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RIA-linked microdialysis sampling in the awake rat: application to free-drug pharmacokinetics of hydrocortisone*

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Abstract: The purpose of this research was to combine microdialysis sampling techniques with a highly sensitive radioimmunoassay (RIA) to study the *in vivo* kinetic response of pharmacologically important substances. This technique allowed for a dense sampling regimen from an awake, free-roaming experimental animal with no loss of blood and with rapid analysis of the dialysate. An important methodological criterion for accurate quantitation of a test drug in the extracellular space was knowledge of the relative recovery of the sampling system at the time of experimentation. Accordingly, the factors which influenced the recovery of drug during dense *in vivo* microdialysis sampling were examined and an analytical technique was developed to measure the instantaneous recovery of drug from the extracellular space. This information was applied to *in vivo* (*iv*) sampling experiments on anaesthetized and awake, free-roaming rats following bolus and multiple long-term *iv* administrations of the highly protein bound steroid (i.e. >90%), hydrocortisone-21-phosphate. These studies indicated that unbound hydrocortisone levels as determined by the RIA-linked microdialysis (RIALM) technique fluctuated rapidly between each 2-min sampling interval, but nevertheless decreased to predose endogenous concentrations in a first-order fashion ($t_{½}$ = 17–29 min). The rapid fluctuations of unbound hydrocortisone may reflect real pharmacokinetic or pharmacodynamic phenomena, attributed, perhaps, to reequilibration of the unbound drug pool with proteins and tissues in the blood.

Keywords: *Microdialysis; radioimmunoassay; pharmacokinetics; cortisol.*

Introduction

Microdialysis is a sampling technique by which the concentration and chemical composition of biochemically important substances existing in the extracellular space can be continuously monitored *in vivo* [1, 2]. The principle of this technique is the positioning of a micro-membrane that allows diffusion of solutes between the region of study and a continuously renewed perfusion solution [3]. The diffusion of solutes is limited by a molecular weight maximum that is characteristic of the membrane material. The exiting perfusion solution (dialysate) is sampled either for *in situ* or *ex situ* analysis.

Early microdialysis sampling techniques were introduced to study neurotransmitter release in the brain interstitial space [3]. A sealed permeable sack filled with saline solution was implanted in the brain of the animal model and was later removed for analysis, thus only a single sample could be obtained. Continuous sampling capabilities were later introduced with the advent of microbore dialysis tubing which, when sealed at one end, could be used as a miniature sack containing two cannulas; one inlet cannula to allow entrance of the perfusion fluid and an outlet cannula for sampling of the dialysed perfusate.

Refinements to this configuration led to an improved design that was originally conceived by Ungerstedt *et al.* [4, 5] and later marketed by Carnegie Medicine AB, Solna, Sweden. The Carnegie Medicine microdialysis probe (henceforth referred to as the CM probe) consists of two concentric cannulas, one slightly shorter than the other, inserted into a tubular dialysis membrane (Fig. 1). The function of the internal cannula is to lead the perfusion fluid onto the internal surface of the dialysis membrane where diffusion of substances from the extracellular space occurs. The dialysed perfusate then flows upwards in

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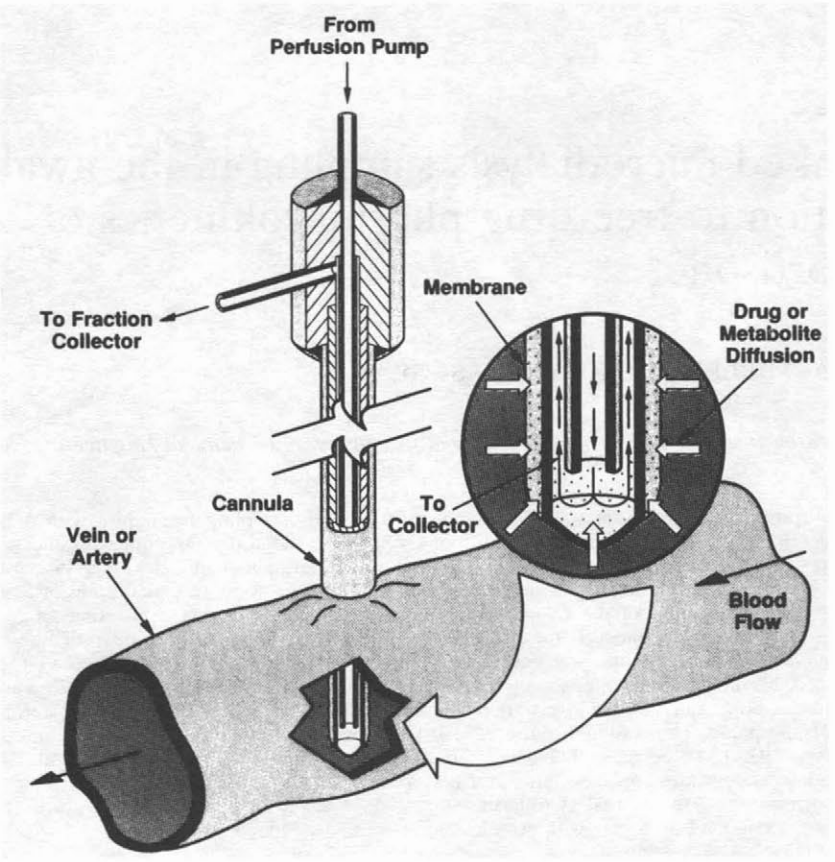

Figure 1
Diagrammatic representation of *iv* microdialysis for pharmacological applications showing the CM probe.

the space between the membrane and the outer cannula. Sampling of the perfusate is achieved via a small tube extending at a right angle from the probe.

Several studies involving microdialysis sampling have been cited in the literature. The vast majority of these studies have implemented rather laborious *ex situ* analytical methodologies. Typically, the collected dialysate is analysed for the substance(s) of interest using HPLC, albeit with little or no sample preparation, in conjunction with electrochemical or spectrophotometric detection [3]. Most notably, Ungerstedt (1984) performed brain microdialysis in the awake rat to correlate neurotransmitter release with behaviour. In this study the CM probe was inserted stereotactically into the stratum to permit monitoring of amphetamine-induced release of dopamine using HPLC with electrochemical detection. Levine and Powell [6] adapted a pituitary microdialysis system to measure neuroendocrine peptides again by HPLC with electrochemical detection. Only Lindefors *et al.* [7] has described a microdialysis technique requiring no chromatographic separation. Here, microdialysis was combined with a specific and highly sensitive (picomolar level) RIA procedure to determine levels of neuropeptides in the n. accumbens region of the brain.

Whereas microdialysis has been limited to biochemical events in the brain, the potential of this technique as applied to *iv* pharmacological studies in the rodent has been largely unexplored. From an analytical point of view, the success of this technique as applied to kinetic studies in animal models depends on whether or not the evolution of chemical events that rapidly occur in the blood and extracellular space can be accurately quantified when subject to the drug recovery and dialysate volume constraints of a dense sampling regimen. Thus, a fundamental prerequisite for *iv* studies of extracellular levels of pharmacologically important substances is a highly sensitive detection method. For this microdialysis was linked with RIA methodol-

ogies to analyse dialysate samples because of the low sample volume requirements and superior sensitivity of the assay method.

In past studies involving microdialysis sampling, the recovery performance of the microdialysis membrane, which must be known in order to arrive at extracellular concentrations, has frequently been determined by *in vitro* methodologies prior to experimentation [8]. These methods, however, provide only an average estimate of relative recovery, and if implemented for pharmacokinetic applications, this estimate must be assumed to be relevant to each and every sampling point in the kinetic profile. In view of the fact that the recovery of drug, among other factors, is a function of its concentration outside the dialysis membrane, then average recovery estimates as determined by *in vitro* methods may not properly correct for rapid and transient changes in blood plasma drug concentration that are typical of pharmacokinetic drug profiles. Hence, a more precise technique must be employed through which the instantaneous recovery of the drug may be determined. This paper demonstrates the utility of introducing an internal standard, preferably a radiolabelled analogue of the drug to be dialysed, incorporated in the perfusion fluid as a means of determining the relative recovery of drug for each dialysate sample collected in a dense sampling regimen.

A microdialysis probe inserted intravenously allowed for multiple kinetic sampling of the systemic circulation without depletion of blood volume. In considering pharmacokinetic implications of this type of sampling device, there are several apparent advantages to this methodology linked with RIA. Principally, the sampling method can augment pharmacokinetic data analysis with statistically powerful results because of the high sampling density. This is particularly beneficial when complex pharmacokinetic profiles are limited by the number of individual concentration–time points for nonlinear regression analysis. The microdialysis technique also prevents collection of drug which is bound to plasma proteins such as serum albumin or α_1-acid glycoprotein. The implication is that this technique provides an efficient and powerful means of studying the distribution and elimination kinetics of the unbound fraction of an experimental drug. Until now, the study of free-drug disposition has presented a considerable challenge to kineticist primarily due to the difficulty associated with developing suitable blood-sample preparation procedures that reflect real unbound drug concentrations.

This paper describes the successful application of a microdialysis technique linked with RIA to study the free-drug kinetic response of anaesthetized and awake, free-roaming rodents to bolus- and multiple *iv* administrations of hydrocortisone-21-phosphate (henceforth referred to as cortisol-21-phosphate and whose hydrolysis product is cortisol). Prior to these studies numerous *iv* microdialysis experiments were performed to establish the ideal conditions for the analytical and surgical techniques. Accordingly, only those experiments are presented here which, from experience, not only reflect optimized experimental parameters, but also transcend previous efforts in this research field.

Experimental

Materials

Chemicals. Cortisol-21-phosphate was obtained from Sigma Chemical Co. (St Louis, MO) in greater than 99% purity as determined by HPLC and TLC. For *in vitro* and *in vivo* recovery studies, [1,2-^{3}H]-hydrocortisone (54.6 Ci mmol^{-1}) and the internal standard [7-^{3}H]-pregnenolone (25 Ci mmol^{-1}), respectively, were obtained from New England Nuclear (Du Pont, Medical Products Dept., Boston, MA) in greater than 99% purity as determined by HPLC and TLC. RIA kits for the determination of cortisol were purchased from NEN and Diagnostic Products Corp. (Los Angeles, CA).

Apparatus. The microdialysis system consisted of a Carnegie Medicine AB (Bioanalytical Systems, West Lafayette, IN) variable flow rate syringe pump (Model CMA/100), a microfraction collector (Model CMA/140) and, for awake animal experiments, a clear Plexiglass animal containment system (Model CMA/120) with tethering balance arm and a dual channel liquid swivel. A rodent shoulder vest (Kaplan Laboratory Animal Supplies, San Jose, CA) was also used for awake animal experiments to facilitate tethering the animals to the balance arm of the animal containment system. The CM microdialysis probes (BAS, Model CMA/10) used in conjunction with this system were comprised of a 20 mm cannula and a 4 ×

0.5 mm polycarbonate-ether dialysis member (20,000 dalton cutoff).

Methods

*Animal procedure**. Male Sprague–Dawley rats (Charles River, Cincinnati, OH and Harlan Sprague–Dawley Inc., Indianapolis, IN) at 8–10 weeks of age (250–300 g) were fasted for 12–18 h prior to surgery and dose administration. Surgical implantation of microdialysis probes and arterial blood-collection catheters were performed with 50 mg kg^{-1} *ip* pentobarbital or ether anaesthesia for the anaesthetized and awake animal sampling experiments, respectively. Microdialysis probes were implanted into the external jugular vein, and in one case, via the common ileac vein into the vena cava by making a longitudinal incision in the skin of the neck or abdomen to expose the vein. While maintaining flow through the sampling system to prevent collapse of the membrane, the probe was then traversed through a small incision in the vein such that the membranes resided in a direction opposite cardiac blood flow, for sampling at the external jugular, or orthogonol to cardiac blood flow, for sampling at the vena cava. A suture around the vein and probe cannula was used to secure the probe to the sampling site, thus occluding blood flow approximately 10–15 mm posterior to the probe membrane. Inlet and outlet tubes of the probe were exteriorized for perfusion and sample collection. For the multiple constant *iv* infusion experiment, an indwelling venous catheter was implanted into the femoral vein for simultaneous collection of whole blood.

Drug administration and sample collection. Prior to drug administration, duplicate samples were collected from the sites indicated in Table 1. Blood samples (Experiment 1 only) were immediately centrifuged to separate plasma and frozen in a 2-propanol/dry ice bath until assayed. For dialysis, normal saline was perfused at flow rates of 5 μl min^{-1} (Experiment 2) and 10 μl min^{-1} (Experiments 1, 3 and 4) to collect 10- and 20-μl dialysate fractions, respectively, every 2 min into polypropylene microvials. The microvials were then frozen and stored as described for blood samples. After collection of predose samples, an appropriate volume of a sterile solution containing 10 mg ml^{-1} cortisol-21-phosphate in saline was administered via the indwelling jugular catheter using a calibrated infusion pump (Sage Instruments) for the multiple infusion study (Experiment 1), and via manual bolus injection into the penile vein (Experiments 2–4). Blood samples (0.1 ml) for Experiment 1 were collected concomitantly with dialysate samples at 25, 35, 65, 75, 105, 115 and 125 min postadministration.

Relative recovery determinations. The relative recovery response, defined as the ratio between the concentration of drug in the dialysate to that in the environment outside the dialysis membrane (i.e. the extracellular space), was individually characterized by *in*

Table 1
Summary of experimental parameters for *in vivo* RIALM sampling

	Dose regimen				
Experiment no.	Infusion no.	Rate (μg min^{-1})	Infusion times (min)	Total dose (mg)	Site of dialysis
1†	1	2400	0–0.5	1.2	Jugular
	2	40.0	0.5–40	1.6	
	3	85.0	40–80	3.4	
	4	166.7	80–120	6.67	
2†	1	Bolus	—	2.7	Vena cava
3‡	1	Bolus	—	5.0	Jugular
4‡	1	Bolus	—	2.8	Jugular

†Anaesthetized animal.
‡Awake, free-roaming animal.

*The laboratory animal facilities and animal care program of Southwest Research Institute have been reviewed and accredited by the American Association for Accreditation of Laboratory Animal Care according to standards set forth in the *Guide for the Care and Use of Laboratory Animals*, DHEW Publications NIH 85–23.

vitro and *in vivo* methods for each microdialysis probe implemented in the animal dosing experiments. In Experiment 2, relative recovery was determined *in vitro* just prior to surgical implantation of the probe. Duplicate dialysate samples were collected over 2 min intervals from reservoir standards containing 0.5, 2.5 and 5.0 μg ml^{-1} cortisol prepared in normal saline and maintained at a constant 37°C. The ratio of the concentration determined in the dialysate to that remaining in the reservoir sample as assayed by RIA was used as a measure of relative recovery. Alternatively, relative recoveries could also be determined *in vitro* by reinforcing a series of cortisol standards with approximately 100 μCi of [1,2-^{3}H]-hydrocortisone and collecting dialysate fractions as previously described. At a later time the ratio of radioactivity counts (β-emission) in the dialysate fraction to that which remained in the reservoir could be determined by liquid scintillation counting (LSC). For either *in vitro* method, the relative recoveries derived from these measurements were described by a fourth-order polynomial expression. This expression was subsequently used as a transform function to arrive at the estimated venous levels of unbound cortisol using the measured dialysate concentrations from *in vivo* sampling of Experiment 2.

In Experiments 1, 3 and 4, we explored a method by which the recovery of the probe during *in vivo* sampling was determined radiometrically for each sampling point by means of reverse dialysis of an analogue compound. To accomplish this a cortisol analogue, [7-^{3}H]-pregnenolone, was used as an internal standard. Approximately 0.1 μCi of this radiolabelled analogue was introduced into the perfusion fluid of the microdialysis sampling system, which consisted of isotonic saline. The principle of this technique is dependent upon the assumption that the diffusion of radiolabelled analogue across the probe membrane from the perfusion fluid to the extracellular space mimics the diffusion of cortisol from this space to the perfusion fluid. If this assumption holds true, the relative recovery of each sampling point can be determined by monitoring the change in specific activity of the dialysate relative to that of the original perfusion fluid. Accordingly, the specific activities of a 2–10 μl aliquot of each dialysate fraction and the original perfusion fluid were determined by LSC coincidentally with the RIA cortisol determinations.

RIA analysis. A 10 μl aliquot of plasma or dialysate was combined with 500 μl of approximately 0.04 μCi of ^{125}I-labelled cortisol and 500 μl of a cortisol antiserum complex. Following incubation of this mixture at 37°C for 15 min, the contents were centrifuged at 1000*g* for 10 min. The supernates were then discarded and γ-emission from the resulting bound pool was counted with an Isoflex dual crystal automatic gamma counter (ICN Micromedics). The manufacturer reported cross reactivity of cortisol antiserum to pregnenolone, progesterone and tetrahydrocortisol was 0.004, 0.16 and 1.10%, respectively.

Plasma ultrafiltrates. Unbound drug concentrations for Experiment 1 were independently determined by RIA analysis of plasma sample ultrafiltrates. The ultrafiltration apparatus consisted of an Ultrafree-MC polysulphone filter unit and microcentrifuge tube (Millipore, Bedford, MA). A 100 μl aliquot of plasma was centrifuged through the filter unit at 15,600*g* for 5 min using a standard benchtop microcentrifuge. The recovered ultrafiltrate was subsequently analysed by the RIA procedure previously described.

Data reduction. The concentration of cortisol was determined by unweighted least squares analysis of a series of six standards ranging 20–500 ng ml^{-1}. A linear response was obtained after transformation of the RIA count data to the logit response variable:

$$\text{Logit } B = \ln \frac{B}{1 - B} \qquad (1)$$

where B is the ratio of net radioactivity counts of sample to that of the blank standard (maximum binding of radiolabelled antigen). A plot of this variable versus the log of the standard concentration typically yielded coefficients of determination (r^2) greater than 0.995.

Best estimates of pharmacokinetic parameters were determined for single bolus *iv* experiments by fitting one- and two-exponential expressions (2 and 3) to the dialysate concentration–time data using a least squares procedure (Marquardt–Levenberg algorithm,

SigmaPlot™, Jandel Scientific, Corte Madera, CA).

$$C(t) = Ae^{-k_{el}t} \quad (2)$$

$$C(t) = Ae^{-\alpha t} + Be^{-\beta t}. \quad (3)$$

Results

Anaesthetized animal experiments

Multiple constant IV infusion. Figure 2 illustrates the pharmacokinetic response of the animal model to multiple constant *iv* infusions. Blood samples drawn from the femoral catheter were acquired at times where the plasma levels of cortisol were predicted to be at steady-state conditions. The predicted steady-state concentrations were determined by best-fit analysis of concentration–time data of total cortisol plasma levels following bolus *iv* administration. Uncorrected dialysate data are plotted on a linear scale in Fig. 2 (top panel). Also included are the instantaneous relative recoveries as determined by the internal standard technique described elsewhere. The corrected data are plotted on a semi-log scale in Fig. 2 (bottom panel) along with the measured and predicted plasma concentration (total cortisol) profiles. Additionally, unbound drug concentrations as determined by the ultrafiltration method are shown to demonstrate the accuracy of the RIALM sampling technique.

As indicated in Fig. 2 (top and bottom panels), free cortisol concentrations were significantly perturbed by the simultaneous blood draw and saline flush of the femoral catheter at the times corresponding to acquisition of

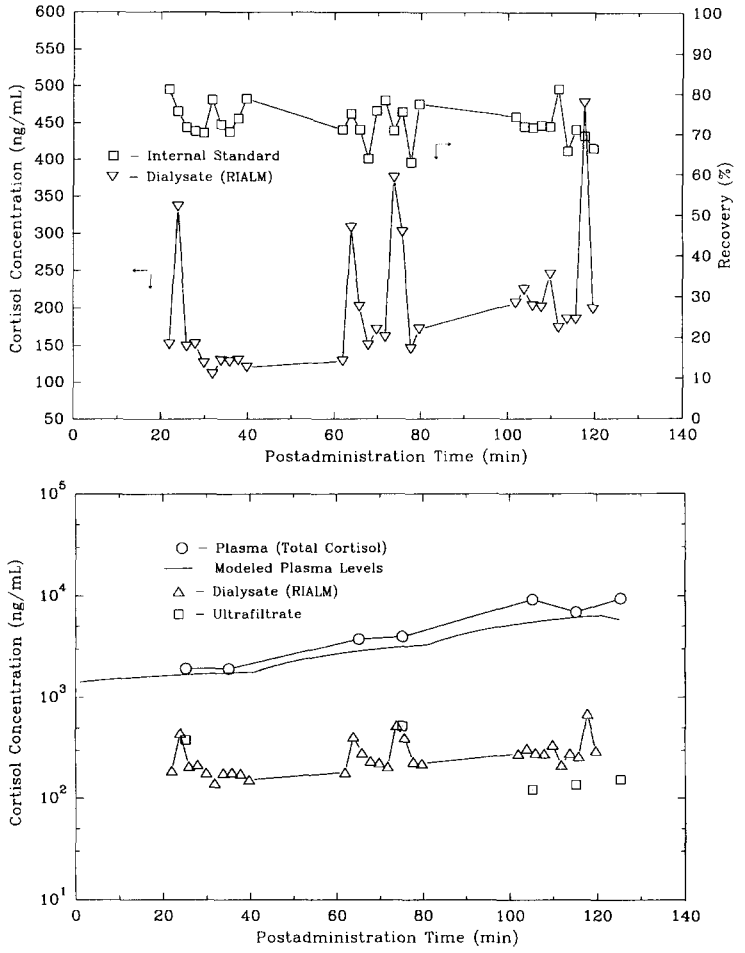

Figure 2
Concentration–time profiles of free (as determined by RIALM sampling at the external jugular vein of the anaesthetized rat) and total cortisol (RIA analysis of plasma) following multiple *iv* administrations of cortisol-21-phosphate. Top panel: comparison of instantaneous recovery and uncorrected dialysate levels. Bottom panel: comparison of plasma and corrected dialysate levels.

plasma samples for total cortisol determinations. In fact, the observed results for free cortisol do not agree with classical pharmacokinetic principles. Although a four-fold increase in the infusion rate resulted in a corresponding increase of plasma cortisol levels (total cortisol) at steady-state, free cortisol levels as determined by RIALM or ultrafiltration methods increased by only 30%.

Bolus IV administration. The dialysate concentration–time profile of cortisol following bolus *iv* administration via the penile vein and dialysis sampling via the common ileac vein at the vena cava is shown in Fig. 3. Dialysate levels plotted in Fig. 3 represent approximate venous concentrations of unbound cortisol as estimated by the relative recovery determination previously described for this experiment. The periodicity of the profile with respect to the rapid concentration minimas that occur at 30, 45 and 60 min postadministration is important to note at this time. Similar features were observed in subsequent bolus *iv* experiments performed on the awake, free-roaming animal.

A biexponential equation, representing a two-compartment model with bolus *iv* input and first-order output, was fitted to the concentration data of Fig. 3. The apparent terminal elimination rate constant and its corresponding half-life were the pharmacokinetic parameters of interest. For comparison with the awake animal experiments, in which a one-exponential expression adequately described the profiles, the pharmacokinetic parameters are shown in Table 2.

Awake animal experiments

Figures 3 and 4 display concentration–time profiles of unbound cortisol observed for awake, free-roaming rats following single bolus *iv* administrations via the penile vein and dialysis at the external jugular vein. Extracellular levels of cortisol declined monophasically, but with similar periodicity as that

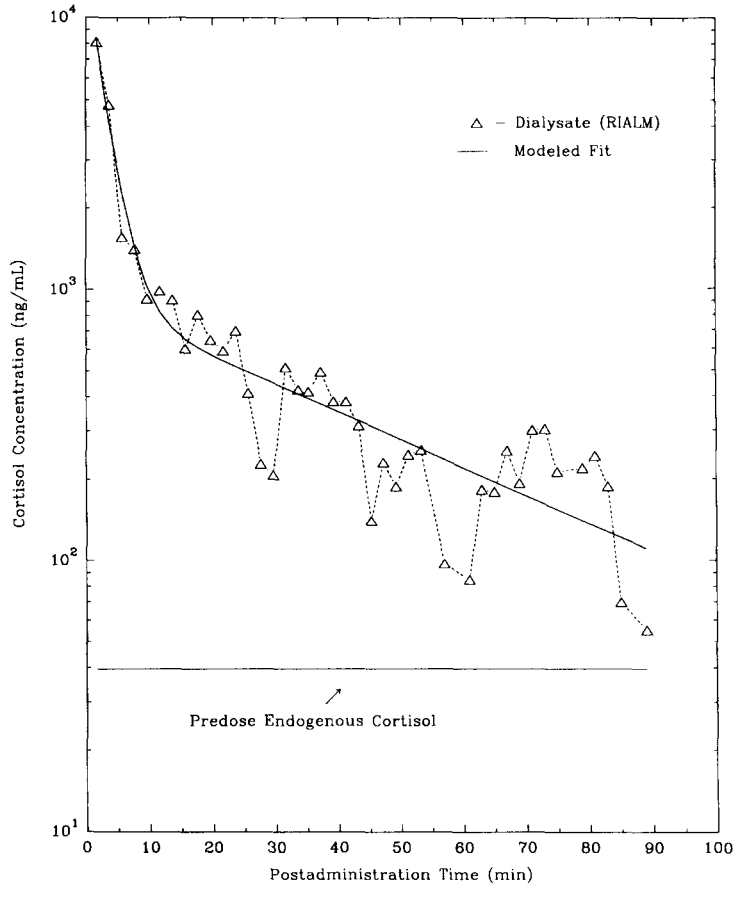

Figure 3
Concentration–time profile of free cortisol as determined by RIALM sampling at the vena cava of the anaesthetized rat following single bolus *iv* administration of cortisol-21-phosphate.

Table 2
Comparison of pharmacokinetic parameters

Experiment no.	Parameter					
	A (ng ml^{-1})	B (ng ml^{-1})	α (min^{-1})	β (min^{-1})	k_{el} (min^{-1})	$t_{1/2}$ (min)
2	14235	906	0.398	0.0236	—	29.3
3	232	—	—	—	0.0238	29.1
4	104	—	—	—	0.0410	16.9

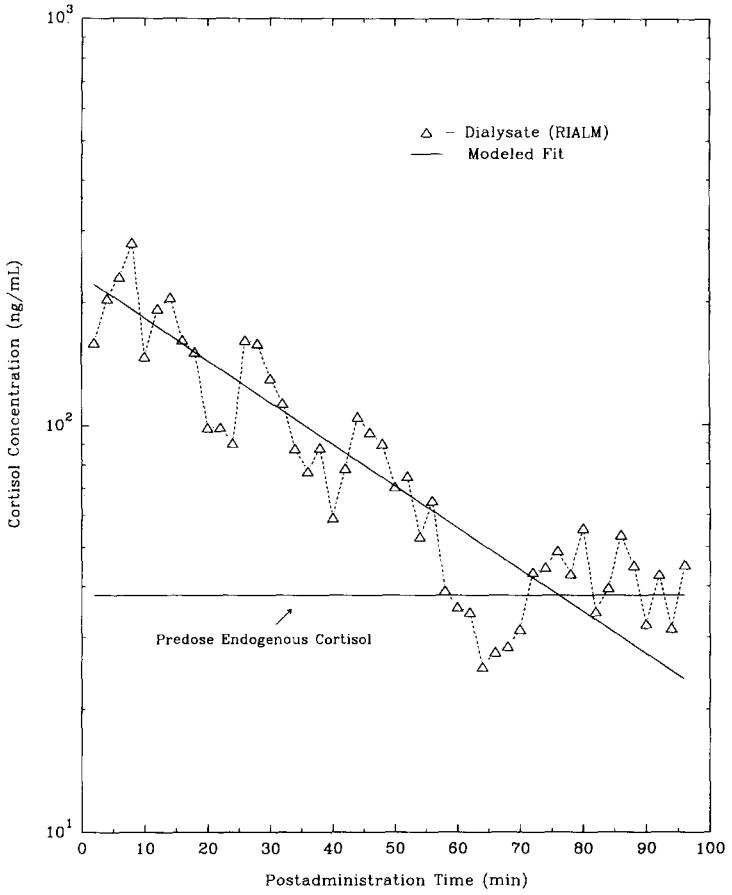

Figure 4
Concentration–time profile of free cortisol as determined by RIALM sampling at the external jugular vein of the awake, free-roaming rat following single bolus *iv* administration of cortisol-21-phosphate.

observed for the anaesthetized animal experiment. Although a distribution phase was not observed for Experiments 3 and 4, the elimination phases, as indicated in Table 2, were remarkably similar for all three experiments. The monophasic response of the awake animal experiments is probably related to the site of dialysis sampling (external jugular vein). A periodic response was again observed for both experiments, occurring at approximately 25, 35 and 65 min.

Discussion

This work demonstrates a unique application of *in vivo* microdialysis sampling to study the free-drug kinetics of cortisol in the rodent. The propensity for cortisol to bind to plasma proteins was exploited as a means of testing the sensitivity of the RIA-linked microdialysis sampling technique to rapid changes in free-drug levels following multiple long-term or single bolus *iv* administrations. RIA is ideally

suited for quantification of dialysate fractions collected over a dense sampling regimen because of the low sample volume requirements (10 μl) and superior assay sensitivity (20 ng ml^{-1}) over most other analytical methods. Notwithstanding the attributes of RIA-linked microdialysis, one must recognize that a suitable method of determining the instantaneous relative recovery of the membrane during sampling (i.e. *in vivo*) is by far the most important analytical requirement of the technique when applied to pharmacokinetic studies, especially if the analyte does not readily dialyse across the probe membrane. For the most part, the internal standard method utilized in these studies seems to take into account many of the physiological and physicochemical factors that may influence dialysis of unbound drug from the extracellular space as well as any mechanical variability associated with the instrumental apparatus. However, these data may also be significantly influenced by the surgical implantation technique. Minimal contact of the probe membrane with the venous wall at the site of dialysis and the accessibility to a recirculating pool of blood that exhibits stable, non-turbulent fluid dynamics are both important factors which must be considered in the implantation procedure. In the present studies, positioning the probe membrane at a venous site either orthoganol to a large vein (e.g. vena cava) via a smaller vein (e.g. common ileac), Experiment 2, or in a direction opposite to cardiac output with occlusion of blood flow near the site of dialysis, Experiments 1, 3 and 4, are configurations which, although they may not circumvent all of the physiological influences of venous dialysis, provided less ambiguous results than other configurations previously attempted. The second procedure (Experiments 1, 3 and 4) is thought to provide more stable blood flow at the dialysis site by reducing the linear velocity of blood that is in contact with the membrane.

An interesting feature of multiple *iv* infusion

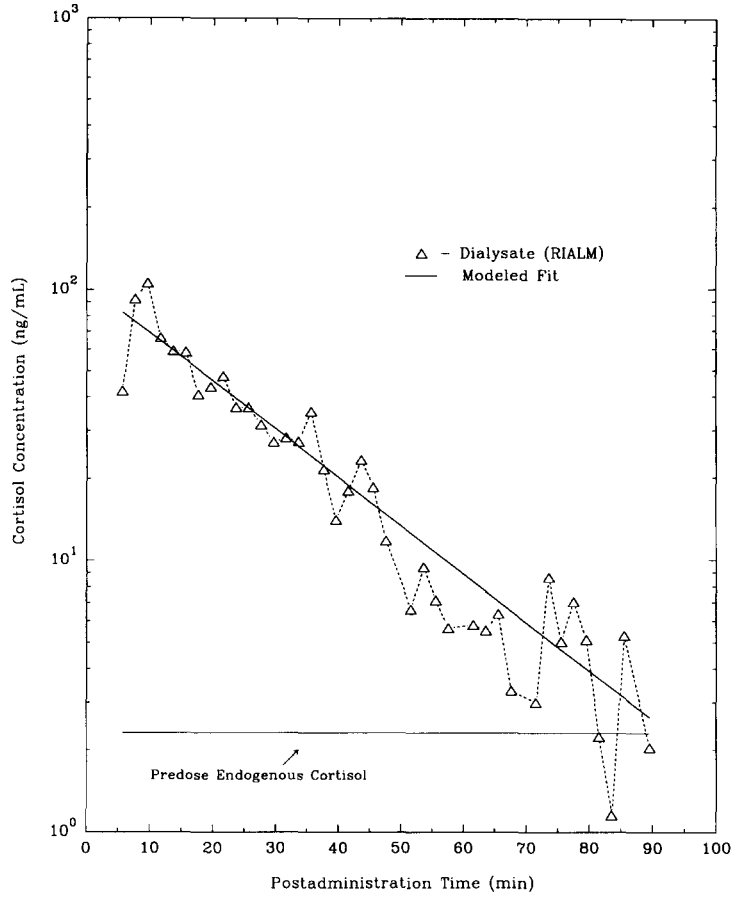

Figure 5
Concentration–time profile of free cortisol as determined by RIALM sampling at the external jugular vein of the awake, free-roaming rat following single bolus *iv* administration of cortisol-21-phosphate.

experiments with simultaneous blood drawing was the seemingly aberrant behaviour of dialysate concentrations that occur at the times corresponding to simultaneous blood drawing (Fig. 2, top panel). The implication is that RIALM is sensitive to physiological perturbations such as rapid changes in cardiac flow and pressure caused by a blood draw and associated saline flush of the femoral catheter. These perturbations could have profound effects on unbound drug concentrations which would easily be detected by the dense sampling regimen of the microdialysis experiment. Under these conditions, caution must be taken when interpreting results of bound versus free concentration data. To account for the observed perturbations of free drug levels, future experiments comparing free- and bound-blood levels should be conducted in separate animals; one animal with simultaneous blood and dialysate sampling and a second with dialysis sampling alone.

Concentration–time profiles (Figs 3, 4 and 5) indicate fluctuations of unbound drug that appear as periodic minimas and which may reflect rapid redistribution of free drug to protein bound state. One may be inclined to speculate that this response is again attributed to aberrant behaviour of the microdialysis probe. However, the fact that the observed periodicity is characteristic of all single bolus experiments, regardless of the site of dialysis, and that reverse dialysis of the internal standard should account for any mechanical variability associated with the instrumental apparatus (e.g. flow rate), seems to suggest that these observations are attributed to real pharmacokinetic or pharmacodynamic phenomena. It is important to emphasize that, for drugs with high protein binding characteristics (such as cortisol), relatively small changes in the fraction of drug bound to protein will result in large variations in free-drug levels [9–11] which may not have been observed by kineticists due to the low density of sampling and lack of free-drug analysis.

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