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STUDY ON THE ANALOGY OF GEL PERMEATION CHROMATOGRAPHY AND CUPROPHAN DIALYSIS FOR THE ISOLATION OF UREMIC 'MIDDLE MOLECULES' USING MODEL COMPOUNDS

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SUMMARY

Selectivities of substituted nitrobenzenes in gel permeation chromatography and cuprophan membrane dialysis were compared. Glucuronide-, glucoside-, acetic acid- and lactoside-substituted p-nitrobenzenes were chosen as model compounds for so-called 'middle molecules' in uremia. It was found that there was not a single linear relationship between the substituent effects in both processes. This was because of the anomalous behaviour of the model compounds in gel permeation. A combination of adsorption and ionic rejection in the latter technique, for the various solutes, was not encountered in cuprophan membrane dialysis, which appeared to be governed only by the molecular mass or volume of the solute. Therefore, gel permeation seems to be inappropriate for the analysis or isolation of so-called middle molecules in sera of uremic patients. Dialysis or filtration on relatively inert membranes is less susceptible to anomalies.

INTRODUCTION

In the past fifteen years many attempts have been made to test the so-called 'middle-molecule' (MM) hypothesis for uremic toxicity, as proposed by Babb et al. [1]. According to this hypothesis, certain solutes that behave in a hemodialyser as though they have intermediate molecular masses (between 300 and 1500 daltons) are thought responsible for the symptoms encountered in uremia, especially uremic neuropathy. No conclusive evidence of their importance has yet been found [2]. Biochemical analysis has been performed mainly by gel permeation chromatography (often named gel filtration) coupled with
ion-exchange chromatography. In recent years a number of solutes present in MM gel permeation chromatography fractions of sera of patients with chronic renal failure have been identified as glucuronide derivatives of aromatic acids. These compounds with rather low molecular masses [3–6] had smaller elution volumes on Sephadex G-15 gels than expected from their molecular mass. An explanation of the anomalous retention of these solutes is found in the rejection of these acidic compounds by carboxyl groups present in the gel matrix. It has been reported that MM peak 7c [7], mainly consisting of o-hydroxyhippuric acid glucuronide \((M_r = 371)\) [4], is dialysed as if it had a higher molecular mass. A clearance value of 36 ml/min on a Gambro Optima (13.5 μm) hemodialyser was reported [8], which is close to the value for vitamin B\textsubscript{12} \((M_r = 1355)\). It was suggested that the anomalous retention of this acidic compound in gel permeation chromatography is also reflected in its diffusive transport through dialyser membranes [9], i.e. it will not be cleared according to its molecular mass.

Anomalous dialysis behaviour has been observed for phosphate, which has a significantly lower dialyser clearance than would be expected from its molecular mass. This is probably due to solvation. This effect, which concerns the effective molecular volume, is also found in gel permeation chromatographic analysis. Some other solutes show exceptional properties on dialysis with cellulose membranes [10]. Alternatively, it has been demonstrated that cuprophane membrane resistances depend linearly (log–log) on solute molecular mass of a wide range of acidic, basic and neutral compounds with different chemical structures in the molecular mass range between 60 and 2000 daltons [11], which demonstrates the relative inertness of the cuprophane membrane.

This report examples the behaviour of commercially available model compounds in gel permeation chromatography and dialysis through cuprophane membranes, in order to study and compare the selectivity of glucuronide-, glucoside- and carboxyl-substituted aromatics in these processes. The chosen model compounds were expected to exhibit adsorption, rejection and size-exclusion behaviour in gel permeation chromatography, to different proportions.

According to Hammett [12], in a series of \(m\)– and \(p\) substituted benzene derivatives, the logarithms of the substituent effects in one process or reaction are, in general, linearly related to those in another reaction. This phenomenon was interpreted as a linear free energy relationship (LFER) [12,13]. The so-called Hammett equation representing this observation is:

\[
\log \frac{K}{K_0} = \rho \log \frac{K'}{K'_0}
\]

where \(K\) and \(K_0\) are equilibrium or rate constants of a substituted and an unsubstituted benzene derivative, \(K'\) and \(K'_0\) are these constants for the corresponding substituted and unsubstituted benzoic acids, and \(\rho\) is a proportion-
ality constant corresponding to the reaction involved. The logarithmic term on the right-hand side of the equation is called the Hammett substituent constant ($\sigma$) and serves as a reference. If the behaviour of the substituted benzene derivatives in one process (A) depends on the nature of the substituent in a similar way as in another process (B), there should be a linear relationship between \( \log \left( \frac{K_{s,i}}{K_0} \right)_A \) on one hand and \( \log \left( \frac{K_{s,i}}{K_0} \right)_B \) on the other for various substituents $i$ \( (K_{s,i}) \) is a rate or equilibrium constant for the compound with substituent $i$), when a single reaction centre (e.g. the aromatic ring) \([14,15]\) is maintained.

**EXPERIMENTAL**

**Materials**

The model compounds used in gel permeation chromatography and dialysis were: nitrobenzene \((M_r = 123)\), p-nitrophenylacetic acid \((M_r = 181)\), p-nitrophenylglucoside \((M_r = 301)\), p-nitrophenylglucuronide \((M_r = 315)\) and p-nitrophenyllactoside \((M_r = 463)\); they were all purchased from Sigma (St. Louis, MO, U.S.A.). Additionally, we used the following compounds for gel permeation chromatography: \(^2\text{H}_2\text{O} \) (99%) \((\text{Janssen, Beerse, Belgium})\), creatinine \((M_r = 113)\), vitamin B\(_{12}\) \((M_r = 1355)\), benzene \((M_r = 78)\) \((\text{Merck, Darmstadt, F.R.G.})\), phenylglucuronide \((M_r = 270)\) and phenylglucoside \((M_r = 256)\) \((\text{Sigma, St. Louis, MO, U.S.A.})\).

**Gel permeation chromatography**

We used a 1.6 cm I.D. column packed with ca. 40 ml (bed height 20 cm) of Sephadex G-15 \((\text{Pharmacia, Uppsala, Sweden})\), swollen in Tris–HCl buffer \((10 \, \text{mM, pH 8.6})\). \( V_0 \) (dextran blue) was 18.5 ml and \( V_t \) \((^2\text{H}_2\text{O}) \) was 40 ml. The flow-rate was 14.2 ml/h, using a peristaltic pump \((\text{LKB, Bromma, Sweden})\). UV absorbance detection was applied at a wavelength of 254 nm \((\text{Jasco Uvidec II, Tokyo, Japan})\). We used refractive index detection \((\text{Knauer, Bad Homburg, F.R.G.})\) for determination of the elution volume of \(^2\text{H}_2\text{O}\). The column, equipped with a water jacket, was maintained at 25°C.

**In vitro dialysis**

In the dialysis experiments a Gambro hemodialysis unit \((\text{Gambro, Lund, Sweden})\) was used. The test solutes were dissolved in and dialysed against fresh dialysate solution \((\text{obtained from concentrate})\) at pH 7.4. The volume of the ‘blood-side’ recirculation container was 2 l. The test solution in this container was maintained at 37°C. Flow-rates of the test solution and dialysate fluid were 200 and 500 ml/min, respectively. Dialysate solution was not recirculated, but directed to waste after single passage. A Gambro parallel plate dialyser \((\text{Lundia 3N, 0.8 m}^2\, \text{effective surface area, 10 μm membrane thickness})\) was
used. For the determination of dialyser clearances, samples were taken from the recycle container at 10-min intervals. Concentrations of test solutes were determined by reversed-phase liquid chromatography.

**Calculations**

Elution in gel permeation chromatography is expressed in terms of a distribution coefficient $K_d$ as follows:

$$K_d = \frac{V_e - V_0}{V_i} \quad (2)$$

where $V_e$, $V_0$ and $V_i$ are peak elution volume, void volume (elution volume of a totally excluded marker, e.g. dextran blue) and the total internal fluid volume of the gel. The value of $V_i$ was determined from the elution volume of $^2\text{H}_2\text{O}$ corrected for isotope exchange of hydroxyl hydrogen from the gel [14]:

$$K_d = 1.075 \frac{V_e - V_0}{V_{^2\text{H}_2\text{O}} - V_0} \quad (3)$$

The membrane dialyser clearance ($K_B$), by definition, equals the amount of solute $i$ removed from the blood-side per unit time, $\dot{M}$, divided by the blood-side inlet concentration ($C_{Bi}$):

$$K_B = \frac{\dot{M}}{C_{Bi}} \quad (4)$$

The blood-side fluid is recycled in a closed loop from a container of volume $V$. The concentration drop as a result of pure dialysis (no ultrafiltration) is:

$$\ln \frac{C}{C_0} = - \frac{K_B}{V} t \quad (5)$$

where $K_B/V$ is a first-order rate constant.

Samples were taken at 10-min intervals from the recycle container. Clearances were determined by regression of $\ln C/C_0$ versus $t/V$.

**Statistics**

For the calculation of dialyser clearances, linear regression analysis was performed using the SAS procedure REG (SAS Institute, Cary, NC, U.S.A.) [16].

**RESULTS AND DISCUSSION**

Gel permeation distribution coefficients, dialyser clearances and substituent effects for the substituted nitrobenzenes in both processes are given in Table I. A plot of the group selectivities (substituent effects) in gel permeation chromatography versus those in dialysis reveals that there is not a single linear
### TABLE I

**DISTRIBUTION COEFFICIENTS ($K_d$), DIALYSER CLEARANCES ($K_B$), AND GROUP SELECTIVITIES IN BOTH PROCESSES FOR THE SUBSTITUTED NITROBENZENES**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$M_r$</th>
<th>$K_d$</th>
<th>$\log\left(\frac{K_d}{K_{d,nbz}}\right)^a$</th>
<th>$K_B$ (ml/min)</th>
<th>$\log\left(\frac{K_B}{K_{B,nbz}}\right)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrobenzene</td>
<td>123</td>
<td>3.75</td>
<td>0</td>
<td>125</td>
<td>0</td>
</tr>
<tr>
<td>$p$-Nitrophenylacetic acid</td>
<td>181</td>
<td>0.65</td>
<td>-0.761</td>
<td>95</td>
<td>-0.119</td>
</tr>
<tr>
<td>$p$-Nitrophenylglucoside</td>
<td>301</td>
<td>2.28</td>
<td>-0.216</td>
<td>75</td>
<td>-0.222</td>
</tr>
<tr>
<td>$p$-Nitrophenylglucuronide</td>
<td>315</td>
<td>0.49</td>
<td>-0.884</td>
<td>67</td>
<td>-0.271</td>
</tr>
<tr>
<td>$p$-Nitrophenyllactoside</td>
<td>463</td>
<td>1.85</td>
<td>-0.307</td>
<td>55</td>
<td>-0.357</td>
</tr>
</tbody>
</table>

*nbz = nitrobenzene.

![Fig. 1](image_url)

Fig. 1. Plot of functional group selectivities in gel permeation chromatography versus those in cuprophan dialysis. lct, gls, glr and ac denote $p$-nitrophenyllactoside, -glucoside, -glucuronide and -acetic acid, respectively. (a) Acidic solutes; (b) neutral solutes.

The relationship for the four substituted nitrophenyl compounds (Fig. 1). However, approximately parallel lines can be drawn through the data points of the ‘acidic’ (lower line) and ‘neutral’ (upper line) substituents, suggesting a linear relationship within these solute groups. The carboxylic acid group contribution to gel chromatographic retention appears to be larger than expected from its contribution to molecular mass. This effect has been described earlier [17] and is explained by ionic rejection with carboxyl groups in the gel matrix.

The addition of an acidic group (carboxyl or glucuronide) apparently changes...
Fig. 2. Logarithm of cuprophan membrane dialyser clearances ($K_B$) (left) and Sephadex G-15 gel permeation distribution coefficients ($K_d$) (right) as a function of the molecular mass of the substituted nitrobenzenes. Note the range of axes. Open symbols denote data taken from manufacturers' documentation, filled symbols denote our own measurements. Solute identification ($M_i$): crea, creatinine (113); nbz, nitrobenzene (123); bz, benzene (78); nac, p-nitrophenylacetic acid (181); ngls, p-nitrophenylglycoside (301); nglr, p-nitrophenylglucuronide (315); nolct, p-nitrophenyllactoside (463); gld, phenylglucoside; glr, phenylglucuronide; B12, vitamin B$_{12}$ (1355).

Lines a and b, neutral substituted nitrobenzenes; lines d and e, acidic substituted nitrobenzenes; line c, Sephadex G-15 molecular mass calibration line.

the primary reaction centre. In this case the Hammett equation is not applicable. Rejection becomes the prevailing factor governing retention, instead of adsorption ($K_d > 1$) and size-exclusion ($0 < K_d < 1$), the former of which is commonly found in gel permeation chromatography of aromatic solutes [18].

In Fig. 2 the membrane dialyser clearances and gel permeation distribution coefficients are plotted against molecular mass on a log–log scale. In gel permeation benzene, phenylglucoside and phenylglucuronide were analysed in addition to the nitrobenzenes. It can be seen that in membrane dialysis solute clearance is linearly (log–log) dependent on molecular mass regardless of the nature of the substituent. The linear regression line for diffusive transport in the dialyser, fitted to the log-transformed data, was (see also Fig. 2):

$$\log K_B = 3.309 - 0.587 \log M_i$$

with a correlation coefficient $r = 0.995 \ (P < 0.0001)$. In this regression line vitamin B$_{12}$ is not incorporated. At higher molecular mass (i.e. vitamin B$_{12}$, $M_i = 1355$) a regression line seems to have a smaller slope than the value of $-0.587$, which applies to molecular masses up to 500 daltons. This would be in accordance with the data reported by Wilke and Chang [19], who demonstrated a linear dependency of the solute diffusion coefficient on solute molal volume (log–log) with slope $-0.6$ between 40 and 500 cm$^3$/mol, and a value of
—0.3 at higher molal volumes, the latter of which also follows from the Stokes–Einstein equation. It can be seen from Fig. 2 that in dialysis no significant adsorption or rejection is observed for the substituted nitrophenyl compounds.

Conversely, in gel permeation chromatography the neutral substituted nitrobenzenes (i.e. glucoside and lactoside) (lines a and b) have larger \( K_d \) values than creatinine and vitamin \( B_{12} \) (line c), which is due to adsorption. Line c represents a part of the Sephadex G-15 selectivity curve, which applies to solutes that have no significant adsorption or rejection. In contrast to the neutral compounds the acidic substituted compounds (lines d and e) have \( K_d \) values smaller than expected from the selectivity curve c. Therefore, it can be concluded that molecular permeation of substituted aromatic solutes is not the same in gel permeation analysis as it is in membrane dialysis.

In the past fifteen years it has been thought that uremic MMs could be peptides resulting from protein catabolism [20]. It is likely that oligopeptides with different numbers of aromatic amino acids, and with different acidity, may have very different elution volumes in gel permeation chromatography, owing not only to differences of conformation but also to chemical interaction.

In conclusion, a general statement that anomalous elution behaviour in gel permeation chromatography will also be reflected in cuprophan dialysis evidently is not valid. The substituted nitrobenzenes behave differently in gel permeation chromatography (using Sephadex gels) and in cuprophan dialysis. In gel permeation chromatography an additional acidic interaction occurs. All this may be generalized as follows: the different factors governing elution in gel permeation chromatography do not necessarily work out the same way in membrane dialysis. Elution volumes of (uremic) serum fractions in Sephadex gel permeation chromatography cannot be used to predict the dialyser behaviour of the (unknown) solutes in these fractions. Conversely, gel permeation chromatography elution volumes cannot be predicted from given dialyser clearances (e.g. those for MMs). The case of phosphate, where anomalous effects are analogous, represents those solutes with anomalous permeation behaviour governed solely by (deviating) molecular volume (be it as a result of solvation or of conformation) and not those solutes that show some form of chemical interaction. The results of this study support our earlier conclusion [2,21,22] that analysis and isolation of anticipated MMs by gel permeation chromatography is not the most rational approach. Dialysis or filtration on relatively inert membranes is less susceptible to anomalies.

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