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In-vivo Calibration of Microdialysis Probe by Use of Endogenous Glucose as an Internal Recovery Marker: Measurement of Skin Distribution of Tranilast in Rats

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Abstract

To estimate the absolute concentration of substrates surrounding a microdialysis probe in-vivo, we developed a simple calibration method using endogenous glucose as an internal recovery marker and determined the skin distribution of tranilast (*N*-(3,4-dimethoxycinnamoyl)anthranic acid), an anti-allergic agent, in rats.

This calibration method was based on the assumption that the concentration of glucose in the extracellular fluid of skin tissues is the same as that in plasma and that the in-vivo recovery ratio of glucose to tranilast by microdialysis is the same as that estimated in-vitro. Based on these assumptions, the dialysate concentrations of tranilast and glucose recovered from cutaneous microdialysis, glucose concentration in plasma, and in-vitro recovery ratio of tranilast to glucose by microdialysis were determined for the estimation of absolute unbound concentration of tranilast in the extracellular fluid of skin tissues. In an in-vitro study employing plasma containing tranilast, the unbound concentration of tranilast in plasma estimated from the dialysate concentration was just comparable with that determined by ultrafiltration methods. Also in an in-vivo study under steady-state plasma concentration of tranilast in rats, the estimated concentration of tranilast in the skin extracellular fluid was the same level as the unbound concentration of tranilast in plasma. Using the present calibration method, the skin distribution of tranilast administered into the intestinal loop or transdermally was continuously monitored in a quantitative manner.

In-vivo microdialysis is a simple technique for sampling unbound substances dissolved in extracellular fluids and biological fluids, by inserting a microdialysis probe consisting of a semi-permeable membrane into a target site and perfusing an appropriate perfusate into the probe. In the field of pharmacokinetic studies, this technique has been widely applied to various tissues including brain, skin, liver, lung, skeletal muscle, adipose tissue, eye, vein and bile duct, to monitor the drug distribution and metabolism (Muller et al 1995; Cimmino & Geloen 1997; Elmquist & Sawchuk 1997). However, the in-vivo recovery percentages for substances (the ratio of the dialysate concentration to that in the extracellular fluid surrounding the probe) vary with different substances, probes and tissues. Also, the recovery percentage of a probe changes with time during in-vivo experiments (Sauerheimer et al 1994). Thus, the amount of substance recovered in the dialysate is only a fraction of that present in the extracellular fluid surrounding the probe.

To estimate the absolute concentration of a substance at a target site from the dialysate concentration precisely, in-vivo calibration of microdialysis probe is essential. Several calibration methods have been reported: extrapolation to zero flow rate (stop-flow); slow perfusion flow rate; point of no net flux (zero net flux); retrodialysis (reverse dialysis); and recovery marker (internal reference) methods (Larsson 1991; Lonnroth 1991; Menacherry et al 1992; Wang et al 1993; Le-Quellec et al 1995; Elmquist & Sawchuk 1997). Recovery marker methods involve the use of exogenous substrates such as tritiated water and tritiated glucose and endogenous substrates such as

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urea, although urea has only been examined in an in-vitro study (Larsson 1991; Eisenberg & Eickhoff 1993; Lonnroth & Strindberg 1995). Methods of extrapolation to zero flow rate, slow perfusion flow rate and point of no net flux can determine the concentration of substrates being kept at a constant level at the target site. In contrast, retrodialysis and recovery marker methods, especially the endogenous substrate method, are applicable to monitor the serial change in the concentrations of exogenously administered substances such as drugs.

In the present study, a simple calibration method of microdialysis probe in-vivo by use of endogenous glucose as an internal recovery marker was developed to determine the skin distribution of tranilast (*N*-(3,4-dimethoxy-cinnamoyl)anthranic acid) in rats. This calibration method is based on the assumption that the concentration of glucose in the extracellular fluid of skin tissues is the same level as that in plasma (Krogstad et al 1996; Wilkins & Atanasov 1996), and that the in-vivo recovery ratio of glucose to tranilast by microdialysis is the same as that estimated in-vitro. The in-vivo calibration employing endogenous glucose as a recovery marker was carried out in the same manner as reported by Larsson (1991), in which tritiated water was employed as an exogenous recovery marker. Tranilast is an anti-allergic drug and has been clinically used in Japan for improving bronchial asthma, atopic dermatitis and allergic rhinitis. This drug is also useful for the oral treatment of keloid and hypertrophic scars (Waseda et al 1984; Yamada et al 1995).

Materials and Methods

Materials

Tranilast was obtained from Kissei Pharmaceutical Co. Ltd (Matsumoto, Japan). Glucose CII Test WAKO was purchased from Wako Pure Chemicals (Osaka, Japan). All other reagents used were of the highest purity available.

Microdialysis study

Animal experiments were carried out in accordance with the Guide for Animal Experimentation, Hiroshima University, and the Committee of Research Facilities for Laboratory Animal Sciences, Hiroshima University School of Medicine. Microdialysis was carried out using a microdialysis device obtained from Carnegie Medicine (Stockholm, Sweden), which consisted of a micro-infusion pump (CMA 100), a micro-fraction collector (CMA 142), microdialysis probes (CMA 10), micro-syringes (CMA 105) and the in-vitro stand with accessories (CMA 130), as reported previously (Murakami et al 1998a). Briefly, the semipermeable membrane (length 4 mm, o.d. 0·5 mm) of the microdialysis probe is made of a polycarbonate polymer with a molecular cut-off level at 20 kD. Krebs–Ringer solution (pH 7·4) was perfused as dialysate at a flow rate of 4 μL min^{-1} in a single perfusion manner by means of a micro-infusion pump.

In-vitro recovery ratio (K) by microdialysis. To estimate the in-vitro recovery ratio of glucose to tranilast by microdialysis, recovery percentages for glucose ($R_{G,\,vitro}$) and tranilast ($R_{D,\,vitro}$) were determined. The microdialysis probe was positioned in Krebs–Ringer solution (1·5 mL) containing various concentrations of glucose (0–2 mg mL^{-1}) and tranilast (0–400 μM). Dialysate was collected periodically every 15 min up to 60 min, and subjected to the analysis. From the slope of the concentrations of glucose and tranilast in dialysate plotted against those in the medium, $R_{G,\,vitro}$, $R_{D,\,vitro}$ and the in-vitro recovery ratio ($K = R_{G,\,vitro}/R_{D,\,vitro}$) were estimated.

In-vitro microdialysis. Male Sprague-Dawley rats, 220–250 g, were anaesthetised with urethane (1·5 g kg^{-1}, intraperitoneally). Blood was collected 60 min after administration of tranilast intravenously at a dose of 133·4 nmol per rat, and plasma (approx. 3 mL) was obtained after centrifugation. A sample of plasma (1 mL) was analysed for the concentration of glucose ($C_{G,\,plasma}$) and unbound concentration of tranilast by ultrafiltration (Ultrafree-CL, Nihon Millipore Ltd, Tokyo, Japan). Simultaneously, the microdialysis probe was positioned in plasma (1·5 mL) and dialysate was collected periodically every 15 min. The dialysate was analysed for the concentrations of glucose ($C_{G,\,MD}$) and tranilast ($C_{D,\,MD}$). From $C_{D,\,MD}$, the absolute unbound concentration of tranilast in plasma ($C_{D,\,plasma}$) was estimated by the following equation:

$$C_{D,\,plasma} = C_{D,\,MD} \times K/R_{G,\,plasma} \qquad (1)$$

where $R_{G,\,plasma}$ denotes the recovery for glucose from plasma into dialysate ($C_{G,\,MD}/C_{G,\,plasma}$) and K is in-vitro recovery ratio ($R_{G,\,vitro}/R_{D,\,vitro}$) as described above. The estimated $C_{D,\,plasma}$ was compared with the unbound concentration of tranilast in plasma determined by the ultrafiltration method.

In-vivo cutaneous microdialysis. Male Sprague-Dawley rats weighing 220–250 g were used. Rats were anaesthetized with urethane and in-vivo cutaneous microdialysis was carried out in the same manner as reported previously (Murakami et al 1998a). Briefly, the hair of the dorsal skin was

removed with clippers and cannulation (polyethylene tubing, PE-50) was made at a femoral vein and at a femoral artery for the administration of tranilast and for the sampling of blood, respectively. The rat was fixed on a surface kept at 37 °C in a prone position. A guide cannula was inserted intracutaneously into the shaved dorsal skin as superficially as possible, and a dummy probe attached in the guide cannula was substituted for a microdialysis probe such that the semi-permeable membrane was exposed to the skin tissue. The probe was fixed in place with surgical tape and the perfusion into the microdialysis probe was started. After pre-perfusion of perfusate for 30 min, tranilast was injected intravenously via the femoral vein cannula at a dose of 133·4 nmol per rat, followed by a constant infusion at a rate of 400·0 nmol min^{-1} to make a steady-state plasma concentration of tranilast of about 100 μM. Blood and skin dialysate were collected periodically every 15 min up to 90 min. Plasma was separated from the blood and was subjected to the analysis of the concentration of glucose ($C_{G,plasma}$), and unbound concentration of tranilast by ultrafiltration. Skin dialysate was subjected to the analysis of glucose ($C_{G,MD}$) and tranilast ($C_{D,MD}$) concentrations. From $C_{D,MD}$, the absolute unbound concentration of tranilast in the skin extracellular space ($C_{D,skin}$) was estimated by the following equation:

$$C_{D,skin} = C_{D,MD} \times K/R_{G,skin} \quad (2)$$

where $R_{G,skin}$ denotes the in-vivo recovery for glucose from skin extracellular fluid to dialysate ($C_{G,MD}/C_{G,skin}$), and the $C_{G,skin}$ value is assumed to be the same as the plasma glucose level. The estimated $C_{D,skin}$ was compared with the unbound concentration of tranilast in plasma determined by ultrafiltration.

Similarly, tranilast was administered intraluminally or transdermally, and cutaneous microdialysis was carried out to determine the skin distribution of tranilast. For intraluminal administration, tranilast was dissolved in a mixture of 1% $NaHCO_3$ and ethanol (7 : 3 v/v) at a concentration of 13·7 μmol mL^{-1}, and 1·0 mL of the solution was administered into a jejunal loop 10 cm in length. For transdermal application, tranilast was dissolved at a concentration of 46 μmol mL^{-1} in ethanol containing 5% polyvinylpyrrolidone (PVP), 20% oleic acid and 10% propylene glycol. PVP was used to increase the viscosity of the dosing solution and the combination of oleic acid and propylene glycol was used as a potent penetration enhancer for tranilast (Murakami et al 1998b). The tranilast solution (0·1 mL) was topically applied on the skin surface at a dose of 4·6 μmol tranilast at the point where the microdialysis probe was cutaneously implanted in advance.

Analysis

Glucose. The concentration of glucose in dialysate and plasma was determined with the glucose oxidase-mutase method using Glucose CII Test WAKO.

Tranilast. The concentration of tranilast in dialysate, ultrafiltrate, and plasma was analysed by high performance liquid chromatography (HPLC) employing a reverse-phase TSK gel ODS-80TM column (Tosoh, Tokyo, Japan) as reported previously (Murakami et al 1998b). Briefly, plasma (50 μL) was mixed with water (50 μL) and acetonitrile (100 μL), and the mixture was centrifuged. The supernatant was injected onto the HPLC column. Dialysate and ultrafiltrate samples were directly injected onto the HPLC column. The mobile phase was a mixture of water containing 1% acetic acid and acetonitrile (1:1 v/v) at a flow-rate of 1 mL min^{-1}. Detection was made at 360 nm.

Results and Discussion

In-vitro recovery ratio of glucose to tranilast

Concentrations of tranilast and glucose in dialysates were plotted against those in the medium (Figure 1). A linear relationship through an origin between them indicates that the recovery percentages for tranilast and glucose by microdialysis are constant in the concentration range employed. From the slop of the line, the recovery for tranilast ($R_{D,vitro}$) and glucose ($R_{G,vitro}$) in-vitro were estimated as 13·9 ± 0·3 and 12·1 ± 0·2%, respectively. Thus, the recovery ratio of glucose to tranilast in-vitro ($K = R_{G,vitro}/R_{D,vitro}$) was estimated as 0·87.

Estimation of plasma unbound concentration of tranilast in-vitro

The feasibility of the endogenous glucose as a recovery marker for calibration of microdialysis probe to estimate the plasma unbound concentration of tranilast was examined firstly in-vitro. The plasma unbound concentration of tranilast determined by ultrafiltration method, plasma dialysate concentration of tranilast and estimated unbound concentration of tranilast in plasma from the plasma dialysate with our method were 0·78 ± 0·02, 0·110 ± 0·004 and 0·74 ± 0·03 μM, respectively. Thus, the estimated unbound concentration of tranilast in plasma after microdialysis was found to be comparable with that determined by the ultrafiltration method.

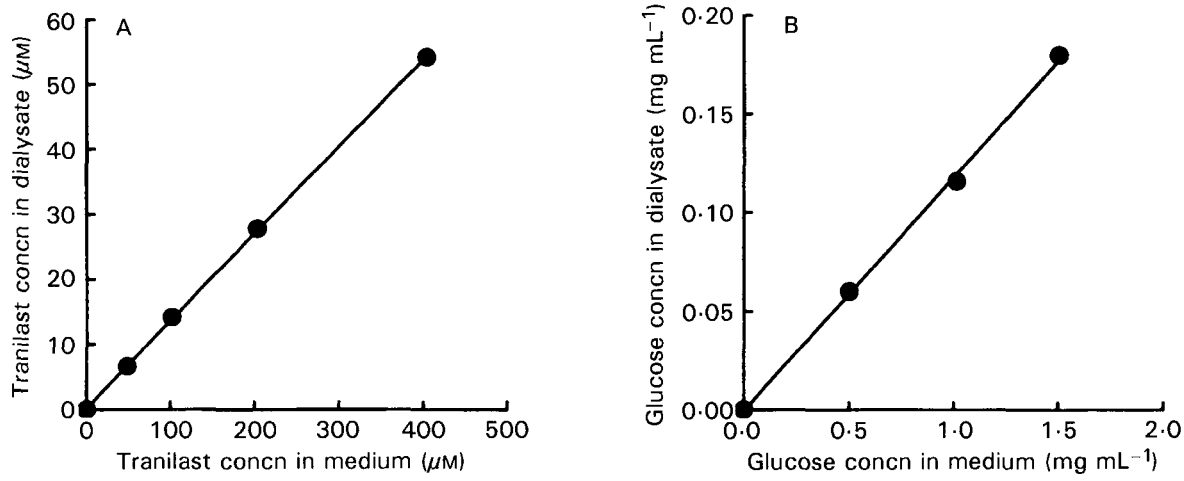

Figure 1. Recovery of tranilast (A) and glucose (B) by microdialysis in-vitro. Each value is the mean of results from three experiments.

In-vivo microdialysis

It is reported that the in-vivo recovery percentages for tritiated water in dialysates from the lung and blood are different and that the recovery percentage changes with time (Larsson 1991). Accordingly, the change in recovery for glucose from the skin was examined as a preliminary study. In some rats, the plasma concentration of glucose varied, to a relatively large extent, with time during the experiment (120 min). However, the skin dialysate concentration of glucose also varied in parallel with plasma level, and resulted in a constant recovery percentage for glucose in-vivo (6·26 ± 0·24%, mean ± s.e.m., n = 4). These results indicate that the glucose level in the skin extracellular fluid is closely related to plasma glucose level.

The unbound concentration of tranilast in the skin extracellular fluid at a steady-state plasma condition was estimated, based on the assumption that the in-vivo recovery ratio of glucose to tranilast is the same as that estimated in-vitro. This assumption would be correct as suggested by Van-Belle et al (1993), who found that the ratios of the relative recovery of carbamazepine-10, 11-epoxide and trans-10, 11-dihydroxy-10, 11-dihydro-carbamazepine to the relative loss of 2-methyl-5*H*-dibenz (b, f) azepine-5-carboxamide (internal standard) are constant in-vitro and in-vivo. As shown in Figure 2, the skin dialysate concentration of tranilast was very much lower than the total plasma level of tranilast. However, the estimated concentration of tranilast in the skin extracellular fluid was the same as the plasma unbound concentration of tranilast determined by ultrafiltration. It is generally accepted that the concentration of substrates with low molecular weights in the extracellular fluid is the same as the unbound concentration of the substrate in plasma. The apparently low recovery percentage of tranilast by microdialysis compared with the total plasma concentration would be due to the high plasma-protein binding of tranilast (98·5%).

Based on these in-vitro and in-vivo microdialysis studies, the unbound concentrations of tranilast in plasma and extracellular fluid of skin tissues were found to be correctly estimated by using endogenous glucose as a recovery marker.

Skin distribution of tranilast after intraluminal and transdermal administration

Tranilast is given orally in clinical use in Japan. Recently, tranilast given transdermally by iontophoresis was found to have a beneficial effect in relieving the pain and itching of keloid and hypertrophic scars at a much lower dose than that required orally (Shigeki et al 1997). Thus, the skin distributions of tranilast after administration into the jejunal loop (dose: 13·7 μmol tranilast per rat) and transdermal administration (dose: 4·6 μmol tranilast per rat) were compared by determining the unbound concentration of tranilast in the skin extracellular fluid with cutaneous microdialysis. Time profiles of tranilast concentrations in plasma and skin dialysates, and estimated unbound concentrations of tranilast in the skin extracellular fluid after intraluminal and transdermal administrations are shown in Figures 3 and 4, respectively. In Figure 3, the plasma unbound concentrations of tranilast, determined by the ultrafiltration method, are also shown. The estimated unbound concentrations of tranilast after microdialysis were in good agreement with plasma unbound concentrations, as found in the constant-rate infusion study (Figure 2). Total plasma concentrations of tranilast after intraluminal administration were much higher

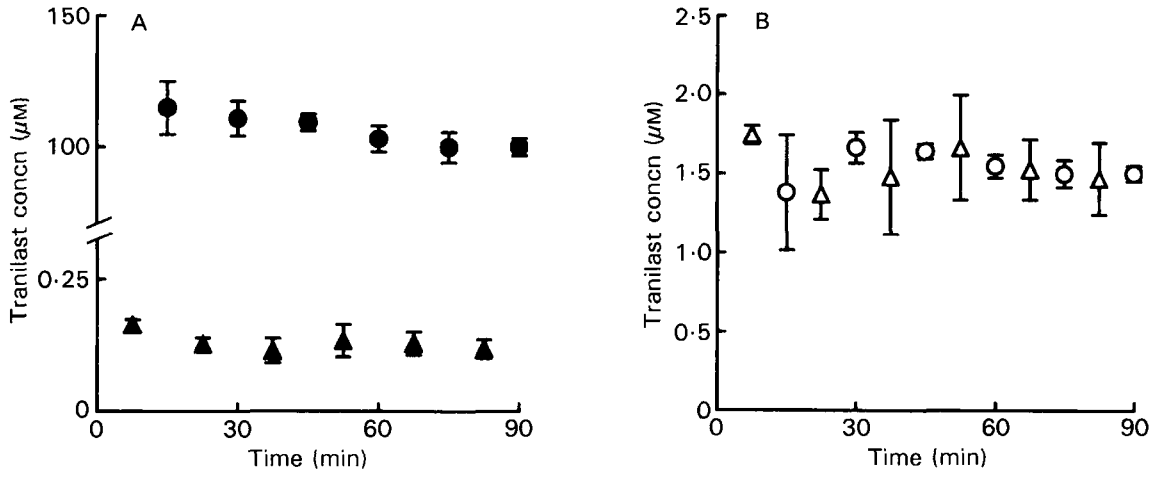

Figure 2. Evaluation of the present calibration method employing endogenous glucose as an internal recovery marker under steady-state plasma concentration of tranilast in rats. A. ●, total plasma concentration; ▲, plasma dialysate concentration. B. ○, unbound plasma concentration (ultrafiltration); △, estimated unbound plasma concentration (microdialysis). Each value is the mean ± s.e.m. of results from four experiments.

than those after transdermal application. On the contrary, the estimated unbound concentrations of tranilast in the skin extracellular fluid were much higher following transdermal application, irrespective of the lower dose (one-third that used for intraluminal administration). In transdermal application, the unbound concentration of tranilast in plasma was less than the detection limit by HPLC (0·05 μM). The area under the concentration–time curves (AUC) between 0 to 120 min of tranilast in the skin extracellular fluid normalized by unit dose were 6·80 ± 2·48 μM min per 1-μmol dose after intraluminal administration, and 296·9 ± 61·6 μM min per 1-μmol dose after transdermal application, respectively. Thus, the advantage of transdermal application of tranilast over oral administration (in addition to less systemic effects) for treatment of skin diseases such as keloid and hypertrophic scars was also revealed from the characteristic skin distribution of tranilast.

In the present study, we attempted to develop a simple calibration method of microdialysis probe in-vivo by use of endogenous glucose as an internal recovery marker. A similar calibration method to the present study has been reported employing exogenously administered tritiated water (Larsson 1991). However, the use of endogenous glucose would be more convenient and applicable to any animal species including human subjects. As a preliminary study, we also compared the present calibration method employing glucose with the method of point of no net flux to determine the

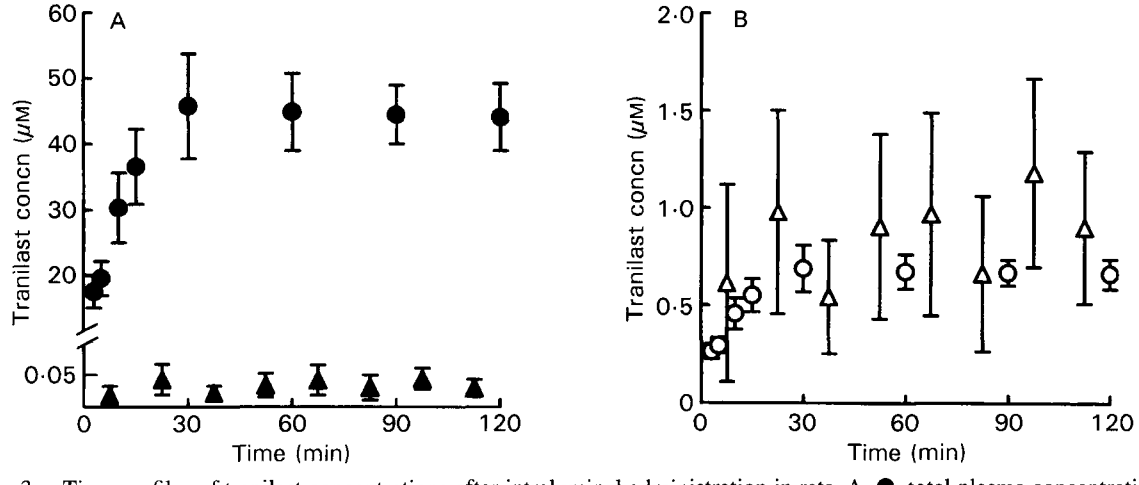

Figure 3. Time profiles of tranilast concentrations after intraluminal administration in rats. A. ●, total plasma concentration; ▲, skin dialysate concentration. B. ○, unbound plasma concentration (ultrafiltration); △, estimated unbound skin concentration (microdialysis). Tranilast was administered into a jejunal loop, 10 cm in length, at a dose of 13·7 μmol per rat. Each value is the mean ± s.e.m. of results from four experiments.

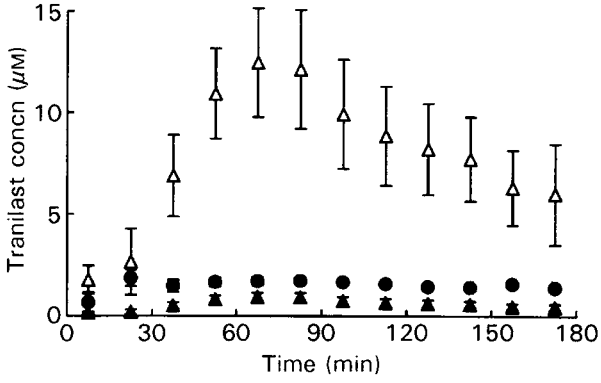

Figure 4. Time profiles of tranilast concentrations after topical application in rats. ●, Total plasma concentration; ▲, skin dialysate concentration; △, estimated unbound skin concentration (microdialysis). Tranilast was dissolved in ethanol containing oleic acid and propylene glycol, and the solution was applied to the skin surface at a dose of 4·6 μmol per rat. Each value is the mean ± s.e.m. of results from four experiments.

concentration of tranilast in an aqueous solution. A more correct value was estimated by the present calibration method (data not shown). In microdialysis studies, the estimation of absolute concentration of substrates of interest surrounding the probe and the change in recovery percentage of substrates with time during the experiments have been major problems to overcome. The present calibration method employing endogenous glucose helps to overcome such problems.

In conclusion, the use of endogenous glucose as an internal recovery marker proved to be a useful simple technique to calibrate the microdialysis probe in-vivo.

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