acebutolol (15 mg/L), 0.015 for acetamide (20 mg/L), 0.014 for aniline (5 mg/L), and 0.023 for a mixture of the three.

Extraction with dichloromethane had no effect on the values obtained during the preparation of the normetanephrine standard curve.

Metanephrine determinations on urines of patients given acebutolol therapy showed either a negative or a negligibly small difference between blank and sample absorbances. After extraction with dichloromethane, in all the cases the blank absorbance decreased (Table 1); this decrease occurred in both the blank and the sample, but was much more pronounced in the blank. The metanephrine concentrations measured after extraction were in the same range as in patients not taking acebutolol (i.e., 300 to 1500 µg/24 h).

Our results demonstrate that acebutolol and its two main urinary metabolites show a significant absorbance at pH 12 to 13 in the 350–360 nm region. Furthermore, hot acidic hydrolysis leads to an increase in absorbance, confirming that acebutolol is unstable in these conditions (3); therefore, the acidic step of the Pisano method might be an important factor in the development of drug-related interference, by releasing compounds that absorb at 350 and 360 nm.

That the absorbance of the mixture of the three compounds is lower than the absorbance of the acetyl acetabolol alone might be attributed to the butyramidie moiety of acetabolol.

When this procedure is applied to urine of patients taking acebutolol, the drug and its metabolites do not interfere and the absorbance differences observed correspond only to urinary metanephrine content in the physiological range. We conclude that dichloromethane extraction can be used routinely for determinations of urinary metanephrines in patients who are undergoing therapy with acebutolol.

Table 1. Absorbance Readings for Urines of 15 Patients Receiving Acebutolol, with and without Dichloromethane Extraction

<table>
<thead>
<tr>
<th>No</th>
<th>Extraction</th>
<th>Metanephrine, µg/24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.064</td>
<td>1170</td>
</tr>
<tr>
<td>0</td>
<td>0.099</td>
<td>1380</td>
</tr>
<tr>
<td>0</td>
<td>0.071</td>
<td>980</td>
</tr>
<tr>
<td>0.005</td>
<td>0.015</td>
<td>220</td>
</tr>
<tr>
<td>0.005</td>
<td>0.011</td>
<td>140</td>
</tr>
<tr>
<td>0</td>
<td>0.026</td>
<td>490</td>
</tr>
<tr>
<td>0</td>
<td>0.082</td>
<td>1300</td>
</tr>
<tr>
<td>0</td>
<td>0.053</td>
<td>750</td>
</tr>
<tr>
<td>0</td>
<td>0.023</td>
<td>350</td>
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<tr>
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<td>0.015</td>
<td>210</td>
</tr>
<tr>
<td>0.002</td>
<td>0.011</td>
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<tr>
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<tr>
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<td>0.013</td>
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<tr>
<td>0.018</td>
<td>0.017</td>
<td>290</td>
</tr>
<tr>
<td>0</td>
<td>0.009</td>
<td>150</td>
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</table>

References

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Factors Affecting Ultrafiltration-Assessed Values for Drug Binding

To the Editor:

Several techniques have been reported for assessing drug binding to plasma proteins, the two most popular being equilibrium dialysis and ultrafiltration. Both of these procedures rely on a membrane that is permeable to small (drug) molecules but impermeable to macromolecules; however, they differ considerably with regard to their underlying assumptions, method of separation, and speed of assay. The literature on the binding of the anti-asthmatic drug, theophylline, in human serum or plasma as assessed by equilibrium dialysis (1, 2) and ultrafiltration (3–6) reveals large intra- and inter-technique variability, with binding values ranging from 28 to 80%. Several investigators have alluded to the possibility that nonspecific drug binding may distort the values obtained in both equilibrium dialysis (7–10) and ultrafiltration (3, 11, 12). However, these methodological considerations are almost always disregarded when such data are used to explain clinical observations. We therefore compared the two techniques with respect to the binding of four drugs in human serum and plasma. The materials (and their sources) were as follows: theophylline and acetaminophen (Sigma Chemical Co., St. Louis, MO 63178); warfarin (Frosst, Pointe, Claire, P.Q. H9R 4P8, Canada); mannitol (J. T. Baker Chemical Co., Phillipsburg, NJ 08865); [14C]mannitol (New England Nuclear, Lachine, P.Q. H8T 3C9); HPLC-grade solvents (Cal-
edon, Georgetown, Ont. L7G 4R9, Canada; Fisher Scientific, Don Mills, Ont. M3A 1A8; and J. T. Baker); dialysis tubing, 12-mm (o.d.) cellulose, molecular exclusion limit 12 000 daltons (A. H. Thomas, Philadelphia, PA 19105). All other chemicals were reagent grade, from various suppliers. Serum and plasma from ostensibly healthy volunteers were obtained from the Blood Bank of Kingston General Hospital and stored at −20 °C.

We determined theophylline concentrations by using the liquid-chromatographic assay of Nakatsu et al. (19), with which standard curves are linear and reproducible from 0.1 to 60 mg of theophylline per liter. Warfarin was assayed by the liquid-chromatographic method of Tasker and Nakatsu (14), which was linear and reproducible from 0.05 to 20 mg/L. Acetaminophen was extracted from all media as follows: equal volumes (40 to 200 µL) of sample (saturated with Na2SO4) and chloroform/t-amyl alcohol/HCl, 11.3 mol/L (60/40/0.25 by vol) were added to 0.5- or 1.5-mL disposable microscale centrifuge tubes. The tubes were capped and shaken in a horizontal shaker for 6 min, then centrifuged for 4 min. A 20-µL aliquot of the clear organic (upper) phase was then injected onto the silica gel column. The mobile phase was hexane/isopropyl alcohol/water (80/19/1 by vol), the flow rate 1.5 mL/min. Assay results were linear and reproducible from 0.25 to 20 mg of acetaminophen per liter.

Concentrations of [14C]mannitol were determined by liquid scintillation counting, with external-standards ratio quench correction. The stock solution contained 10 mg of mannitol per liter, with an activity of 10⁴ dpm/40 µL. Samples and blanks (40 µL) were mixed with 10.0 mL of Scintiverse LSC cocktail (Fisher Scientific) in standard glass vials, and this radioactivity was counted for 10 min or 40 000 counts, whichever came first; mannitol concentrations were calculated after background subtraction.

Equilibrium dialysis was conducted as follows: the serum (adjusted to pH 7.4 where necessary, with O2/CO2, 95/5 by vol) or buffer sample (2.0 mL), contained in a bag of dialysis tubing tied at each end, was equilibrated against 20.0 mL of Krebs-Ringer phosphate or Hank’s 1949 buffers (pH 7.4) for 16 h at 37 °C, with mild agitation. After this incubation, duplicate samples from both compartments were removed for drug concentration measurement.

Ultrafiltration was conducted on 2.0-mL samples of serum (pH 7.4), plasma (pH 7.4), or buffer, each of which had been incubated at 37 °C for 1.5 h in a test tube before filtration. After incubation, the sample was transferred to the reservoir of the filtration apparatus (15) and centrifuged (250 × g, 20 min) to yield about 60 µL of protein-free filtrate. Filtrate volume was controlled by adjusting the duration of centrifugation, although volumes in excess of 150 µL required centrifugation at 1000 × g. Buffer samples were always centrifuged at 250 × g, and filtrate volume was again controlled by adjusting the duration of centrifugation. After ultrafiltration, duplicate samples of both filtrate and retentate were removed and assayed for drug, except in the case of filtrate volumes <6 µL, where only one 40-µL sample of filtrate could be assayed. The concentration of all drugs under all conditions was 10.0 mg/L in the original sample. Ultrafiltration membranes were type PTGC (Millipore, Mississauga, Ont. L4V 1M5, Canada), except where noted.

We found that apparent binding values for theophylline added to 10 human serum samples were 20.4 (SD 3.79)% and 76.7 (SD 7.07)%, as assessed by equilibrium dialysis and ultrafiltration, respectively. Similarly, apparent binding of acetaminophen in 10 serum samples was 33.5 (SD 8.58)% and 85.3 (SD 1.96)% by these two techniques. These discrepancies were significantly different for both theophylline (t(19) = 5.98, df; = 8; p < 0.001) and acetaminophen (t(19) = 13.28, df; = 8; p < 0.001). Further investigation showed that these discrepancies were not the result of serum storage, inter-individual differences, dilution phenomena, or unequal equilibration times. Dialysis of buffer against buffer containing drug revealed no appreciable distortion, by the dialysis tubing, of the established equilibrium for theophylline, acetaminophen, or warfarin. Mean apparent binding values were 2.25 (SD 2.85)% for theophylline, −0.5 (SD 2.40)% for acetaminophen, and 1.0 (SD 1.18)% for warfarin (n = 4).

Ultrafiltration of drug solutions in distilled water, however, yielded apparent binding values of 50.2 (SD 3.98)% for theophylline, 86.7 (SD 1.90)% for acetaminophen, and 83.2 (SD 5.24)% for warfarin (n = 4). The effects of altered filtrate volumes on the drug concentration in the ultrafiltrate (apparent binding) for four drugs are shown in Figure 1. Each sample contained 10 µg of theophylline, acetaminophen, warfarin, or mannitol per liter, in Hans’ 1949 buffer (pH 7.4), and each point represents the means of at least three filtrations. Volumes were within 10 µL of the stated volume for all filtrations. Theophylline, acetaminophen, and warfarin all demonstrated substantial apparent binding, whereas mannitol showed no apparent binding at any volume tested. As shown in Figure 2, plasma binding values for theophylline and acetaminophen also depend on filtrate volume. Both curves appeared to plateau at about 200 µL of filtrate (10%), where the apparent percentages bound are 40% for theophylline and 22% for acetaminophen.

We believe that the apparent binding when three of the four drugs were stud-
We conclude that ultrafiltration is not a universally appropriate technique for estimating the extent of drug binding to plasma macromolecules. It appears to be most appropriate in cases in which the drug being studied is hydrophilic and ordinarily is used at relatively high concentrations in plasma. Because there is no control against which one can compare any of the current techniques, all such data must be interpreted with caution.

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References


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Tris Carbonate Interferes with Certain Methods for Protein and CO2

To the Editor:

Evaluating a lot of Fisher "Sera-Chem" normal human control material (lot no. 311-010), reconstituted with Fisher abnormal diluent II (no. 293576; Fisher Scientific Co., Orangeburg, NY 10902), we found discrepancies in the bicarbonate and total protein concentrations, as determined with different analytical systems. The diluent contains no carbon dioxide and an unspecified concentration of tria(hydroxymethyl)aminomethane (Tris) carbonate. A second diluent used in this study was distilled water and tetramethyl ammonium bicarbonate (Hyland, Costa Mesa, CA 92626).

Total carbon dioxide was determined with a SMAC (Technicon Instruments Corp., Tarrytown, NY 10591), in which the carbon dioxide released after addition of acid is absorbed by an alkaline-buffered solution containing phenolphthalein (1). The red color of the phenolphthalein is proportional to the total carbon dioxide content of the sample. We also used a PVA-4 electrolyte analytical system (Photovolt Corp., New York, NY 10010), which determines bicarbonate as follows: a strong acid is added to the sample, liberating carbon dioxide, and an amount of acid equivalent to the carbon dioxide released is used to neutralize the sample. The residual free acid is backtitrated to pH 7.4 and the bicarbonate concentration computed.

We determined total serum protein with the SMAC, which includes the biuret reaction and measurement of absorbance of the resulting complex at 550 nm (1); an aca (Du Pont Co., Instrument Products Div., Wilmington, DE 19898), which also involves the biuret reaction but measures the absorbance of the complex at 540 nm (2); a manual