Pharm Pharmacol Lett (1992) 2 : 139–142

Pharmaceutical and Pharmacological Letters

Microdialysis sampling in vitro: the effect on recovery of probe length, flow rate and temperature

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Received 28 August 1992

Abstract Aqueous and human plasma solutions of theophylline and tetracycline were sampled using probes constructed from narrow diameter tubular microdialysis membranes. The recovery of both drugs was found to be inversely dependent on the perfusate flow rate and directly dependent on the length of the probe. There was a linear dependence of drug recovery on temperature over the range of 4°C to 37°C. A reproducible *in vitro* recovery of 92.0 ± 4.3% was obtained using microdialysis probes (25 mm length, 1.6 μl/min flow rate). The plasma protein binding of theophylline, determined by microdialysis (49.1 %) was not significantly different from that determined by ultrafiltration (47.8 %). It was noted that the plasma protein binding of theophylline was temperature dependent, changing from 49.1 % at 20°C to 23.5 % at 37°C.

In circumstances where there is no constraint to probe length it is suggested that improved recovery and reproducibility are possible by the use of longer microdialysis probes of between 10 to 50 mm. Our experiments indicate that careful control of perfusate flow rate and sample temperature will improve the accuracy and reproducibility of the microdialysis sampling technique.

Introduction

The development of the microdialysis probe technique has made it possible to directly sample extracellular fluid *in vivo* [1]. This technique involves perfusing a short length of narrow diameter tubular dialysis membrane with a carrier solution. Low molecular weight components in the sample can diffuse across the dialysis membrane into the perfusate where they may be collected and analysed. The microdialysate sample is free of high molecular weight components, and is therefore amenable to immediate analysis by high-performance liquid chromatography (HPLC), or a similar technique, without the necessity for any sample processing. This greatly simplifies and speeds up the analytical procedure. Furthermore, microdialysis measures only the free fraction of the drug in blood (i.e. that not bound to plasma proteins) which is more relevant from a pharmacokinetic and pharmacological perspective.

Despite the widespread use of microdialysis for the measurement of neurotransmitters in the brain its potential advantage for measuring compounds in other body tissues such as blood has only recently been exploited [2-6]. Historically, the length of microdialysis probes has been constrained by the need to sample from very discrete areas of the brain. However the use of short probe lengths of 0.5-2 mm can lead to relatively low recovery of analyte (typically 5-25%) and poor reproducibility. The use of short probe lengths are not obligatory when sampling blood, and we have therefore investigated whether a longer microdialysis probe would lead to significant improvements in both recovery and reproducibility.

We have examined several types of tubular dialysis membrane using microdialysis probes fabricated in our laboratory. An assessment has been made of the effect of probe length, perfusate flow rate and temperature on the recovery of tetracycline and theophylline, as test drugs, from buffered aqueous solution and human plasma *in vitro*. The plasma protein binding of theophylline, determined by microdialysis, was compared with the value obtained by the established technique of ultrafiltration. In addition, the effect of temperature on the plasma protein binding of theophylline was determined by microdialysis.

Materials and Methods

Materials. Theophylline hydrochloride and tetracycline hydrochloride were supplied by Sigma Chemical Co. Ltd. (Poole, UK). All other chemicals were of HPLC grade or analytical grade , as appropriate.

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Human plasma was obtained from the blood bank at the Nottingham University Hospital. Solutions of the test drugs were prepared in Krebs- Ringer solution for the experiments in aqueous solution, and in human plasma with 0.02% sodium azide added as a preservative. The concentrations of theophylline (10 μg/ml) and tetracycline (4.0 μg/ml) were chosen to give a final concentration within the normal range of the *in vivo* plasma concentration. Tubular microdialysis membranes (0.2 mm internal diameter) were obtained commercially: Cuprophan (Medicell International Ltd., London, UK) Gambro Alwall (Gambro Ltd, Sidcup, UK.,) and Hospal (Sandoz Hospal, Rugby, UK).

Microdialysis. Microdialysis probes with lengths of tubular dialysis membrane varying from 2 mm to 51 mm were constructed from 18 gauge stainless steel tubing and narrow bore polythene tubing connected with hot melt glue according to the design in Figure 1. A Harvard-11 microprocessor syringe driver (Harvard Apparatus, Kent, UK) was used to pump distilled water or Krebs-Ringer buffer through the microdialysis probes at an accurately controlled flow rate. Microdialysis sampling was performed by suspending the probe in a 10 ml volume of drug test solution contained in a jacketed vessel which permitted temperature control *via* a thermostatted water circulator. The test samples were continuously stirred throughout the experiment by means of a magnetic stir-bar to ensure adequate mixing.

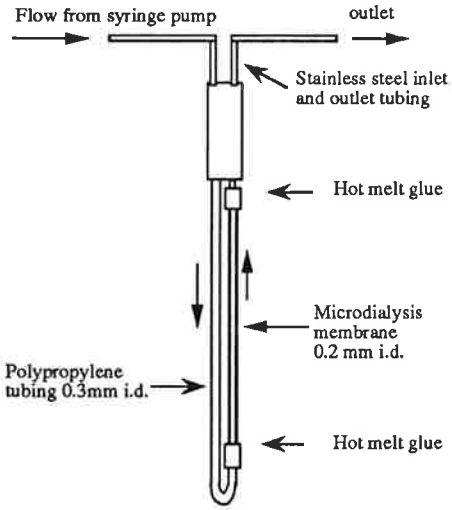

Fig. 1 Design of home-made microdialysis probe for *in vitro* use.

Analysis. For the experiments with aqueous solutions of the test drugs the perfusate from the microdialysis probe was connected directly to a low-volume flow-through cell in an ultraviolet absorption detector . The concentration of test drug was determined from the ultraviolet absorbance at 280 nm. For plasma solutions and Krebs-Ringer buffer solutions, the concentration of theophylline in the perfusate was analysed by HPLC. The perfusate was sampled at ten minute intervals by connecting the outlet of the probe to a loop injection valve for automated HPLC analysis. The HPLC column was an Hypersil ODS (Shandon Scientific Ltd., Cheshire, UK), 50 x 4.6 mm with 3 μm particle size. The mobile phase was 9% acetonitrile in 0.05 M potassium phosphate buffer (pH 2.5) and the flow rate 1.0 ml/min. Theophylline was detected using a Gilson 115 ultraviolet absorbance detector (Gilson Medical Electronics, Villiers-de Bel, France) at 280 nm and the data was collected using an electronic integrator. The retention time for theophylline was 2.0 min.

Determination of recovery. The recovery of the drugs by the microdialysis probes was determined by dividing the concentration of the drug in Krebs-Ringer buffer measured after microdialysis, by the concentration measured in a standard solution, the standard having the same concentration as the test samples. The effect of length of dialysis tubing (2.0 - 55 mm), flow rates (1.6 -40 μl/min) and temperatures (4 - 37°C) on recovery was determined. The recovery of theophylline by the microdialysis probe in plasma was assumed to be the same as that determined in Krebs-Ringer solution.

Protein binding experiments. Theophylline was added to human plasma to give a final concentration of 10.0 μg/ml, which is within the normal therapeutic plasma concentration of the drug in adults. The sample was mixed thoroughly, allowed to equilibrate for 2 hours at the experimental temperature and then divided into two portions for analysis by microdialysis and ultrafiltration. Ultrafiltration was performed with a Centricon-3 microconcentration system (Amicon Ltd., Gloucester, UK). The spiked plasma sample was divided into 1 ml aliquots which were ultrafiltered by centrifugation (3000g, 10 min) at 20°C. The resulting ultrafiltrate was analysed for unbound theophylline using the HPLC method described above. Microdialysis was performed as described above, using a probe calibrated for recovery and a flow rate of 1.6 μl/min. Samples of microdialysate were analysed by HPLC at 10 minute intervals until three or more consecutive samples gave a consistent theophylline concentration. The drug concentration of the microdialysate was subsequently determined using a correction for the recovery of the probe.

Results and Discussion

Microdialysis in buffered aqueous solution. Typical data are presented for the effect of flow rate on the recovery of theophylline (Table 1) and the effect of probe length on the recovery of tetracycline (Fig. 2). The recovery of the test solutes was inversely dependent on flow rate and directly dependent on probe length. This relationship between flow rate, recovery and length closely conformed to mathematical predictions derived from Fickian diffusion.

Table 1: Effect of perfusate flow rate on the recovery of theophylline from Krebs Ringer buffer.

Flow rate (μl/min)	Recovery (%) mean ± s.d.	Coefficient of variation (%)
16.8	20.4 ± 0.61 (n=21)	3.0
8.3	35.5 ± 1.31 (n=19)	3.7
4.3	59.7 ± 2.27 (n=9)	3.8
1.6	90.5 ± 5.6 (n=9)	6.2

(25 mm probe length, 37°C)

Microdialysis probes of equal length gave a reproducible recovery at different flow rates and there was little variation in recovery of theophylline or tetracycline between the dialysis probes made from materials from different manufacturers. Figure 3 shows the flow rate-recovery profiles of theophylline using probes of equal length made from tubular dialysis membranes from three different manufacturers. A recovery of 92.0 ± 4.3 % (mean ± s.d.; n=9) was noted for theophylline using dialysis fibres of 25 mm length and a flow rate of 1.6 μl/min.

The relationship between temperature and recovery (Figure 4) was linear between 20°C and 37°C ($R^2 = 0.99$, $p < 0.01$). It is evident that the microdialysis process is highly temperature dependent as would be expected from the known thermodynamics of diffusion. This temperature dependence can lead to errors in the estimation of sample concentration by microdialysis. For example, it is standard practice to calibrate individual microdialysis probes in solution, prior to *in vivo* use, owing to the difficulty of determining recovery *in vivo*. Unless this

calibration is performed at the same temperature as the *in vivo* experiment, there will be an error in the correction factor which will in turn lead to an incorrect estimation of drug concentration *in vivo*. Consideration should also be made of the temperature difference between the core and peripheral venous drainage.

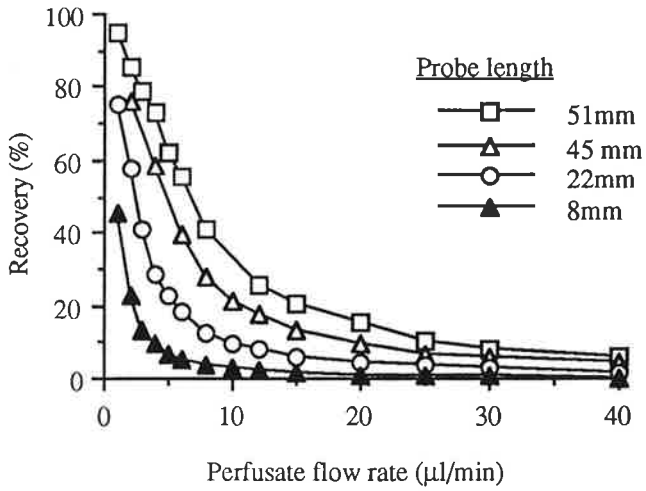

Fig. 2. Effect of flow rate and microdialysis probe length on the recovery of tetracycline (4.0 µg/ml) from aqueous solution.

Theoretically, at recoveries of greater than 90% perfusate drug concentration becomes relatively independent of small fluctuations in flow rate, membrane length or relative permeability. The near maximal recoveries obtained with extended probe lengths should therefore result in improved reproducibility compared with the traditional probe length of 1 or 2 mm.

In previous reports of the use of microdialysis in plasma or blood (5-9) probe lengths of 2-5 mm with perfusate flow rates of 2-5 µl/min, achieved recoveries of between 5-23% for a variety of drugs.

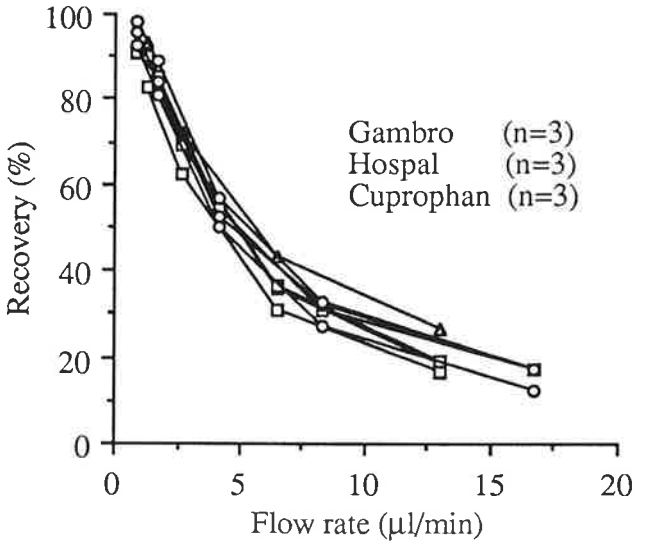

Fig. 3. Reproducibility of flow rate-recovery profiles for three types of tubular dialysis membranes. Test drug theophylline (100 µg/ml in Krebs-Ringer buffer), probe length 25mm, 22°C.

These low recoveries can lead to poor reproducibility due to minor variations in flow rate or probe permeability and also reduce the sensitivity of the method. It is suggested that improved reproducibility and sensitivity could be obtained for the microdialysis technique by increasing the probe length to achieve a recovery value of 90% or greater. In smaller experimental animals, such as the rat, it should be possible to use probes of 10-15 mm in length for *in vivo* blood sampling. In larger animals (including man) it is likely that probes of length 15-50 mm could be used.

Determination of free theophylline concentration in plasma

The free theophylline concentration in plasma at 20°C was found to be 50.9 ± 4.1% of the total theophylline concentration when determined by microdialysis using a correction for recovery assessed in Krebs-Ringer solution.

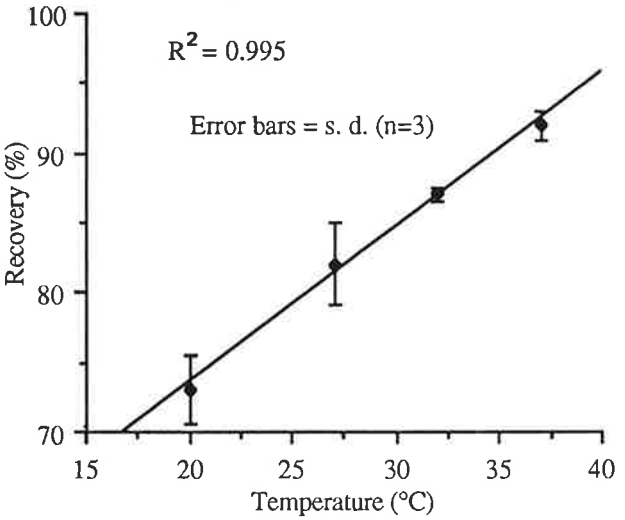

Fig.4. Effect of temperature on the recovery of theophylline (10 µg/ml) from Krebs-Ringer solution. Probe length 25 mm, flow rate 1.6 µl/min.

This compares well with the value of 52.2 % ± 3.5% for the same sample determined by the established technique of ultrafiltration. The similarity of these values indicates that the recovery correction factor determined from Krebs-Ringer buffer is also valid for plasma solutions. It is important to establish the equivalence of recovery between a microdialysis probe used *in vitro* and *in vivo* since, without this equivalence, microdialysis becomes a non-quantitative sampling method. The data presented in this study support the assumption that recoveries determined in Krebs-Ringer solution and in human plasma are equivalent. However, doubt has been expressed about the validity of this *in vitro-in vivo* equivalence in other tissues, such as brain, fat and muscle [1,10], due to differences in diffusion rates of drugs through these tissues compared with those in the aqueous environments of plasma or blood. In addition, local depletion effects may also invalidate data obtained from tissues with a small extracellular volume [11].

Determination of protein binding of theophylline

Microdialysis gave a value of 49.1 ± 4.1% (mean ± s.d., n=8) for theophylline protein binding at 20°C compared with a value of 47.8 ± 3.5% (mean ± s.d., n=6) determined by ultrafiltration. Both of these values are consistent with values reported in the literature and emphasize the validity of the microdialysis technique for the determination of drug plasma protein binding *in vitro* [12]. The data presented here support the results of previous workers [13] which has shown a good agreement between drug plasma protein binding determined by microdialysis compared with the established techniques of equilibrium dialysis or ultrafiltration.

Table 2: The effect of temperature on human plasma protein binding of theophylline determined by microdialysis.

Temperature (°C)	Protein binding (%) mean ± s.d (n=3.)
20	49.1 ± 2.0
27	41.0 ± 2.5
32	34.0 ± 4.7
37	23.5 ± 4.5

(25 mm probe length, flow rate 1.6 μl/min)

The significant effect of temperature on the protein binding of theophylline is readily determined by microdialysis (Table 2). This effect is difficult to determine by ultrafiltration because of the problems associated with accurate temperature control during the centrifugation step. This ability to precisely regulate the temperature of protein binding experiments when using a microdialysis probe permits a more accurate determination of binding equilibria to be made than can be achieved using the ultrafiltration method.

Conclusions

The results from the present *in vitro* studies indicate that the recovery of a drug using microdialysis with a narrow tubular probe is sensitive to changes in probe length, temperature and perfusate flow rate. To obtain reproducible and accurate analytical data from this technique it is necessary carefully to control these variables. Where probe length is not an experimental limitation we therefore recommend the use of microdialysis probes of greater length (up to 50 mm) than are in current use. The higher recovery obtained by these longer probes leads to improved reproducibility and accuracy in the estimation of analyte concentration in solution.

Acknowledgements. The authors thank Glynis Brown and Rachel Wilson for their expert technical assistance.

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