a value for the concentration of the sample of 0·199 $\pm$ 0·005. This result agrees with our other determinations and confirms that the determinations showed no deviation greater than 3% from the mean.

TABLE 3. CALIBRATION DATA FOR THE RADIOPAQUE AGENTS ON THE DOUBLE COLUMN

Sample placed on the column	Vol. removed from neutral column	% Transmission at the peak of the effluent fraction*			
		Sample conc. 0·1336 mg/ml H_2O	Sample conc. 0·1336 mg/ml plasma	Sample conc. 0·2506 mg/ml H_2O	Sample conc. 0·2506 mg/ml plasma
Acetrizoate-Na ..	70	72 (0·6)	72·4 (0·6)	55·2 (1·2)	
Creatinine	70				99 (0·3)
Iodopyracet-Na ..	100	84 (0·7)	83·3 (0·6)	75·5 (0·8)	
Creatinine	100				100 (0·1)
Iodohippurate-Na ..	80	83·2 (0·3)	82·3 (0·4)	71·4 (0·5)	
Creatinine	90				99 (0·2)
Diatrizoate-Na ..	60	64 (0·4)	64·5 (0·6)	45·8 (1·0)	
Creatinine	60				99 (0·3)

In all cases 0·6 ml sample was placed on the column. Each determination was done in quadruplicate.
* Mean value; the range is given in parentheses.

An important aspect of the data in Table 3 is that the concentrations of the radiopaque agents measured in the plasma samples were found to be identical to those in the water samples. This means that a total dissociation of the radiopaque agent from the proteins takes place and that with this method the total concentration, bound and unbound, of radiopaque agent is measured.

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