Methodological Aspects of the Use of a Calibrator in *In Vivo* Microdialysis–Further Development of the Retrodialysis Method

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Purpose. To investigate the performance of two alternative retrodialysis recovery methods and to describe the influence of different recoveries on the reliability in estimating unbound extracellular concentrations of morphine.

Methods. Unbound concentrations of morphine in striatum and in blood were determined by microdialysis after a 10 min i.v. infusion in freely moving rats. *In vivo* recovery of morphine was determined by morphine itself, *retrodialysis by drug*, and by the calibrator nalorphine, *retrodialysis by calibrator.*

Results. The low calibrator recovery in striatum (8.6%) resulted in large variability in the estimation of unbound extracellular concentrations when *retrodialysis by calibrator* was used. In blood, where the recovery was higher (36%), the variability was smaller. Also, when retrodialysis by drug was used, the variability remained low. This difference is caused by the propagation of errors in the way retrodialysis recovery is determined. Therefore, calibrator recovery values ≥20% are preferable in concentration estimations using *retrodialysis by calibrator.*

Conclusions. When no time-dependent change in recovery is observed, retrodialysis by drug determined before the systemic administration is the best method. The calibrator is valuable as a quality control during the experiment.

KEY WORDS: microdialysis; morphine; nalorphine; retrodialysis; calibrator.

INTRODUCTION

In order to correctly estimate the true unbound extracellular drug concentration based on its microdialysate concentration in pharmacokinetic experiments, a reliable *in vivo* calibration method is required (1–3). As a result of continuous perfusion of a microdialysis probe, only a fraction of the drug concentration in the extracellular fluid is reflected in the dialysate. This fraction is referred to as the relative recovery. Several methods have been developed to estimate the *in vivo* recovery. One method commonly used in microdialysis (MD) is a simplified version of the difference method (3). This method is based on the diffusive loss of the drug of interest from the perfusion solution into the tissue surrounding the probe prior to its systemic administration. This method has been called reverse dialysis (4). The advantage of reverse dialysis is that the recovery is based on the drug of interest. The disadvantage is that the recovery is determined prior to systemic administration and therefore may not be relevant during the following experiment. This problem has been addressed by introducing a calibrator to the perfusion solution (5–8). The approach was named retrodialysis by Wong *et al.* (7). The concentration of the drug of interest in the tissue is determined by the degree of loss of the calibrator from the perfusion solution during the entire experiment.

The lack of a defined nomenclature of the *in vivo* recovery methods has led to confusion in the literature. In order to diminish the confusion, we suggest the general term *retrodialysis* for recovery methods which reflect the loss of a substance from the perfusion solution. When the loss of the drug of interest is studied, the method can be called *retrodialysis by drug*. When the loss of a calibrator is studied, the method can be referred to as *retrodialysis by calibrator.*

All estimated drug concentrations include an analytical error that propagates differently depending on the handling of the data. The recovery calculated by the retrodialysis method is estimated from the difference between incoming (C_{in}) and dialysate (C_{out}) concentrations. The smaller that difference is, the more likely it is that the analytical error in the individual estimations of C_{in} and C_{out} will cause a lower accuracy in the recovery estimations between individual sampling intervals.

In the present paper a combined retrodialysis approach is suggested to determine the unbound concentrations of morphine. The relative recovery of the calibrator, nalorphine, is determined in the presence of the drug of interest, morphine, before systemic administration using *retrodialysis by calibrator* and *retrodialysis by drug* simultaneously. In the subsequent experimental period the *in vivo* probe recovery is monitored continuously with *retrodialysis by calibrator.*

The main objective of this study was to compare the different methods for retrodialysis recovery estimations, and to look at their performances during different recoveries. A further developed retrodialysis method is suggested in which each rat serves as its own control regarding the recovery of drug vs. calibrator.

MATERIALS AND METHODS

Drugs and Reagents

Morphine hydrochloride (10 mg/ml) was purchased from Pharmacia Upjohn AB (Stockholm, Sweden). Hypnorm® was obtained from Janssen-Cilag (Beerse, Belgium). Nalorphine hydrochloride and low molecular weight heparin were provided by Sigma Chemicals (St Louis, USA). Solvents were of HPLC grade, and all other chemicals were of analytical grade.

Probes and Perfusion Solutions

All MD experiments were performed with CMA/12 probes (3 mm, Stockholm, Sweden) and CMA/20 probes (10 mm, Stockholm, Sweden) for brain and blood, respectively. The perfusion solution for the striatal probe was prepared as described previously (9). The perfusion solution for the blood probe was similar to the one for striatum except for the addition of 2 mM HEPES. Both solutions were kept at −20°C before

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usage. Before each experiment the perfusion solutions were adjusted to room temperature and degassed for 5 min in an ultrasonic bath.

Animal Surgery

Six male Sprague Dawley rats with a body weight of 280–335 g were used (Møllegaard, Denmark). After a 7 day acclimatisation period each rat was anaesthetised with 0.2 mg/kg fentanyl (Hypnorm®) intramuscularly. Two indwelling cannulae, PE-10 connected to PE-50, were implanted into the femoral artery for blood sampling and into the femoral vein for drug infusion. A heparinised saline solution (100 IU/ml) was maintained in the arterial cannula to prevent clotting. The flexible CMA/20 probe, perfused with a 0.1% solution of low molecular weight heparin, was placed in the right jugular vein through a guide cannula and anchored to the pectoral muscle via two sutures. The anaesthetised rat was mounted in a stereotaxic instrument (David Kopf Instruments, Tujunga, USA) for the implantation of the striatal probe. A midsaggital incision was made to expose the skull and the CMA/12 guide cannula was implanted into the striatum with the co-ordinates 2.7 mm lateral and 0.8 mm anterior to bregma and 3.8 mm ventral to the brain surface. After insertion the guide cannula was anchored to the skull with a screw and dental cement (Serviton, De Trey Ltd, UK). A 15 cm piece of PE-50 tubing was looped subcutaneously distal to the posterior surface of the neck, allowing the perfusion solution to adjust to body temperature before it entered the striatal probe (10). Protruding ends of all cannulae and probe tubings were led subcutaneously to the posterior surface of the neck where they were protected by means of a plastic cap sutured to the skin. During the surgical procedure the rat body temperature was maintained at 38°C by means of a heating pad. The rats were transferred into a CMA/120 system for freely-moving animals and were allowed to recover for 24 hrs. The rats had free access to water and food.

Experimental Design and Recovery Calculations

On the day of the experiment, the 3 mm CMA/12 probe was inserted into the striatal guide. After insertion both MD probes were perfused with their respective blank perfusion solutions, at a flow rate of 2 μl/min, by means of a CMA/100 precision infusion pump (CMA Microdialysis, Sweden). Samples were collected at 10 min intervals. After a 60 min stabilisation period, the perfusion solutions were switched to solutions containing nalorphine at a concentration of 250 ng/ml, and morphine at concentrations of 100 ng/ml (blood) and 200 ng/ml (brain). Twenty μl dialysate fractions leaving the probes were collected and analysed for their contents of both morphine and nalorphine (C_{out}), during 70 min, after which the morphine and nalorphine concentration in the perfusion solution entering the probes (C_{in}) was sampled. The simultaneous estimation of the retrodialysis of morphine and nalorphine is referred to as the *reference period*. Following the reference period the perfusion solutions were changed to contain only the calibrator, nalorphine, at a concentration of 250 ng/ml. A washout period of 70 min before the start of the drug infusion was allowed to ensure the complete removal of morphine from the probes.

Retrodialysis by Drug

The relative recovery of morphine was estimated in each rat by retrodialysis of morphine during the reference period and was determined as the concentration in the perfusion solution leaving the probe (C_{out}) relative to the concentration of morphine entering the probe (C_{in}) according to:

$$\text{Recovery}_{\text{in vivo, ref}} = \left(\frac{C_{in} - C_{out}}{C_{in}}\right) \quad (1)$$

where C_{out} is the concentration of morphine in the perfusate leaving the probe and C_{in} is the concentration of morphine in the perfusion solution entering the probe.

Retrodialysis by Calibrator

The experimental recovery of morphine was determined in each rat using continuous retrodialysis by calibrator (nalorphine), corrected for the ratio of retrodialysis recovery of morphine and nalorphine from the reference period in the same rat according to:

$$\text{Recovery}_{\text{in vivo, exp}} = \left(\frac{C_{in} - C_{out}}{C_{in}}\right)_{\text{calibrator}} \times \left(\frac{\text{Recovery}_{\text{in vivo, drug}}}{\text{Recovery}_{\text{in vivo, calibrator}}}\right)_{\text{ref}} \quad (2)$$

where C_{out} is the concentration of nalorphine in the perfusate leaving the probe and C_{in} is the concentration of nalorphine in the perfusion solution entering the probe both during the experiment, while the ratio of the recovery between morphine and nalorphine is obtained from the reference period.

Intravenous Administration of Morphine

Rats 1–3 and 4–6 received 10 min infusions of 10 and 40 mg/kg morphine (34 and 136 μmol/kg), respectively. Blood and striatal extracellular fluid were continuously sampled by MD from the start of the infusion and continued for 190 min. At the end of the experiment the concentration of nalorphine entering the probes during the experiment (C_{in}) was sampled. The microdialysate fractions were stored at −20°C pending analysis.

Analysis of Morphine and Nalorphine

The concentrations of morphine and nalorphine in the dialysates were determined by reversed phase HPLC. Sample volumes of 17 μl were directly injected (Triathlon, Spark Holland BV, The Netherlands) and chromatographic separation was achieved using a Nucleosil C-18 column (150 × 4.6 mm i.d., 5 μm, Chrompack, Sweden). The mobile phase consisted of 520 ml 0.01 M phosphate buffer (pH 2.1) containing 0.2 mM dodecylsulphate and 480 ml methanol, which was delivered at a flow rate of 1.0 ml/min (ESA 580, ESA Inc., USA). Detection of morphine and nalorphine was accomplished using an electrochemical detector (Coulochem II, ESA Inc., USA) with a high sensitivity dual analytical cell (ESA model 5011, ESA Inc., USA) and a guard cell (ESA model 5020, ESA Inc., USA). The potential of the guard cell, placed before the auto injector, was set at 600 mV, while the potentials of analytical cell 1 and

cell 2 were set at 0 and 450 mV, respectively. Chromatographic data were recorded and processed with an integrator (Shimadzu CR-5A, Shimadzu Europe, Sweden). The peak height was used for quantification. Under the conditions described, the lower level of detection for morphine was 2 ng/ml. Standard curves in the range between 3.9 and 250 ng/ml showed good linearity ($r > 0.999$). The coefficients of variation at 15 ng/ml and 200 ng/ml morphine were 3.4 and 1.2% (striatum) and 5.3 and 3.6% (blood).

Calculations of Unbound Concentrations in Striatum and Blood

The concentration-time profile of morphine in striatum and in blood were calculated according to three different approaches.

Recovery Estimated from Retrodialysis by Drug (Method I)

The unbound concentrations of morphine in striatum and blood were calculated from the dialysate concentrations corrected by the recovery of morphine estimated during the reference period (Eq. 1).

Recovery Estimated from Retrodialysis by Calibrator (Method IIa)

The unbound concentrations in both tissues were calculated from the dialysate concentrations corrected both for the recovery ratio morphine:nalorphine during the reference period and by the nalorphine recovery at each collection interval (Eq. 2).

Recovery Estimated from Retrodialysis by Calibrator with Moving Average (Method IIb)

The unbound concentration-time profiles were determined as described in method IIa with the exception that an average value of the recovery of the calibrator nalorphine, from five subsequent fractions centralised around the sampling interval, was used instead of the individual values.

Pharmacokinetic Analysis

The individual half-lives in blood and striatum were estimated from the individual unbound concentration-time profile obtained with the three different recovery methods. The elimination half-lives were estimated from the last eight observed MD points from the disposition curves using linear regression.

Statistical Analysis

The recovery of morphine and nalorphine was analysed by a paired *t*-test. Differences between the recoveries and the pharmacokinetic parameter, half-life, obtained from the three methods were tested using a paired two-tailed t-test. The computer programme Statview® was used for all statistical calculations. All data are expressed as mean ± standard deviation (SD) unless otherwise stated.

Simulation

A simulation was performed to study the influence of different degrees of variation (SD) on the magnitude of the variability (CV) in the estimated recovery. Four different levels of SD were used to generate the corresponding CV value with varying recovery. The CV was calculated as SD/Recovery × 100. The simulated values for SD were 2, 5, 10 and 20%.

RESULTS

The average recovery of morphine in striatum was 8.3% during the reference period (Eq. 1). The recovery of nalorphine in striatum was 8.1% during the reference period and 8.6% during the experiment (Table I). The average recovery of morphine in blood was 31% before the experiment and the average recovery of nalorphine in blood was 32% during the reference period and 36% during the experiment (Table II). No significant difference was found between the recovery values of morphine and nalorphine in striatum and in blood. There was no significant difference between the nalorphine recoveries estimated during the reference period and during the rest of the experiment. The recovery ratio between morphine and nalorphine during the reference period was 0.96 in striatum and 0.99 in

Table I. Comparison of Retrodialysis Recoveries of Morphine (M) Versus Nalorphine (Nal) in Striatum for Individual Rats (3 mm probe)

	Reference period (n = 3)[b]			During experiment (n = 16)[b]	
Rat[a]	M %	Nal %	Ratio (M/Nal)	M[c] %	Nal %
1	7.6 ± 0.5	7.8 ± 0.8	0.98	8.1 ± 3.7	8.3 ± 3.8
2	7.0 ± 0.7	7.5 ± 0.5	0.93	10.9 ± 7.7	11.7 ± 8.2
3	9.7 ± 0.4	11.0 ± 2.8	0.88	8.7 ± 1.9	9.9 ± 2.1
4	10.8 ± 1.1	11.1 ± 1.7	0.97	6.2 ± 2.1	6.4 ± 2.2
5	7.6 ± 0.9	7.5 ± 1.0	1.00	8.0 ± 2.5	8.0 ± 2.5
6	5.1 ± 0.2	5.2 ± 0.2	0.99	7.5 ± 7.4	7.6 ± 7.5
Average	8.1 ± 2.6	8.3 ± 2.8	0.96	8.2 ± 4.9	8.6 ± 5.2

Note: Mean ± SD.
[a] Rats 1–3 received a 10 mg/kg dose of morphine, while rats 4–6 received a 40 mg/kg dose of morphine.
[b] Number of dialysate fractions.
[c] Estimated according to Eq. 2.

Table II. Comparison of Retrodialysis Recoveries of Morphine (M) Versus Nalorphine (Nal) in Blood for Individual Rats (10 mm probe)

	Reference period (n = 3)[b]			During experiment (n = 16)[b]	
Rat[a]	M %	Nal %	Ratio (M/Nal)	M[c] %	Nal %
1	27.9 ± 1.0	29.4 ± 1.0	0.95	32.8 ± 2.7	34.5 ± 2.8
2	58.6 ± 1.6	57.7 ± 5.6	1.01	55.4 ± 8.0	54.6 ± 7.9
3	31.3 ± 2.8	30.6 ± 1.7	1.02	38.7 ± 9.7	37.9 ± 9.5
4	40.0 ± 0.9	38.8 ± 2.0	1.03	26.6 ± 2.7	25.8 ± 2.6
5	28.3 ± 2.0	28.1 ± 1.8	1.01	29.2 ± 2.5	29.0 ± 2.5
6	23.2 ± 0.6	25.5 ± 1.5	0.91	33.5 ± 4.3	36.7 ± 4.7
Average	31.4 ± 7.5	31.5 ± 6.4	0.99	36.0 ± 11.0	36.4 ± 10.8

Note: Mean ± SD.
[a] Rats 1–3 received a 10 mg/kg dose of morphine, while rats 4–6 received a 40 mg/kg dose of morphine.
[b] Number of dialysate fractions.
[c] Estimated according to Eq. 2.

blood (Tables I and II). As a result, the calculated recovery of morphine during the experiment according to Eq. 2 was 8.2% in striatum and 36% in blood, respectively, which was not significantly different from the morphine recovery during the reference period.

Both the differences in recovery between individual probes and the fluctuations within each probe contribute to the variability found in the average recovery value (Tables I and II). The standard deviation within probes are presented in the data for each rat. In general, a smaller coefficient of variation (CV) was found for the blood recovery than for the striatal recovery. Two examples of morphine and nalorphine recovery over time are presented in Fig. 1. Rat 3 (Fig. 1a) shows moderate fluctuations in recovery, while Rat 6 (Fig. 1b) displays the largest fluctuations in the present experiment.

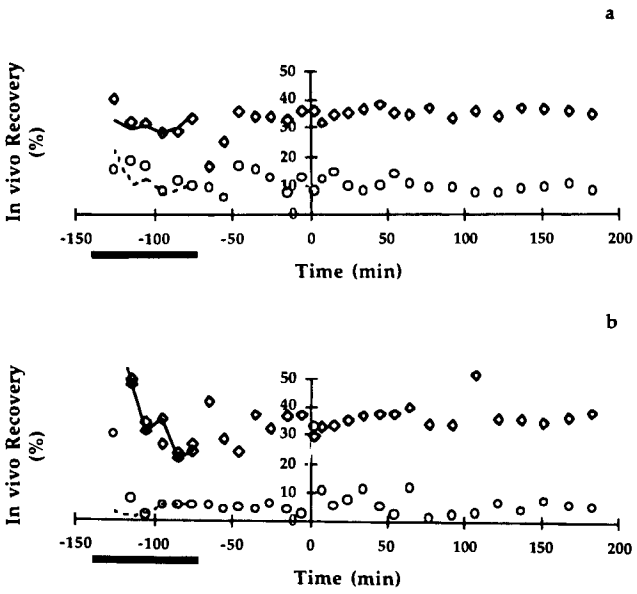

Fig. 1. *In vivo* probe recovery of nalorphine in striatum (open circles) and blood (open diamonds) estimated by retrodialysis during the entire experiment for Rat 3 (a) and Rat 6 (b). The horizontal bar indicates the reference period while the lines represent the retrodialysis recovery of morphine.

The dialysate concentration-time profiles of morphine were generally very smooth with small fluctuations (Fig. 2a-d). Depending on the method used for recovery calculations, the obtained unbound concentration-time curves displayed different degrees of fluctuation. For method I, where morphine recovery from the reference period was used as the correction factor to estimate unbound concentrations, the same profiles as those for the dialysate concentrations were maintained. In method IIa, where the dialysate concentration was corrected for the nalorphine retrodialysis recovery at each collection interval, the variability in nalorphine recovery was propagated to the unbound concentration-time profile. This was especially prominent for the striatal concentration profile in Rat 6 (Fig. 2c). In method IIb, where the nalorphine recovery was averaged over five consecutive fractions, the fluctuations in the profiles grew smaller. The blood profiles were not as influenced by the method used for recovery calculation (Fig. 2b and d).

The larger variability in striatal concentration was also propagated to the determination of the half-lives. However, the average half-life of morphine was not significantly influenced by the method of recovery calculation, even though a marked influence was present in isolated instances (Table III).

As an illustration of the consequences of different magnitudes of recovery in the retrodialysis methods, the experimentally calculated loss (C_{out}/C_{in}) ± SD and recovery values are presented in Table IV, together with the calculated CV. Taking the data for Rat 1 as an example, the SD for the loss equals that of the recovery ($C_{in} - C_{out}/C_{in}$), while the CV is profoundly influenced by the magnitude of the recovery value. As a result of a low striatal recovery of 8.3% and an SD of 3.8%, the CV becomes 46%. For the blood probe with a recovery of 35%, a SD of 2.8% results in a CV of 8.3%. Consequently, the size of the recovery together with the size of the SD will determine the uncertainty in the estimates of the unbound extracellular concentrations. Fig. 3 displays the influence of the size of the retrodialysis recovery in conjunction with the size of the SD in the range of 2–20%. With an increasing SD, a higher recovery is required for reliable results. This is not due to errors in the

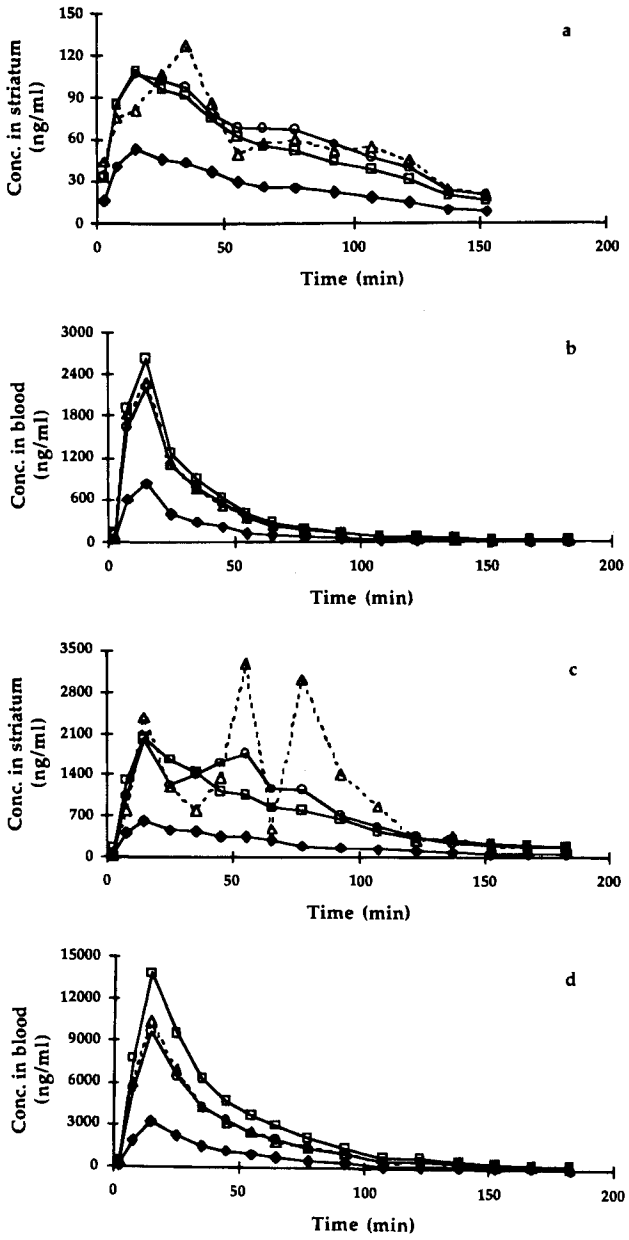

Fig. 2. Unbound concentrations of morphine in (a) striatum and (b) blood following a 10 min infusion of 10 mg/kg in Rat 3. Unbound concentrations of morphine in (c) striatum and (d) blood following a 10 min infusion of 40 mg/kg in Rat 6. Profiles displayed are obtained with method I (-□-), method IIa (-Δ-) and method IIb (-○-), respectively. Brain dialysate concentrations (-◆-) depicted were multiplied by 5 to be shown more clearly.

microdialysis experiment itself, but rather a result of the inherent propagation of errors with the retrodialysis method.

DISCUSSION

The main purpose of the present study was to investigate the performance of two alternative retrodialysis recovery methods and to describe the influence of different recoveries on the reliability in the estimation of unbound extracellular concentrations of morphine.

The major problem in estimating true unbound concentrations in tissues using microdialysis concerns the choice and reliability of the recovery method. The *in vitro* recovery value cannot be used for *in vivo* calculations due to different transport characteristics in these situations (11–13). Therefore, when determining unbound extracellular concentrations with microdialysis, it is necessary to calibrate the probes *in vivo*. The calibrator serves as a quality control of the probe function. The ideal calibrator in microdialysis experiments is the substance of interest itself. However, individual recovery estimates cannot be made with the substance of interest present in the perfusion solution when that substance is also administered systemically under non-steady state conditions. The availability of radioactive or deuterated substances to be used as alternative calibrators is limited. Therefore, a calibrator similar to the drug of interest added to the perfusion solution is a promising approach (4,5,7,8). The value of a calibrator lies in the fact that it provides a means of obtaining information about the recovery throughout the experiment in individual subjects. In the present study the recovery of the calibrator, nalorphine, was estimated simultaneously in striatum and blood using the method of retrodialysis either with or without the drug, morphine, being present in the perfusion solution. Morphine did not influence the recovery of nalorphine. In addition, the *in vivo* recovery of morphine was in good agreement with those obtained for nalorphine in striatum as well as in blood, suggesting that the substances have similar microdialysis characteristics. Other examples of calibrators that have been used are AZdU for AZT (8) and nalorphine for codeine (14).

The retrodialysis method is a very simple and convenient method for recovery determinations *in vivo*. Nevertheless, the method has previously been applied either before or during the experiment. In this study a combined approach was tested. The unbound concentrations obtained using method I are based on retrodialysis by drug, while retrodialysis by calibrator is used in method II. When method I is used, the recovery determination is carried out before the systemic administration of the drug, assuming that the recovery remain constant. Sjöberg *et al.* reported a time dependent recovery of tritiated water in blood but not in brain (15). Within 5 hours the recovery in blood gradually decreased by 20%. Therefore an experimental design similar to methods IIa and IIb is valuable for monitoring and if necessary compensating for changes in probe efficiency over time. In the present study no changes in *in vivo* probe recovery in striatum or in blood were observed within the time frame of the experiment (5h). This observation suggests that the morphine recovery estimated before the systemic administration is

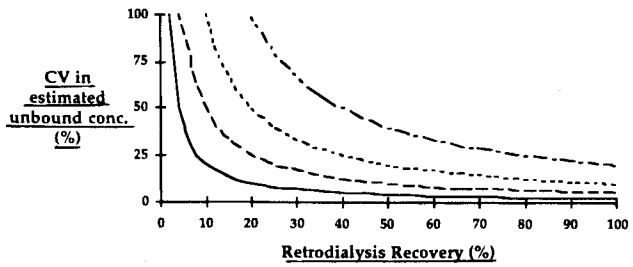

Fig. 3. Effect of different levels of SD on the magnitude of the CV with varying recovery. The SD's were set at values of 2% (——), 5% (– – –), 10% (- - - - -) and 20% (-·-·-).

Table III. Individual Half-lives of Morphine in Blood and Striatum Calculated After Correction with the Three Different Recovery Methods

	$t_{1/2,striatum}$ (min)			$t_{1/2,blood}$ (min)		
	Method			Method		
Rat[a]	I	IIa	IIb	I	IIa	IIb
1	35.7	40.5	33.4	30.0	27.9	30.1
2	54.5	46.3	40.4	25.0	25.8	26.0
3	48.0	56.5	45.5	26.2	27.2	26.1
4	49.0	42.5	50.9	28.0	27.5	26.9
5	42.6	48.8	47.6	31.2	27.9	32.3
6	44.4	21.8	29.3	26.1	27.0	26.8
Average ± SD	45.7 ± 6.4	42.7 ± 11.7	41.2 ± 8.4	27.8 ± 2.5	27.2 ± 0.8	28.0 ± 2.6
CV (%)	14.0	27.4	20.6	9.0	2.9	9.3

[a] Rats 1–3 received a 10 mg/kg dose of morphine, while rats 4–6 received a 40 mg/kg dose of morphine.

sufficient for the estimation of reliable unbound concentrations in striatum and in blood. A stable calibrator recovery was also found for AZdU, gabapentin and codeine (8,14,16).

Sources contributing to the observed variability are of bioanalytical nature, such as measuring low concentrations in small sample volumes. If the concentration in the outgoing perfusate was determined accurately one would expect an estimate of the unbound extracellular concentrations to be of low variability. As exemplified in the present study this is not true in all cases. When applying method IIa to the data, variability in the recovery of the calibrator leads to fluctuations in the unbound concentration time profiles of morphine in blood and striatum. For method IIb, the fluctuations were reduced. These fluctuations were not present in the dialysate concentration time profiles of morphine. The explanation for this discrepancy is not to be found in the retrodialysis method as such, but are a consequence of low retrodialysis recoveries in general. When the recovery is low, the concentration in the perfusate leaving the probe is close to the incoming concentration. A small change in the analysed calibrator concentration in the perfusate leaving the probe will inherently have a large impact on the calculated recovery. As the recovery value is directly used in the calculation of the unbound concentration, the propagation of this variability in recovery leads to fluctuations in the concentration time profiles. This phenomenon is illustrated by the dialysate concentration profiles of morphine, which are smooth despite a low recovery. The accuracy depends on both the magnitude of the SD of the recovery estimation and the value of the recovery of the calibrator itself. As is shown in Fig. 3, assuming a SD of 5%, a calibrator recovery of at least 20% is required to obtain a variability of less than 25% in the estimated unbound concentrations. A CV of 50% will result in a 3-fold range in the concentration estimates within one experiment. Decreasing the CV to 20%, this range is reduced to 1.5-fold. This is important because the uncertainty in the unbound concentrations will to a large extent determine the accuracy of the estimated parameters of the concentration time curves. Unbound concentrations obtained with higher recovery values will be more accurate. In general, it is worthwhile to aim at higher recoveries in microdialysis studies. When retrodialysis by calibrator is to be used for the recovery determination a calibrator recovery of >20% is recommended for more reliable estimation of the extracellular unbound concentration. If the calibrator recovery is lower, factors such as flow rate, membrane material and membrane length should also be considered as possible modes for increasing the recovery. Also, the study design, including different rates of drug administration, can be used. Preferentially, if they are in line with the purpose of the study,

Table IV. Relative Loss Versus Calculated Recovery Values (Eq. 1) for Nalorphine in Striatum and Blood During the Experiment

	Striatum				Blood			
Rat[a]	$Loss_{in\ vivo}$[b] (%)	CV (%)	$Rec_{in\ vivo}$[c] (%)	CV (%)	$Loss_{in\ vivo}$[b] (%)	CV (%)	$Rec_{in\ vivo}$[c] (%)	CV (%)
1	91.8 ± 3.8	4.2	8.3 ± 3.8	46.2	65.5 ± 2.9	4.4	34.5 ± 2.9	8.3
2	88.3 ± 8.2	9.3	11.7 ± 8.2	70.3	45.4 ± 7.9	17.4	54.6 ± 7.9	14.5
3	90.1 ± 2.1	2.4	9.9 ± 2.1	21.5	62.1 ± 9.5	15.3	37.9 ± 9.5	25.0
4	93.6 ± 2.2	2.3	6.4 ± 2.2	34.1	74.2 ± 2.6	3.5	25.8 ± 2.6	10.0
5	92.6 ± 2.5	2.7	8.0 ± 2.5	31.2	71.0 ± 2.5	3.5	29.0 ± 2.5	8.6
6	91.6 ± 7.4	8.1	7.6 ± 7.5	97.6	63.3 ± 4.8	7.5	36.7 ± 4.7	12.9
Average	91.3 ± 5.2	5.7	8.6 ± 5.2	60.2	63.6 ± 10.8	17.0	36.7 ± 10.8	29.6

Note: Mean ± SD (n = 16).
[a] Rats 1–3 received a 10 mg/kg dose of morphine, while rats 4–6 received a 40 mg/kg dose of morphine.
[b] $Loss_{in\ vivo} = C_{out}/C_{in}$.
[c] $Recovery_{in\ vivo} = (C_{in} - C_{out}/C_{in})$.

steady state conditions should be aimed at when recovery problems are present. If this is not possible, a calibrator with a higher recovery should be used to monitor probe efficiency over time, and recovery by drug should be the method of choice for the estimation of unbound extracellular concentrations.

Microdialysis offers the advantage of being able to determine the $t_{1/2}$ from more data points as compared to traditional estimations of half-life. Therefore, the determination of the half-life from microdialysis data is less sensitive to deviating points. However, the errors introduced by the fluctuating retrodialysis recovery also propagate to some extent to the half-life (Table III). The half-life in blood determined with microdialysis from this study agrees well with earlier reports and show a small variability between rats independent of the recovery method used (17).

In summary, the microdialysis recovery of morphine can be monitored using retrodialysis by drug or retrodialysis by calibrator. Retrodialysis by calibrator has the advantage of being able to monitor recovery continuously and to correct for changes in probe recovery. However, small recovery values of the calibrator results in large errors in the estimation of the unbound extracellular concentration mainly due to the mathematical processing of the data. To improve the accuracy in the estimated concentrations and eventually in the pharmacokinetic parameters, a recovery of the calibrator of 20% or higher is preferable if retrodialysis by calibrator is to be used. If the calibrator recovery shows no trends during the experiment, the estimated recovery of the drug during the reference period is sufficient to use for the estimation of reliable unbound extracellular concentrations.

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