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In vitro recovery of triamcinolone acetonide in microdialysis

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The purpose of this study was to assess the factors affecting the calibration of the microdialysis probe for the *in vitro* recovery of triamcinolone acetonide (TA). Recoveries of TA were determined in microdialysis, retrodialysis, and no-net flux methods. Experiments were performed at room temperature or 37 °C while the reservoir medium was either stirred or unstirred. The effect of the viscosity of the medium on the recovery was studied using methylcellulose gel spiked with TA. Recovery was also calculated by the no-net-flux method in Ringer's solution and in plasma. Stirring the medium increased the recovery of TA by 30%. The recovery was higher at 37 °C under stirred or unstirred conditions and was same in either direction of dialysis. Increasing viscosity of the reservoir medium decreased the recovery (55% in Ringer's solution to 14% in 20% methylcellulose gel). Recovery from spiked plasma under stirred conditions was only 15% and this shift which was also seen in no-net-flux method was accounted for by the protein binding. Binding of TA, determined by ultrafiltration, was 20% in 5% gel and 81% in plasma. The recovery determined by the no-net-flux method was similar to the retrodialysis result. Stirring, temperature, viscosity and protein binding in the reservoir medium affected the *in vitro* recovery of TA.

1. Introduction

Microdialysis is a technique of measuring the unbound drug concentrations in tissue and body fluids *in vivo*. This technique uses the dialysis principle employing a membrane permeable to water and small solutes. A concentration gradient is created by the continuous perfusion of the probe, causing diffusion of substances from the interstitial space into the dialysate (and vice-versa). Microdialysis takes place in non-equilibrium conditions and hence, the calibration of the microdialysis probe or the recovery of the drug (the fraction of the free drug at the site of sampling which dialyses into the probe) is essential in estimating the free drug levels in the tissue. Two common methods for calculating the recovery involve retrodialysis and no net-flux method. Retrodialysis is the general term for recovery methods that reflect the loss of a substance from the perfusate where the direction of diffusion is reversed as compared to the *in vivo* microdialysis experiments. The recovery calculated by the retrodialysis method is estimated from the difference between perfusate (C_{in}) and dialysate (C_{out}) concentrations [1]. A modified recovery estimation method involves the addition to the perfusate of an internal standard with similar characteristics as the test compound and measuring the rate of loss of the internal standard during microdialysis sampling under the assumption that the recoveries of the test compound and the internal standard are equal [2–4]. An advantage of the use of the internal standard is that absolute tissue concentration can be calculated for each dialysate throughout the entire experiment. Differences in recovery, due to changes in tissue of probe characteristics, can be detected immediately. Nevertheless using the internal reference method, one has assumed that the recovery of the test compound equals the delivery of the internal standard [3–5].

Another method for calculating the recovery is the no-net-flux method wherein, a constant drug concentration is maintained in the tissue or reservoir solution into which the probe is inserted, and the perfusate concentration is varied below and above this concentration. The direction of diffusion depends on the concentration gradient across the microdialysis membrane and the concentration at which no net flux occurs is calculated. Linear regression analysis of the plot of initial perfusate concentration, against change in concentration of the dialysate, allows the calculation of the point where no diffusion occurs and the slope of the regression line represents the recovery [4, 6].

These two methods for calculating the recovery of the drug operate under the assumption that the recovery does not depend on the direction of diffusion. Factors that complicate the extrapolation of this assumption to the *in vivo* experiments include the temperature of the system, stirring condition, viscosity or composition of the surrounding medium, and protein binding. The objective of the present study was to assess the effect of temperature, stirring, viscosity and protein binding on the estimation of the *in vitro* recovery of triamcinolone acetonide (TA).

2. Investigations and results

2.1. Microdialysis experiments

The recovery of TA was always higher under stirred conditions at both room temperature and 37 °C ($p < 0.05$). Table 1 shows the recoveries of TA at 2, 5 and 10 μg/ml drug concentrations. Also, the recovery was higher at 37 °C when the medium was stirred ($p < 0.05$). Fig. 1A shows the recovery results at 5 μg/ml at different study conditions. When the internal standard budesonide (Bud) was added to the perfusate, the recoveries of TA and Bud were similar (63 ± 2.9 and $57.3 \pm 2.1\%$) when the reservoir solution was stirred. Recovery of TA from plasma was 15 ± 1 and $11 \pm 1\%$, when plasma was stirred or not stirred, respectively.

2.2. Retrodialysis experiments

The recoveries of TA at different drug concentrations were similar in the retrodialysis and microdialysis experiments at room temperature (Fig. 1B). However, the recovery at 37 °C was higher in retrodialysis when the medium was not stirred ($p < 0.05$, Table 1). Recovery values changed during the study because a different probe was used. However, statistical analyses when the same probe was used were used for inference.

2.3. Effect of viscosity

Effect of viscosity of the surrounding medium on the recovery of TA was studied by perfusing the Ringer solution with Bud through increasing concentrations of methylcellu-

Table 1: Recovery (%) of TA from microdialysis and retrodialysis under different experimental conditions

Conc. (g/ml)	Microdialysis				Retrodialysis			
	RT		37°C		RT		37°C	
	Stirred	Unstirred	Stirred	Unstirred	Stirred	Unstirred	Stirred	Unstirred
2	68.6 ± 3.3	61.4 ± 4.1	79.5 ± 4.6	64.1 ± 3.4	68.8 ± 1.3	55.1 ± 1.8	81.3 ± 2.3	66.5 ± 4.1
5	68.8 ± 3.6	55.9 ± 4.1	70.1 ± 4.2	46.6 ± 3.9	67.2 ± 4.4	61.4 ± 2.2	77.7 ± 2.2	65.3 ± 2.6
10	55.3 ± 1.5	41.5 ± 1.7	71.8 ± 3.9	43.1 ± 1.6	72.8 ± 3.8	63.8 ± 2.7	80.7 ± 2.1	68.7 ± 1.9

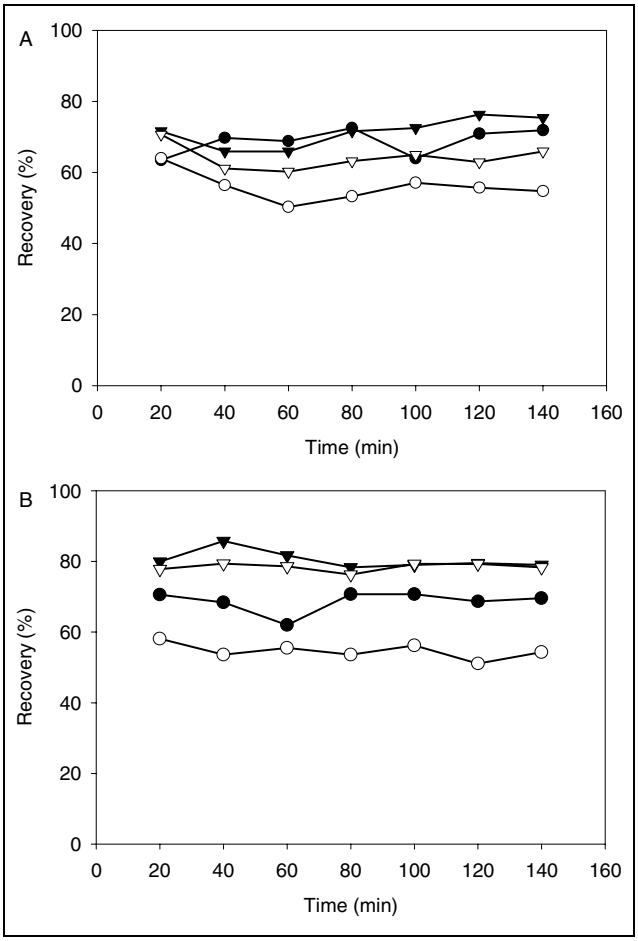

Fig. 1: *In vitro* recovery of TA (5 μg/ml) in A: microdialysis and B: retrodialysis (open symbols: unstirred medium; closed symbols: stirred medium, circle: room temperature, inverted triangle: 37 °C).

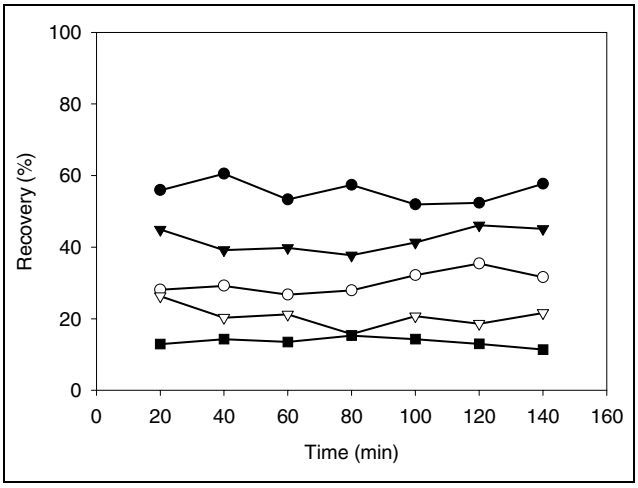

Fig. 2: Effect of viscosity of the medium on the recovery of TA (5 μg/ml) (●, 5%-stirred; ○, 5%-unstirred; and ▼, 10%-stirred; ▽, 10%-unstirred; ■, 20%-unstirred).

lose gel. Fig. 2 illustrates the recoveries of TA in 5%, 10% and 20% methylcellulose at stirred and unstirred conditions. Recovery of TA decreased with increasing gel concentration. The medium could be stirred only at 5 and 10% gel. Recovery was higher under stirred conditions (Table 2).

2.4. Effect of protein binding

Recovery of TA was assessed when blank Ringer solution was perfused through the probe immersed in fresh human plasma spiked with TA. The recovery of TA was higher under stirred condition (14.7 ± 1.1%) than under unstirred condition (10.7 ± 1.3%). The recovery of TA in plasma was significantly lower than in Ringer solution. Binding of TA in either plasma or 5% and 10% methylcellulose gel was determined by ultrafiltration. In plasma, 81.2 ± 2.0% of TA was bound while the binding estimates were 19 ± 3% and 46.1 ± 3.0% in 5% and 10% gel, respectively. Binding could not be assessed in 20% methylcellulose because of high viscosity.

2.5. No-Net-Flux experiments

The no-net-flux method estimates the gain and the loss of drug from the probe to the dialysis medium when chan-

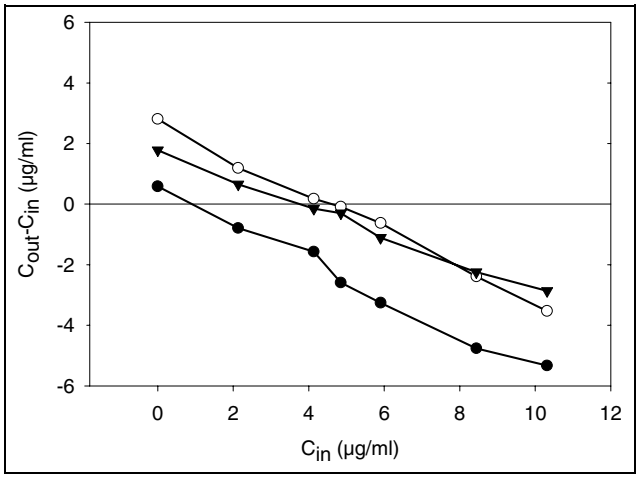

Fig. 3: Recovery of TA (5 μg/ml) by no-net-flux method in Ringer's solution and plasma (○, stirred Ringer's solution; ▼, unstirred Ringer's solution; ●, stirred plasma).

Table 2: Recovery (%) of TA from 5%, 10%, and 20% methylcellulose gel

Study condition	5% gel	10% gel	20% gel	Plasma
Stirred	55.6 ± 3.2	42.0 ± 3.3	–	14.6 ± 1.3
Unstirred	30.0 ± 3.0	20.6 ± 3.2	13.6 ± 1.3	10.7 ± 1.2
% binding	19.2 ± 3.0	46.1 ± 0.3	–	81.2 ± 1.7

ging the direction of the concentration gradient across the dialysis membrane.

Fig. 3 shows the results of no-net-flux method in Ringer's solution and plasma. The slope of the regression line represents the recovery, and the X-axis value at which the regression line crosses the axis represents the no-net-flux point [7]. The recoveries of TA in Ringer solution and plasma were 63.7% and 58.2%, respectively. The marginal lower recovery in plasma might be due to the viscosity differences between Ringer solution and plasma. However, the no-net-flux point was lower and represented only 18% of the total plasma concentration of TA. This shift in the no-net-flux point is explained by 82% binding of TA to plasma proteins, as determined by ultrafiltration. Recovery was higher when Ringer's solution was stirred.

3. Discussion

The present study addresses some issues that are important in the calibration of the microdialysis probe. Since the process of microdialysis proceeds under non-equilibrium conditions, the extraction efficiency of the probe must be known prior to the estimation of the *in vivo* levels of the analyte. Two most commonly used methods of estimating the recovery are retrodialysis and no-net-flux techniques. Large differences in the *in vitro* and *in vivo* diffusion coefficients have been found [8]. The effects of the direction of diffusion, stirring the external medium, temperature, viscosity of the external medium, and binding of the drug to the macromolecules in the external medium were studied. In the present study, experiments were carried out to evaluate ideal conditions at which the *in vitro* calibration results of triamcinolone acetonide (TA) could be extrapolated to the *in vivo* conditions.

The *in vitro* microdialysis or retrodialysis recovery of TA was higher when the medium was stirred. This is because of fluid boundary layer playing an important role in the mass transfer [4]. This could be important in using the *in vitro* calibration results to predict *in vivo* microdialysis recovery when the probe is inserted into a solid tissue where the sink conditions might not be ideal. The experiments were also carried out at room temperature and 37 °C. The recovery of TA was higher at 37 °C. The effect of temperature on recovery is an important consideration in *in vitro* microdialysis studies. Parry et al [9] studied the effect of temperature on *in vitro* relative recovery of catecholamine and indoleamine neurotransmitters increasing the temperature from 0 to 46 °C and demonstrated the importance of controlling temperature in reducing the error when estimating extracellular concentrations of neurotransmitters and metabolites. Larsson [3] reported that blood flow and higher temperature *in vivo* than *in vitro* explain the greater initial recovery obtained in blood *in vivo* compared to the *in vitro* situation, where no stirring took place and the temperature was 22 °C. By controlling temperature, stirring, viscosity and by including an internal standard to correct for instrumental discrepancies during microdialysis studies, the error of *in vitro* calibration, could be reduced. In our experiments recovery was always higher in stirred conditions, which is in accordance with previous observations [4].

Differences between the *in vitro* and the *in vivo* recoveries have been observed due to the different external media resistance of tissue and aqueous solutions [10]. In the present study, the structural stability of the tissues was mimicked by using methylcellulose gel and the recoveries of TA at different gel concentrations were compared. The recovery of TA decreased with increasing viscosity of the medium: 57.3 ± 2.1% in Ringer's solution to 13.6 ± 1.3% in 20% gel, when the medium was not stirred. Stirring a 5% methylcellulose gel increased the recovery of TA from 30.0 ± 3.0% to 55.6 ± 3.2%. Binding of TA to methylcellulose was 19.0 ± 3.0% and 46.1 ± 3.0% at 5 and 10% gel concentrations, respectively. The estimates for the recovery of TA from the gel corrected for its binding at 5% and 10% gel concentrations were 37.4% and 28.3%, respectively. Thus, the decrease in the recovery due to viscosity in 5% gel was 32% and 49% in 10% gel.

The recovery of TA was also estimated by no-net-flux method. Stirring the medium increased the recovery. Recovery from Ringer's solution was comparable to plasma under stirred conditions. However, the no-net-flux point for plasma was lower than that for Ringer's solution and the extent of this shift (82%) corresponded with the plasma protein binding of TA. Only 18% of the total plasma concentration was present in the unbound form which is available for dialysis.

In conclusion, the present study addresses some of the issues of *in vitro* calibration of microdialysis probes. The *in vitro* recovery of triamcinolone acetonide was studied under different conditions. The recovery was higher at stirred condition and higher temperatures. Use of Bud as the internal standard in the perfusate yielded similar recovery results. Replacing the surrounding medium with methylcellulose gel decreased the recovery, by virtue of both viscosity and binding of TA to methylcellulose. Stirring the medium was necessary to obtain the correct results in no-net-flux method. The net recovery of TA compared to the total plasma concentration was lower due to plasma protein binding. The recoveries of TA by microdialysis, retrodialysis, and no-net-flux methods were similar. Thus, maintaining the stirring conditions and the temperature, viscosity of the medium, and binding of the drug need to be considered during *in vitro* calibration of microdialysis probes.

4. Experimental

4.1. Drugs and reagents

Triamcinolone acetonide (TA) and budesonide (Bud) were purchased from Sigma (St. Louis, MO). Ringer's solution containing 189 mM NaCl, 3.9 mM KCl, 3.4 mM $CaCl_2$ (pH 7.2) was prepared in the laboratory. Methanol (HPLC grade) and ethylacetate (Optima grade) were purchased from Fisher (Pittsburgh, PA). Ammonium sulfate was analytical grade and methylcellulose was technical grade.

4.2. Methods

Microdialysis experiments were performed with a flexible microdialysis probe (CMA/10 A, B Stockholm). The probe was connected to a Hamilton syringe, using a Harvard 22-infusion pump. Flow rate of the perfusate was kept at 3 μl/min.

Blank Ringer's solution was perfused (3 μl/min) through the probe immersed in a beaker containing 200 ml Ringer's solution spiked with different concentrations (2, 5, 10 μg/ml) of TA (reservoir medium). Dialysate samples ($n = 7$) were collected in 20-min intervals and analyzed (C_{out}). These experiments were carried out under both stirred and unstirred conditions to study the effect of stirring on the recovery of TA. Stirring was achieved by a magnetic stirrer at 200 rpm. Also, the experiments were carried out at both room temperature and at 37 °C to study the effect of temperature on the recovery.

To study the effect of internal standard on the recovery results, the internal standard Bud (C_{in}, 5 μg/ml) in Ringer's solution was perfused at a flux rate of 3 μl/min through the probe immersed in 200 ml Ringer's solution containing 5 μg/ml TA in the beaker. Dialysate samples ($n = 7$) were collected in 20 min intervals and analyzed (C_{out}) for both Bud and TA. These experiments were carried out at room temperature with and without stirring.

4.2.1. Retrodialysis experiments

In retrodialysis experiments the microdialysis probe was immersed in blank Ringer's solution and perfused with different concentrations of TA in Ringer's solution (2, 5, 10 μg/ml) at a flux rate of 3 μl/min. Samples (n = 7) were collected over 20-min intervals. These experiments were done at room temperature and 37 °C with and without stirring.

4.2.2. Calculation of recovery

In microdialysis experiments recovery (R) was calculated as follows:

$$R = \frac{C_{out}}{C_{in}} \cdot 100$$

Where C_{in} is the concentration in the reservoir solution.
In retrodialysis experiments the recovery (R) was calculated as follows:

$$R = \frac{(C_{in} - C_{out})}{C_{in}} \cdot 100$$

4.2.3. No-net-flux experiments

The no-net-flux method was used to determine mass transport of the analyte across the microdialysis membrane as function of perfused concentrations. The probe was immersed in 200 ml Ringer's solution or in plasma containing 5 μg/ml of TA. The dialysis probe was perfused with Ringer's solution containing varying concentrations (C_{in}) of TA (0, 2, 4, 5, 6, 8 and 10 μg/ml). System was equilibrated for 30 min before the sample collection when the perfusate concentration was changed. Three samples (C_{out}) were collected for each concentration perfusate and analyzed by HPLC.

4.2.4. Effect of viscosity

The influence of viscosity on the recovery of TA was studied using 5, 10 and 20% methylcellulose gel prepared in water. Each gel was spiked with 5 μg/ml of TA. Ringer's solution containing 5 μg/ml Bud was perfused through the probe immersed in the gel and the dialysate samples (n=7) were collected for analysis. These experiments were carried out at room temperature with and without stirring.

4.2.5. Protein binding

Extents of TA binding to the plasma proteins and to methylcellulose were determined by ultrafiltration. Plasma or methylcellulose samples (1 ml) were taken in the ultrafiltration devices (pore size 30 KD, Amicon) and centrifuged at 2000 g in a fixed angle rotor at ambient temperature. The ultrafiltrate (about 100 μl) was collected and analyzed directly by HPLC.

4.2.6. Analytical methods

The concentrations of TA and Bud were determined by HPLC, consisting of Constameter LDC pump, spectromonitor LDC detector connected to a HP 3396 integrator. Chromatography was carried out on a Zorbax ODS C_{18} (150 × 4.6 mm) column at a flow rate of 1ml/min [11]. The mobile phase consisted of methanol and water (70 : 30, v/v). The detector was set at a wavelength of 254 nm for the detection of TA and Bud. The stock solutions of TA (0.5 mg/ml) and Bu (1 mg/ml) were prepared separately in methanol. The standard dilutions of TA (0.2–2 g/ml) and Bud (0.5–5 μg/ml) were prepared in the mobile phase from the stock solution. For the purpose of studying the protein binding and its effect on the recovery, standard dilutions of TA were also prepared in plasma to contain 1–10 μg/ml.
For analyzing the dialysate samples, the calibration curve was constructed in the mobile phase. The plasma samples were analyzed after liquid-liquid extraction [11]. Briefly, 1 ml of plasma was mixed with 1 g ammonium sulfate and was extracted twice by shaking for 15 min with 3 ml portions of ethylacetate. The two layers were separated by centrifugation at 2000 g and the organic phase was evaporated to dryness under a stream of nitrogen gas. The resulting residue was dissolved in 100 μl of mobile phase, centrifuged at 3000 g for 10 min and injected into HPLC system.
The recovery estimates between different experimental conditions were statistically compared by two-tailed t-test.

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