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Cyclosporin: measurement of fraction unbound in plasma

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A reproducible ultracentrifugation method has been developed for the measurement of the fraction of cyclosporin (CyA) unbound in plasma. The sample is centrifuged to remove any particulate matter, ultracentrifuged in polyallomer tubes and then frozen in liquid nitrogen. Appropriate sections are then cut from the tube for determination of the concentration of radioactivity and calculation of fraction drug unbound. Using this method, the fraction unbound has been measured in plasma from renal transplant patients receiving CyA and found to range between 0·04 to 0·122. The binding is temperature-dependent and principally hydrophobic.

Cyclosporin (CyA) is a 'third generation' immunosuppressant used chronically in transplant patients (Borel 1982). Authors disagree on the possibility of relating adverse clinical response, such as nephrotoxicity, to CyA concentration in blood, plasma or serum as measured by either radioimmunoassay (RIA) or HPLC, since rejection sometimes occurs with high CyA concentrations in these fluids and nephrotoxicity with low concentrations (Morris et al 1982; Stiller et al 1982; Lokiec et al 1982; Kahan et al 1982; Calne 1985; Drakopoulos et al 1985; Rogerson et al 1986). Nonetheless, increasingly, blood and plasma concentration monitoring is being used as a guide in CyA therapy.

It is generally accepted that the therapeutic or toxic response to a drug is better related to its concentration in plasma water, than to its concentration in plasma or whole blood (Rowland 1980). Limited in-vitro data indicate that CyA is highly (98%) bound in human plasma (Niederberger et al 1983), particularly to lipoproteins (Sgoutas et al 1986; Lemaire & Tillement 1982; Mraz et al 1983). Difficulties have existed, however, in developing a reliable method to determine the protein binding of CyA. Awni & Sawchuk (1985) found that it either binds to or fails to pass through commercially available semipermeable membranes, thus negating the use of the commonly used techniques of equilibrium dialysis and ultrafiltration.

We now report on a reliable ultracentrifugation method and show its application to binding measurements of CyA in the plasma of renal transplant patients receiving the drug.

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MATERIALS AND METHODS

Plasma samples

Blood, containing EDTA (approximately 1 mg mL^{-1}) as the anticoagulant, was centrifuged at 3500 rev min^{-1} for 4 min at 37 °C and the plasma collected at the same temperature. The plasma samples were allowed to equilibrate with the normal atmosphere and no attempt was made to control the pH. (Preferably, the binding determination should be carried out immediately but if this is not possible the plasma must be stored in the dark at 4 °C where lipoproteins are stable for up to 3 weeks (Bronzert & Brewer 1977).)

Radiolabelled CyA

Tritiated CyA of high specific activity was obtained from Sandoz (Basel, Switzerland) Batch No. RA574-1 (Ch83.0210) with a radiochemical purity >99%. A solution was prepared in methanol containing 110 μCi (61 μg) of CyA mL^{-1}. The specific activity of the CyA was 1·79 μCi μg^{-1}.

Measurement of the radioactivity of the [^{3}H]CyA samples was by liquid scintillation counting (LKB Model 1218, LKB-Wallac, Turku, Finland) with RIA-LUMA scintillator (LKB) in glass vials. Quench correction was by the external standard ratio (ESR) method.

Ultracentrifugation

The spiked plasma samples were centrifuged at 37 °C (±0·75 °C) and 180 000*g* with a high-speed centrifuge (MSE Superspeed 65, MSE Ltd, Crawley, Sussex, UK). A ten-place fixed-angle (35°) titanium head (MSE Part No. 43114-124), capable of taking capped '3 mL' polyallomer tubes (MSE Part No. 34411-105) was used for the samples.

Section freezing and cutting. Each ultracentrifuge tube was separately handled after centrifugation. For freezing, the tube was placed in a holder of 18-gauge copper wire, which allowed it to be lowered slowly into liquid nitrogen. Slicing of the sections, held in a 'V' block after freezing, was by cutting through the tube with a junior hacksaw having a fine, blunted blade to minimize swarf with resultant loss of radiolabelled material.

Procedure

The radiolabelled sample was prepared by adding plasma (4·00 mL) to a glass tube which had been freshly spiked with 5·0 μL of [^{3}H]CyA in methanol and dried. In this way no dilution or solvent contamination of the sample occurred. The CyA was dissolved by frequent gentle mixing of the plasma in a water-bath at 37 °C for 20 min. The sample was then centrifuged at 3500 rev min^{-1} for 4 min to deposit particulate matter. Aliquots (180 μL) were taken, without disturbing any deposit, for counting (in duplicate) and for ultracentrifugation (1·62 mL). The capped polyallomer ultracentrifuge tubes were inserted into the ultracentrifuge head, pre-heated to 37 °C, and centrifuged at 50K rev min^{-1} (≈180 000*g*) for 12–18 h, the centrifuge being allowed to stop without braking, to minimize turbulence. Each sample was then frozen by slow immersion in liquid nitrogen (5 mm every 40 s) to prevent distortion of the various 'bands' of solution. After about 4 min the contents were completely frozen.

Several sections were cut from each frozen tube, the cutting equipment being washed between sections. Then each section, including two of 5 mm thickness from the middle of the clear central zone, was placed into individual capped, preweighed liquid scintillation vials which were reweighed and liquid scintillator added; the polyallomer sections then floated to the top and were removed, washed, dried and weighed to enable calculation of the mass of liquid in each section. The concentration of radioactivity in each section was determined, assuming the density of all aqueous solutions to be 1·00.

The section with the lowest radioactivity was taken to contain protein-free drug. The fraction of CyA unbound (fu) was calculated as follows:

$$\text{fu} = \frac{\text{d min}^{-1}\,\text{mL}^{-1}\ \text{protein-free solution}}{\text{d min}^{-1}\,\text{mL}^{-1}\ \text{plasma}} \quad (1)$$

RESULTS

Distribution after centrifugation

The distribution of radioactivity up the ultracentrifuge tube after a typical run with plasma is shown in Fig. 1. The major concentrations of radioactivity

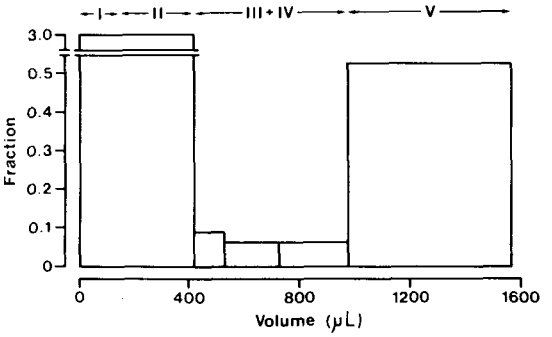

FIG. 1. Distribution of cyclosporin along an ultracentrifuge tube (expressed as fraction of initial concentration) after centrifugation of plasma. Note: zero volume represents the bottom of the tube. See text for meaning of sections I to V.

were in sections II and V which corresponded with albumin + low and high density lipoproteins and with very low density lipoproteins + chylomicrons (to which the drug was found to bind by as much as 30%), respectively. The concentrations of radioactivity in section I, which consisted mainly of gamma-globulin, were similar to that in the original plasma sample. Sections III and IV were the protein-free regions and the lower of the two concentration values determined in these sections was used in the calculation of fraction CyA unbound. The total radioactivity recovered was typically 92–102% of that in the original plasma sample.

The apparent protein content of the sample sections used for estimation of fraction CyA unbound was shown to be <300 μg mL^{-1} by the method of Lowry et al (1951).

Fig. 2 shows the typically obtained shallow gradient of CyA in the tube after ultracentrifugation of

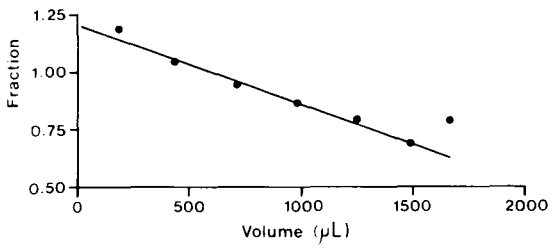

FIG. 2. Distribution of cyclosporin along the ultracentrifuge tube (expressed as a fraction of initial concentration) after centrifugation of plasma ultrafiltrate. Note: zero volume represents the bottom of the sample tube.

the drug in a protein-free plasma ultrafiltrate, prepared by ultrafiltration of control plasma through an Amicon Centriflo System. At the position corresponding to the protein-free region of an ultracentrifuged plasma sample (i.e. Sections III and IV, Fig. 2), the radioactivity was 90–95% of the original activity.

Non-adsorption of CyA to tubes

Experiments demonstrated that the radioactivity seen in the protein-free fraction was not derived from any radioactive material which might have been associated with the polyallomer ultracentrifuge tube wall.

Concentration independence of fraction unbound

As fu measurements were made on patient samples which already contained variable amounts of CyA, fu was determined in blank plasma samples at low and high concentrations over the anticipated concentration range (0–3000 ng mL^{-1}). No evidence of change of fu with CyA concentration was found. The CyA concentration increase from the added spike was approximately 40 ng mL^{-1}.

Reproducibility

During the etablishment of the method, pairs of plasma samples with CyA fu values differing widely (0·0315–0·232) were spiked to give low and high CyA concentrations and were analysed at different temperatures. The mean relative deviation of these samples was 9·7% (n = 25). With the final method, the within-run variability typically had a relative standard deviation of 2·6% (n = 5), which is comparable with that obtained by Verbeeck & Cardinal (1985) for a variety of other drugs using an ultracentrifugation technique.

Temperature dependence of fraction unbound

Table 1 shows the results of experiments carried out at three different temperatures on plasma from three normal subjects. These results show a clear change of fu with temperature, justifying the requirement that measurements should be carried out at physiological temperature to be meaningful.

Patient studies

Measurements of fu were made at frequent intervals on samples obtained over 10 days from kidney transplant patients receiving CyA therapy. The values of fu obtained (Table 2) were variable and in the range of 0·042 to 0·122, a 2·5-fold variation overall.

Table 1. Changes of fraction unbound of cyclosporin with temperature in normal plasma.

Subject no.	Temperature (°C)		
	5	20	37
1	0·173/0·206*	0·067/0·060	0·032/0·031
2	0·232/0·232	0·104/0·112	0·043/0·045
3	0·162/0·202	0·077/0·087	0·034/0·034

* Paired values are two different CyA concentrations (typically 65 and 1000 ng mL^{-1}).

Table 2. Protein binding values (fu) of cyclosporin obtained in plasma from kidney transplant patients by ultracentrifugation at 37 °C.

Patient no.	n	Mean fu	Range of fu
1	32	0·0546	0·043–0·074
2	29	0·0879	0·053–0·122
3	32	0·0685	0·042–0·117
4	31	0·0713	0·061–0·081
5	29	0·0810	0·068–0·091

n = No. of samples.

DISCUSSION

Cyclosporin binds to lipoproteins (Lemaire & Tillement 1982; Mraz et al 1983; Sgoutas et al 1986). As heparin may alter plasma lipoproteins (Piafsky 1980), plasma samples were obtained from blood anticoagulated with EDTA, which has no effect on lipoprotein stability (Bronzert & Brewer 1977). Also, as the distribution of CyA between blood and plasma is temperature-dependent (Follath et al 1983), all blood samples from patients were reheated to 37 °C before separation of the plasma, a procedure known to re-establish the in-vivo conditions (Niederberger et al 1983).

The major prerequisite in determining drug protein binding is that the condition of the in-vitro measurement should simulate those existing in-vivo when the blood sample was taken. This implies that the measurements should be carried out with undiluted plasma and at 37 °C to give meaningful results.

We initially investigated ultrafiltration as well as ultracentrifugation. Ultrafiltration, using both a standard (Amicon Centriflo) system and a micro-ultrafiltration system (Amicon Centrifree system, YMT membrane) gave non-reproducible results. Also, with a solution of CyA dissolved in protein-free plasma ultrafiltrate (obtained by ultrafiltration of plasma through an Amicon Centriflo system) much of the CyA failed to pass through the membranes. Awni & Sawchuk (1985) attempted to measure the fraction CyA unbound in rabbit plasma using ultrafiltration and had similar problems.

Ultracentrifugation is an acknowledged technique used to measure the unbound fraction of drugs. Physiological conditions can be maintained and only relatively small volumes of plasma are needed. Where more than one method has been available for comparison, a good correlation of fraction unbound obtained by ultracentrifugation and by other techniques has generally been found (Neilsen 1969; Weder & Bickel 1970; Oellerich & Müller-Vahl 1984; Verbeeck & Cardinal 1985). Niederberger et al (1983) used an ultracentrifugation method to determine the binding of CyA to plasma proteins, and found binding to be very high. We, too, experienced difficulties in obtaining reproducible results and the major problem was traced to the surface activity of CyA, which tends to concentrate at air–water interfaces, making sampling of the protein-free region prone to error. Additionally the high concentration of CyA in the chylomicron/very low density lipoprotein in the top layer makes sampling by the usual 'drawing-off' method open to contamination error.

Only the most central portions of the clear fraction should be used for the measurement of fraction unbound, as contamination of the wall of the ultracentrifuge tube by protein can occur when a fixed-angle head comes to rest as illustrated in Fig. 3.

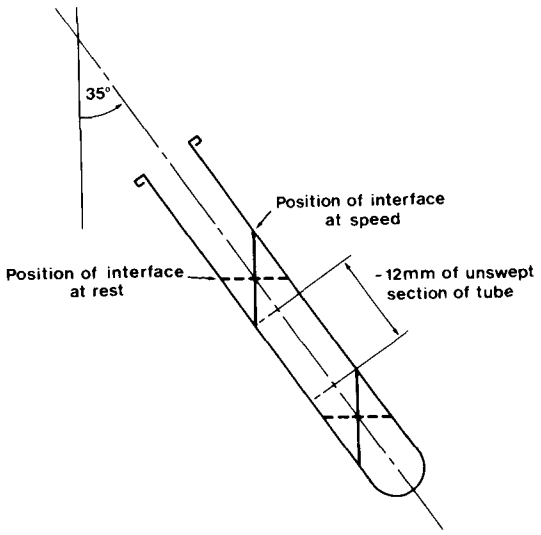

FIG. 3. Position of lipoprotein interfaces in centrifuge tube during and after centrifugation in fixed angle head.

The reliability of the freezing method is evidenced by the extremely low protein concentration in the relevant cut, where the contribution to the fraction unbound from this source is calculated to be <0·005 on values which ranged between 0·04–0·12. The small amounts of material detected by the Lowry method are probably not contaminating protein but small peptides which would reduce the calculated error even further.

The possibility of increased fu values resulting from radiochemical impurities and/or tritium exchange was also considered. The radiolabelled CyA was >99% pure and if it is assumed that most of any impurity would have similar binding characteristics to CyA, then errors from this source could be considered negligible. Also no evidence of tritium exchange was seen or has been reported for this material.

The concentration gradient of CyA in protein-free solution is similar to the gradients seen by Kurz et al (1977) for a variety of drugs, and the concentration of CyA in the corresponding protein-free region of centrifuged plasma was sufficiently close to that of the original protein-free solution (90–95%) for the error in the calculation of fraction unbound to be ignored.

The increase in the fraction CyA unbound as the temperature is lowered from 37° to 4 °C is similar to that reported by Niederberger et al (1983). This temperature dependency goes some way in explaining the rise in the blood/plasma concentration ratio with decreasing temperature (Follath et al 1983), if it is considered that the primary event is the loss of drug from protein as the temperature is lowered. In general, the binding of molecules to specific receptor sites on proteins shows an increase as the temperature is lowered. This is as a consequence of a loss of entropy which results in a negative enthalpy change in the system (i.e. an exothermic reaction). With CyA the reverse is the case. A Van't Hoff plot of a representative binding data set is shown in Fig. 4 and the observed linearity suggests a thermodynamic explanation of the binding phenomenon for CyA. The negative slope implies that the reaction is endothermic and consequently the driving force

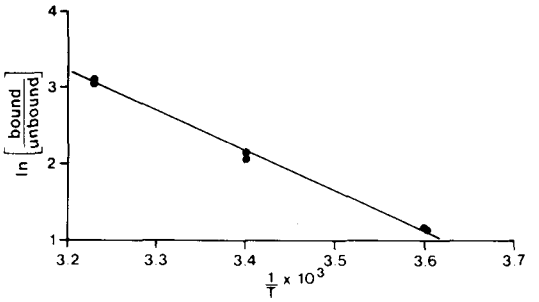

FIG. 4. Van't Hoff plot of representative binding data.

must be entropic and the binding hydrophobic. The thermodynamics are the same as that for the partioning of lipophilic molecules into organic solvents from aqueous solution (Kauzmann 1959). This, and the fact that fraction unbound is concentration-independent, are consistent with the CyA 'binding' being a simple partitioning of drug into the many lipophilic environments existing in plasma.

Although no reference technique is available for the comparison of fu measurements of CyA in plasma, the ultracentrifugation technique developed has proved to be reproducible, a key factor in acceptance, and is recommended as the method of choice for CyA binding measurement.

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