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COMMUNICATIONS

A new method for measuring the free fraction of cyclosporin in plasma by equilibrium dialysis

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The free fraction of cyclosporin in plasma has been measured by equilibrium dialysis in steel chambers. Equilibration time was 16 h. The intra-assay coefficient of variation was 7%. The free fraction remained constant in plasma samples frozen for up to 6 months, and was independent of the total concentration within the therapeutic range.

Cyclosporin is a potent immunosuppressant that is widely used in organ transplantation. It has a narrow therapeutic range, but no clear correlation between plasma (or blood) concentration and clinical effect has been established. For drugs which have a high fraction of the total plasma concentration bound to proteins, the free fraction is considered to correlate better with the clinical effect (Sjöqvist et al 1980). Cyclosporin is highly bound to lipoproteins (Lemaire & Tillement 1982) but the methods used for the determination of the degree of binding are time consuming or not reliable. Several attempts have been made to determine the free fraction, by ultrafiltration (Awni & Sawshuk 1985) and by ultracentrifugation (Lemaire & Tillement 1982). The aim of this study was to develop an easy and reproducible method for determination of the free fraction of cyclosporin in human plasma.

Methods

Chemicals. Tritiated cyclosporin, labelled at amino acid no. 9, with a specific activity of 8·3 μCi $mmol^{-1}$ was a gift from Dr Voges at Sandoz. The active drug was diluted with methanol to give a concentration of 2·5 μg mL^{-1}. Unlabelled drug, from the same source, was used for studies of concentration-dependent binding.

Plasma samples. Blood samples were drawn in vacuum tubes with EDTA as anticoagulant. Separation of the plasma was done at 37 °C.

Binding experiments. Equilibrium dialysis was performed using two-chambered stainless steel dialysis cells, each of 1 mL capacity, constructed at the Karolinska Institute. The cells were separated by a membrane (Spectropor 1, Spectrum Medical Industries, Los Angeles, USA), with a molecular weight cut-off of 6000–8000. To wash the membranes free from glycerol they were boiled for 1 h in distilled water three times, they were then stored at 4 °C in a buffer at pH 7·35 for up to three weeks.

Buffer (Ehrnebo & Odar-Cederlöf 1975), 750 μL, consisting of 4·00 g $Na_2HPO_4.2H_2O$, 0·78 g $NaH_2PO_4.H_2O$ and 5·60 g NaCl L^{-1} solution and plasma were pipetted on the opposite sides of the membrane, and 2 μL tritiated cyclosporin, corresponding to 5 ng, was added to the plasma. Equilibration time was determined by dialysis at 37 °C, and defined as the time required for the free fraction to asymptote the line, FF = constant (as defined below), i.e. equilibrium = 100%. Aliquots of plasma and buffer were pipetted at 0·5, 1, 2, 3, 5, 7, 15 and 24 h, using a glass pipette (SMI, Berkeley, USA), into scintillation vials containing 10 mL scintillation liquid (Lumagel, Lumac, Switzerland). To prevent adsorption of the dialysate to the pipette, this was rinsed with the same volume of methanol, which was added to the vial. The plasma samples were counted for 1 min, and the buffer for 15 min in a beta counter (LKB-Wallac, Turku, Finland). Sample quenching was corrected for by internal standardization. The free fraction, FF, and bound fraction, BF, of the drug were determined by

$$FF = B/P,\ BF = 1 - FF$$

where B and P refer to disintegrations min^{-1} in the buffer and plasma, respectively.

The volume shift after dialysis was measured with a Hamilton syringe. The free fraction was corrected for the shift (Lima et al 1983).

To evaluate concentration-dependent binding, EDTA-plasma from a healthy subject was spiked with cyclosporin to give zero, 100, 250, 500, 1000, 1500, 2000, 2500 and 3000 ng mL^{-1}. The samples, in duplicate, were

dialysed to equilibration and the free fraction calculated as above.

The intra-assay coefficient of variation was determined by analysing the same sample, with a concentration of 100 ng mL^{-1}, 16 times.

EDTA-plasma from a healthy subject was spiked with cyclosporin to give 100 ng mL^{-1}, and frozen in glass tubes at −20 °C, in 1 mL portions. The stability of this sample was studied on 14 different occasions during 6 months. The free fraction was assayed as above.

To find the recovery of the drug after dialysis, three experiments were performed. Plasma samples containing cyclosporin 100 ng mL^{-1} were dialysed together with tracer for 24 h at 37 °C using cells of Teflon, Perspex and of stainless steel. Plasma and buffer were pooled and counted for radioactivity. The chambers and the membranes were rinsed with methanol, and counted.

Results

The time required to reach equilibrium was 16–18 h (Fig. 1). For subsequent experiments a 16 h equilibration time was used.

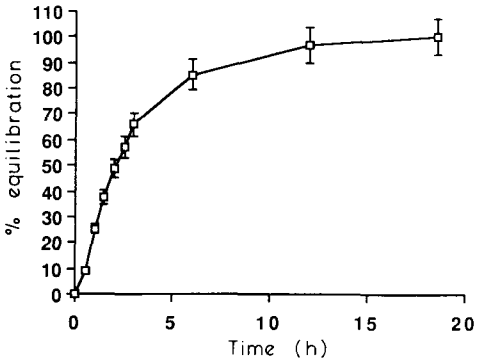

FIG. 1. Time curve of equilibration of cyclosporin between plasma and buffer compartments. The labelled drug was added to the plasma compartment. Mean ± 7% of four experiments.

The intra-assay coefficient of variation was 7% (free fraction %: range 1·2–1·5, n = 16).

The results of the study of concentration-dependent binding showed no change with concentrations up to 3000 ng mL^{-1} (free fraction %: range 1·0–1·3, n = 8 means of duplicates).

Repeated analysis of the original sample over six months revealed no systematic change in the free fraction (free fraction %: range = 1·0–1·5 n = 16, means of duplicates).

When conventional cells of Teflon or Perspex were used, about 98% of the labelled cyclosporin was adsorbed to the membranes and the surfaces. The adsorption to the membranes in the steel cells was 10 ± 3%, but no adsorption to the surfaces was observed. After washing with methanol the total recovery was 60% using the Teflon and Perspex cells, and 98% with the steel cells.

Discussion

The main difficulty in determination of the free fraction of this highly lipophilic drug is its extensive adsorption to surfaces. The first attempt with equilibrium dialysis, for 24 h, using Teflon or Perspex cells gave about 98% adsorption. Rinsing the surfaces and membranes with methanol liberated about 60% of the added labelled drug. This indicated that almost 40% migrated into the polymer. When cells of stainless steel were used, these gave a recovery, without washing, of 90% of the added tracer, i.e. about 10% was adsorbed to the membrane. These findings indicate that the use of plastic devices may give false results.

The method described gives results similar to those quoted by Lemaire & Tillement (1982). But as expected, the free fraction in the present assay differs from those made at other temperatures, since the binding to blood element is dependent on the temperature used.

Pilot studies in renal transplanted recipients of cyclosporin binding to plasma proteins by equilibrium dialysis, as described above, indicated an average free fraction of 1·3% but with at least 8-fold interindividual variation (Henricsson & Lindholm, unpublished).

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