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Journal of Pharmacological and Toxicological Methods 52 (2005) 251–259

Journal of Pharmacological and Toxicological Methods

www.elsevier.com/locate/jpharmtox

Original article

Development of an intravenous microdialysis method for pharmacokinetic investigations in humans

Jan-Peer Elshoff[a], Stephanie Läer[b,*]

[a]*Institut für Experimentelle und Klinische Pharmakologie, Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany*
[b]*Klinische Pharmazie und Pharmakotherapie, Universität Düsseldorf, Universitätsstrasse 1, 40225 Düsseldorf, Germany*

Received 26 October 2004; accepted 6 January 2005

Abstract

Introduction: Limited blood volume is a major problem in pharmacokinetic investigations in specific populations, e.g. children. Intravenous microdialysis might help to obtain improved data sets as it is already successfully done in small animals. Since quantification of drugs is crucial in microdialysis, we developed an in vitro method to produce a workable intravenous microdialysis for human use. **Methods:** A specifically designed microdialysis cell consisting of glass was heated to 37 °C. The cell was filled with Ringer's solution, plasma or whole blood. A microdialysis probe was inserted into the cell and perfused with Ringer's solution with addition of 4% dextran. The β-receptor blocker sotalol served as a test drug. The stepwise in vitro evaluation process addressed issues of loss of dialysate, calibration by retrodialysis and relative recovery. These conditions were then applied in an in vivo pilot study to one single healthy volunteer after written informed consent. **Results:** To address loss of perfusion fluid 4% of dextran was added and high and constant amounts of dialysate were achieved. To account for changes in the relative recovery a continuous use of retrodialysis by the calibrator atenolol was introduced. The recovery of atenolol was comparable to sotalol. The pharmacokinetic analysis revealed that sotalol concentrations from microdialysates were not different from conventional plasma samples (100±11%, n=33) resulting in subsequent comparable pharmacokinetic parameters. **Discussion:** This stepwise approach using an in vitro device enabled us to demonstrate the determination of pharmacokinetic parameters of sotalol. The most important evaluation step is represented by the continuous use of retrodialysis by the calibrator atenolol because it can account for changes in the relative recovery of the drug. This approach should be a starting point to simplify pharmacokinetic studies in special populations, e.g. in small children, to improve drug treatment.

Keywords: Methods; In vitro microdialysis cell; Intravenous microdialysis; Retrodialysis by calibrator; Pharmacokinetics; Children; Sotalol

1. Introduction

Most dosing regimens for children are based on empiricism and are extrapolated from adult studies since pharmacokinetic investigations in infants or neonates have not been performed. Several studies showed that this lack of information is responsible for an increased level of side effects or ineffective therapy (Impicciatore et al., 2001; Turner, Nunn, Fielding, & Choonara, 1988). Thus it is inevitable to carry out pharmacokinetic studies to ensure adequate dosing for a safe and effective drug therapy in children.

The limited blood volume is one of the major problems in pharmacokinetic investigations in children. Without compromising the infants' clinical state blood withdrawals of about 5 ml/kg seem to be safe (Warkentin, 1997). Several data points are necessary to adequately describe pharmacokinetic parameters. In addition, the routine blood analysis has to be performed to define the disease status of the child. Others mentioned that 1 ml of blood in a 1-kg neonate or premature infant is equivalent to removing 70 ml from an adult (Blanchette & Zipursky, 1984). This demonstrates the critical importance of reducing or even avoiding the needed

* Corresponding author.
E-mail address: Stephanie.Laeer@uni-duesseldorf.de (S. Läer).

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doi:10.1016/j.vascn.2005.01.001

blood volume for neonates whereas it will be meaningless for an adult. Sparse data sampling and subsequent population pharmacokinetic analysis has its limitation in the practical application because inaccurate documentation and non-compliance of the patients often prevent a proper analysis and interpretation of data (Urquhart, 1994). The method of intravenous microdialysis might therefore help to obtain a rich data set for pharmacokinetic investigations in children to determine drug concentrations without the necessity of blood samples.

Intravenous microdialysis has been used in several animal studies for pharmacokinetic investigations, mainly in rats dealing with the same problems of limited blood volume as in pediatric patients (Evrard, Deridder, & Verbeeck, 1995; Höcht, Di Verniero, Opezzo, & Taira, 2003; Opezzo, Hötch, & Taira, 2001) The method allows a continuous sampling of drug concentrations. Therefore, a higher temporal resolution can be achieved compared to conventional plasma sampling. Furthermore, microdialysis samples only the unbound drug concentration which is independent of changes in the volume of distribution and most important directly correlated to the drug effect. Therefore, it is even more appropriate for pharmacokinetic/pharmacodynamic modeling than using conventional plasma concentrations.

Only a few intravenous microdialysis studies in humans have been performed. These studies investigated endogenous parameters like drug induced alterations in serotonin plasma levels (Castejon, Páez, Hernández, & Cubeddu, 1999; Páez & Hernández, 1998; Stjernström, Karlsson, Ungerstedt, & Hillered, 1993) or lactate, pyruvate and glucose plasma concentrations in intensive care patients, but did not address pharmacokinetic questions. Recovery (the term *recovery* will be used for *relative recovery*) was assessed either before starting the microdialysis sampling or by conventional blood samples. The drawback of these methods was that changes in recovery during the experiment e.g. due to alterations in blood flow or even mechanical disturbances (Evrard & Verbeeck, 1994) were not monitored and this might have misled results and interpretations. This shortcoming can be overcome by using a continuous internal recovery control as it was introduced by Wong, Wang, and Sawchuk (1992) for brain microdialysis.

To develop a workable in vivo intravenous microdialysis method we used a simple in vitro test device. In clinical microdialysis, however, in vitro relative recovery has been regarded as not predictive for in vivo relative recovery due to the different properties of the tissue surrounding the microdialysis membrane in vivo (Glick, Dong, Keller, & Carlson, 1994). In our study, the "tissue" blood will be studied "ex vivo" and this might be more predictive. However with our in vitro settings we wanted to get a starting point regarding flow rate, constitution of the perfusion fluid and most importantly the assessment of an internal standard to account for changes in the relative recovery. Since we will use the internal standard continuously throughout the in vivo procedure differences between the relative recovery of the in vitro and the in vivo experiments are of minor importance.

Therefore, the objective of this work was to use a simple in vitro test device and to stepwise evaluate issues of loss of dialysate, calibration by retrodialysis and recovery for pharmacokinetic investigations of sotalol. Sotalol is often used for the treatment of life threatening tachyarrhythmia in neonates and infants (Pfammatter & Paul, 1997). As a pilot in vivo investigation, parameters evaluated in vitro microdialysis conditions were tested in one healthy adult male volunteer after oral ingestion of 160 mg sotalol. Sotalol concentrations as well as pharmacokinetic parameters in microdialysate and plasma were compared in the same healthy volunteer and compared to results reported in the literature.

2. Methods

The in vivo application of the microdialysis experiment was performed in accordance with the Declaration of Helsinki and subsequent amendments. The local ethics committee had approved the investigation. The volunteer gave written informed consent prior to participation in the study.

2.1. Drugs and reagents

Pure sotalol and atenolol were supplied by Sigma (St. Louis, MO, USA). For the in vivo experiments sotalol (Sotalex^R) tablets and ampoules (Bristol-Myers Squibb, München, Germany) and atenolol ampoules (Tenormin^R, Astra Zeneca, Wedel, Germany) were used. HPLC-grade methanol, butanon, glacial acid and sulfuric acid were purchased from E. Merck (Darmstadt, Germany). Ethanol was received from the pharmacy of the University Hospital Hamburg-Eppendorf, Germany. Dextran with a molecular weight of 70 kD was ordered from Amersham Pharmacia Biotech (Uppsala, Sweden). Dextranase (EC 3.2.1.11) was received from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). 3,5-Dinitrosalicylic acid and naphthoresorcine were purchased from Fluka Chemie (Buchs, Switzerland).

2.2. Microdialysis cell, microdialysis equipment and procedure

The microdialysis cell (Fig. 1) was a double glass chamber with an inner diameter of 20 mm and was constantly heated to body temperature (37 °C) by liquid water heating. The sample volume contained 15 ml of the media Ringer's solution (147 mM NaCl, 2.25 mM $CaCl_2$, 4.0 mM KCL; Delta-Pharma, Pfullingen, Germany), drug free pooled citrate plasma (Department of Transfusion Medicine, University Hospital Hamburg-Eppendorf, Germany), or drug free whole blood taken from young healthy volunteers. Microdialysis probes were immersed by an

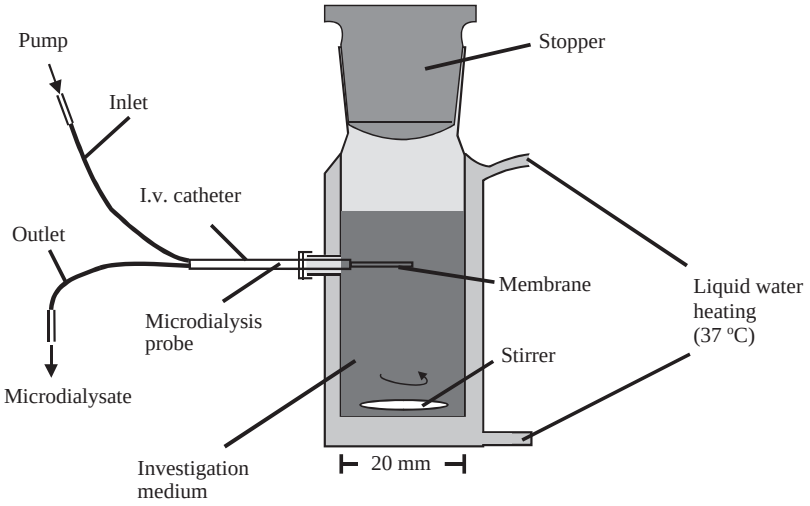

Fig. 1. Microdialysis cell for in vitro evaluation of microdialysis sampling conditions. The microdialysis cell was a double chamber glass device heated by a liquid water heating (37 °C). Ringer's solution, plasma or whole blood was filled into the magnetically stirred sample volume (20 ml) as investigation media. The membrane of the microdialysis probe was immersed into the sample volume by an intravenous (i.v.) catheter. The sample volume was covered with a stopper to prevent the investigation media from evaporation. Perfusion fluid was pumped through an inlet into the microdialysis probe. Microdialysate samples were collected in definite time intervals from the outlet.

intravenous catheter into the respective medium, penetrating a silicon seal at the end of a special insertion canal. A stopper covered the microdialysis cell to prevent the medium from evaporating. The media was stirred magnetically to achieve a constant concentration gradient between external medium and perfusion fluid.

Two types of microdialysis membranes were used. A CMA-70 (CMA/Microdialysis, Stockholm, Sweden) microdialysis membrane was used for optimizing the perfusion medium and the flow rate and for the in vivo investigation. This probe had a polyamide membrane (PO probe), a 20 kD molecular weight cut-off, an outer diameter of 0.6 mm and a length of 20 mm. It had been sterilized using beta radiation for human application. In addition, a MAB 1.30.20 (Microbiotech AB, Stockholm, Sweden) with a cuprophane membrane, a 6 kD molecular weight cut-off (CUP probe), an outer diameter of only 0.24 mm and a length of 20 mm was then tested with the optimized conditions in the in vitro microdialysis cell to demonstrate whether the microdialysis method is adjustable to other sizes and types of membranes. This probe right now is not ready for human application, because it has to be sterilized before human use. The microdialysis probes were connected to a CMA 102 microdialysis syringe pump (CMA/Microdialysis, Stockholm, Sweden). The fluid which was pumped into the microdialysis probe is called the perfusion fluid or perfusate. The perfusion fluid consisted of Ringer's solution with or without 4% dextran, sotalol (1.0 μg/ml) and/or atenolol (1.0 μg/ml) according to the specific experimental design in the in vitro or in vivo investigation. The fluid which was collected after the dialysis process is called microdialysate or dialysate.

At the beginning of the microdialysis procedure the microdialysis probes were flushed at a flow rate of 10 μl/min for at least 10 min with Ringer's solution. Thereafter, the probe was perfused with a perfusion fluid at the flow rate used in the subsequent experiment for at least 60 min before starting the experiment to assure constant perfusion fluid flow through the probe. For each experiment a new microdialysis probe was used. The sampling interval was set to 35 min according to the time course of the sotalol absorption, distribution and elimination to receive a reliable and precise concentration time profile of the test drug sotalol.

2.3. In vitro investigation

Two sets of in vitro investigations were carried out. In the first set of experiments, questions of loss of perfusion fluid and an optimal flow rate were investigated. Therefore, the media were spiked with sotalol at a concentration in the therapeutic range of 1.15 μg/ml of adults (Wong et al., 1992). The experiments were carried out with each of the three investigation media. Every single medium was investigated three times at three different days. In each medium the flow rates were set to 0.3, 0.5, 1.0, 2.0, and 5.0 μl/min. The dialysates were weighed and used to calculate and compare their amounts for the evaluation of the loss of perfusion fluid. For HPLC analysis of sotalol 10 μl of the microdialysate sample were used. At each flow rate six samples were collected before switching to the next flow rate. Microdialysate samples were collected in 0.5 ml tubes and stored at −80 °C until analysis.

For the second set of experiments, the suitability of the calibrator (internal standard) was evaluated. It had to be comparable to sotalol's physico-chemical properties, e.g. molecular size and water solubility to achieve identical recoveries. Therefore, atenolol was selected as a calibrator.

The perfusion fluid was spiked with sotalol (1.0 μg/ml) and atenolol (1.0 μg/ml) and the recovery of sotalol and atenolol from both microdialysis probes using a flow rate of 1 μl/min were compared.

2.4. Assessment of recovery and probe calibration in the in vitro experiments

In vitro recovery for the first set of experiments was determined by calculating the quotient of the sotalol (atenolol) concentration in the dialysates ($C_{\text{Dialysate}}$) and the sotalol (atenolol) concentration in the surrounding investigation medium ($C_{\text{Investigation medium}}$) using the following formula:

$$\text{Recovery } [\%] = C_{\text{Dialysate}}\ [\mu g/ml] \Big/ C_{\text{Investigation medium}}\ [\mu g/ml] \bullet 100[\%]$$

In vitro recovery for the second sets of experiments was calculated from the quotient of the atenolol and sotalol concentrations in microdialysate ($C_{\text{Dialysate}}$) and perfusion fluid ($C_{\text{Perfusate}}$).

$$\text{Recovery}[\%] = C_{\text{Dialysate}}[\mu g/ml] / C_{\text{Perfusate}}[\mu g/ml] \bullet 100[\%]$$

2.5. Digestion of the added colloid

The use of Ringer's solution as perfusion fluid showed a substantial loss of perfusion fluid, comparable to previous microdialysis experiments in adipose tissue and skeletal muscle cell (Hamrin, Rosdahl, Ungerstedt, & Henriksson, 2002; Rosdahl, Ungerstedt, & Henriksson, 1997). It is caused by the oncotic pressure of the proteins in the tissue around the probe. As addition of dextran to the perfusion fluid diminished the loss in adipose tissue and skeletal muscle, we also added dextran (4%) to the perfusion fluid and increased the oncotic pressure to 4 kPa (Edsman & Sundelöf, 1987).

In order to avoid the plugging of the HPLC system, the macromolecule dextran had to be removed before analysis. The removal of dextran was obtained by digestion with dextranase which reduced it to smaller molecules. To optimize the digestion conditions the necessary time of digestion and the amount of added enzyme were evaluated. Therefore, a solution of dextranase in double distilled water was prepared. Dialysates and dextranase solution were mixed in a ratio of 1:1 and incubated for digestion at 37 °C.

The optimal duration of the digestion was evaluated by analyzing samples from half an hour up to 24 h (Fig. 2A). To monitor the digestion process, 3,5-dinitrosalicylic acid was added afterwards, which is reduced to aminonitrosalicylic acid by the digestion products. Absorption of aminonitrosalicylic acid was measured with a UV/VIS spectrometer at 540 nm. To optimize the amount of enzyme, different enzyme concentrations from 1 U/ml up to 200 U/ml were added to the dialysates (Fig. 2B). For the visualization of the digestion procedure, the products of

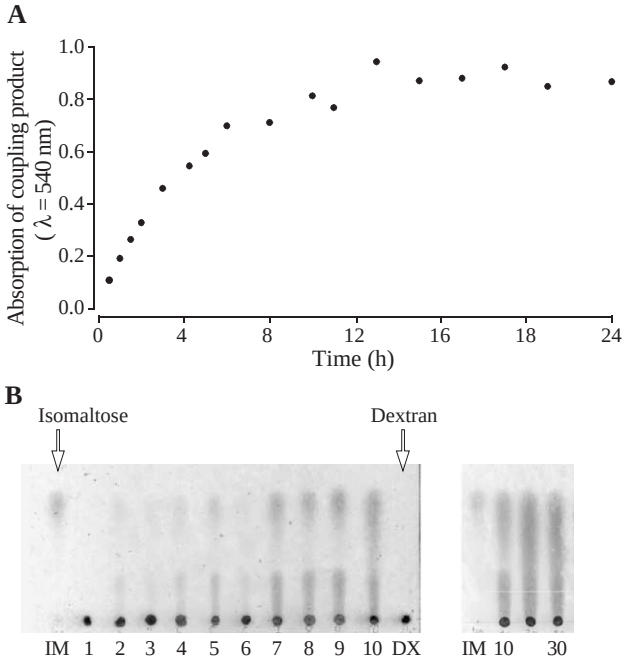

Fig. 2. A. Representative time course of the decomposition of dextran by dextranase over 24 h. To determine the progress of the decomposition 3,5-dinitrosalicylic acid was reduced by the end product isomaltose. The reduction product was quantified by measuring its absorption at 540 nm. B. Representative blot of HPTLC separation of dextran and its decomposition products after treatment with increasing amounts of dextranase (1 to 10, 20, and 30 U/ml) over a period of 12 h at 37 °C. Detection was performed with ethanolic naphthoresorcine and sulphuric acid with subsequent development at 110 °C for 5 min. IM=isomaltose, DX=dextran.

the digestion were analysed on HPTLC sheets (Merck silica gel 60, 20×20 cm, E. Merck, Darmstadt, Germany; elution solvent: glacial acid 20 %, methanol 20%, butanon 60%). Detection was performed by spraying the sheets with 0.2% naphthoresorcine in ethanol and 20% sulphuric acid (ratio 1:1) followed by heating at 110 °C for 5 min.

2.6. In vivo microdialysis

An intravenous catheter was placed into the cubital vein of each arm of the healthy volunteer. In one of the catheters a sterile CMA 70 microdialysis probe was inserted. From the other catheter conventional blood samples were taken. The used perfusion fluid consisted of Ringer's solution with addition of 4% dextran and 1.0 μg/ml atenolol as internal standard. The flow rate was 1.0 μl/min. Baseline dialysates were sampled for at least 2 h to assure that the recovery of the calibrator was stable. Thereafter, the volunteer swallowed 160 mg sotalol. The sampling interval was 35 min for 12 h, after that only once every hour until 24 h after application of the drug. Blood samples for comparison were collected every 35 min in the middle of the collection interval of the dialysates. This was necessary to correct for the lag time of the microdialysate from the probe to the sampling tube. Plasma samples and dialysates were stored at −80 °C until analysis.

To quantify the sotalol concentration the calibrator or internal standard atenolol was used continuously (continuous retrodialysis by calibrator). Because the inward and outward diffusion is equal (Müller et al., 1995) in vivo recovery was calculated from the outward diffusion of the calibrator, this means that the loss of atenolol was used to calculate the sotalol concentration in the in vivo dialysates using the formula:

$$C_{\text{Sotalol Plasma}}[\mu g/ml] = 100 \bullet C_{\text{Sotalol Microdialysate}}[\mu g/ml] \times (100 - \text{Recovery}_{\text{Atenolol}}[\%])^{-1}$$

where $C_{\text{Sotalol Plasma}}$ is the sotalol concentration in plasma, $C_{\text{Sotalol Microdialysate}}$ is the sotalol concentration in the dialysate and $\text{Recovery}_{\text{Atenolol}}$ is the relative recovery of atenolol calculated from the quotient of the atenolol concentration in dialysate and perfusate.

2.7. High-performance liquid chromatography

An LC Workstation Class *VP* from Shimadzu (Kyoto, Japan) with an RF-10XL fluorescence detector was used. Chromatographic separation was performed on a Spherisorb C6 column (150×4.6 mm i.d., 5 μm particle size; Chromatographie Service, Langerwehe, Germany) with a Spherisorb C6 guard column (17×4 mm i.d., 5 μm particle size) at ambient temperature. One hundred microliters of the plasma samples and 10 μl of the dialysates were analysed using a validated HPLC assay with fluorescence detection (Läer, Wauer, & Scholz, 2001b; Läer et al., 2001a). The limit of quantification for sotalol in this assay was 0.09 μg/ml and below 0.1 μg/ml for atenolol. The intra-and inter-assay precision of the assay in different matrices varied between 0.6% and 14.4%, the respective accuracy between 86.1% and 109.8%. Precision and accuracy did not differ between various matrices because the method used an internal standard to control the relative recovery of the matrices (Läer et al., 2001a,b).

2.8. Data analysis and statistics

Data are given as arithmetic means plus/minus standard deviations (S.D.). Intra-day and inter-day variation were determined by repeatedly collecting samples at the different flow rates. Precision is expressed as coefficient of variation (C.V.). Accuracy is expressed as percentage of the amount of dialysate in each sample relative to the expected amount of dialysate. Possible differences between sotalol and atenolol recovery were tested using a Wilcoxon rank test for related samples. A p value lower than 0.05 was regarded as statistically significant.

The maximal plasma concentration of sotalol (C_{max}) and its respective time point (t_{max}), the area under the concentration time curve (AUC) and the terminal half life ($t_{1/2}$) were calculated using a one compartment model with first order absorption and elimination using the software package KINETICA v2.0 (Innaphase S.a.r.l., Champs-sur-Marne, France). Oral clearance (CL/F) was calculated as ratio between applied dose and AUC, the elimination rate constant (ke) from the ratio of the natural logarithm of 2 (ln2) and $t_{1/2}$, and the apparent volume of distribution (Vd/F) after oral administration of a drug from the ratio of oral clearance and ke, F is the bioavailability.

3. Results

3.1. In vitro microdialysis and digestion of dextran in the microdialysates

In vitro experiments were performed in the microdialysis cell. Considerable differences were noted when the expected dialysate volumes over periods of 35 min were compared to the achieved volumes. Whereas in Ringer's solution the expected dialysate volume was reached at all flow rates, in plasma and whole blood the expected volumes were only achieved at 5.0 μl/min with 88% and 93%, respectively (Fig. 3A). The relative dialysate volume exponentially decreased

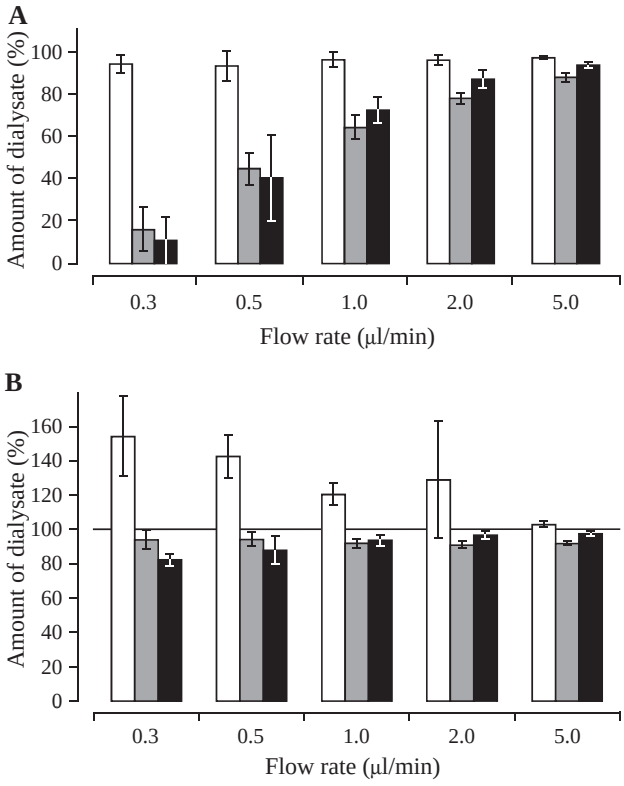

Fig. 3. Relative amount of dialysate (% of the expected amount) in Ringer's solution (white bars), plasma (grey bars) and whole blood (black bars) at flow rates of 0.3 up to 5.0 μl/min. Sampling was performed with a polyamide microdialysis membrane with 20 kD molecular weight cut-off and an outer diameter of 0.6 mm in intervals of 35 min. Data are mean±SD, n=18 each bar number of samples of 3 probes. In (A) Ringer's solution was used as perfusion fluid. In (B) Ringer's solution with addition of 4% dextran was used as perfusion fluid. The horizontal black line indicates 100%.

with lower flow rates and at 0.3 μl/min only 16% in plasma and 11% in whole blood remained (Fig. 3a). After addition of 4% dextran to the perfusion fluid, high and constant amounts of dialysate were achieved in plasma and whole blood from 0.3 μl/min up to 5.0 μl/min ranging from 82% to 98%, respectively (Fig. 3B).

To avoid plugging of the HPLC-system the dextran was digested with dextranase. Fig. 2a shows the rise of the isomaltose concentration (final digestion product) with increasing time of digestion. After 12 h the increase of the isomaltose concentration stagnated and so the breakdown of dextran was completed. Dextranase concentrations higher than 10 U/ml did not lead to an extra effect even though it was raised up to 200 U/ml (Fig. 2b shows until 30 U/ml). Therefore, digestion of dextran in the dialysates was performed with addition of 10 U/ml dextranase over 12 h. Furthermore, Fig. 2b shows several intermediate products on the HPTLC layer indicating that the hydrolysis of the α-1,6-glycosidic bond between the glucose monomers of the dextran to isomaltose was not completed but led to several intermediate polysaccharides.

Neither dextran nor dextranase affected the concentration of sotalol or atenolol. After adding either dextran (4%) or dextranase (10 U/ml) to sotalol (1 μg/ml) and atenolol (1 μg/ml) in Ringer's solution no difference was noted when analysing the sotalol and atenolol concentrations in these samples.

Determining the sotalol concentrations in the microdialysis samples with whole blood as investigation medium gained from the PO probe showed that with decreasing flow rates from 5.0 to 2.0, 1.0, 0.5, or 0.3 μl/min the recovery increased from 50% to 92%, 94%, 105%, or 117%, respectively (Fig. 4). For comparison the respective results for Ringer's solution and plasma were at the respective flow rates: 72% to 92%, 91%, 96%, or 102% (Ringer's solution), and 56% to 79%, 85%, 89%, or 91% (plasma) showing a similar pattern as in whole blood: with decreasing flow rate

Table 1
Recovery of sotalol and calibrator atenolol in different microdialysis probes by retrodialysis

Probe	(*n*)	Sotalol (*S*)	Calibrator (*A*)	Mean difference of recoveries [(*S*−*A*)/*S**100]
PO	25	88.0±1.4%	88.8±0.7%	1.0%
CUP	47	70.8±6.6%	69.8±7.9%	2.3%

Values are mean±SD. PO: Polyamide membrane, 20 kD molecular weight cut-off, outer diameter of 0.6 mm; CUP: Cuprophane membrane, 6 kD molecular weight cut-off, outer diameter of 0.24 mm.

an increase in recovery of sotalol. Why the relative recovery in whole blood was higher at low flow rates than in plasma can only be speculated and might contribute to the fact that in whole blood the additional cell compartment may lead to sotalol concentration gradients which are not present in the cell free plasma under in vitro conditions.

Because the optimization of the in vitro condition were aimed at the blood microdialysis, the corresponding relative amounts of the dialysate at different flow rates were illustrated in Fig. 4. For the in vivo microdialysis conditions, a flow rate of 1.0 μl/min was chosen which should give a high sotalol recovery of about 94% with sufficient dialysate volumes even at short sampling intervals to enable analytical procedures. For the CUP probe the relative recovery was determined using the optimized conditions at 1.0 μl/min. At this flow rate the recovery was 71% and this lower recovery was expected because of the smaller diameter and therefore the smaller surface of the CUP membrane.

With the continuous use of retrodialysis by calibrator, the relative recovery of sotalol was obtained and compared to the relative recovery of atenolol (Table 1). The recovery of atenolol was not statistically different from sotalol ($p>0.05$, Wilcoxon rank test). This suggests that atenolol is a reliable calibrator for the estimation of the in vivo recovery of sotalol. The relative recovery of sotalol from the direct

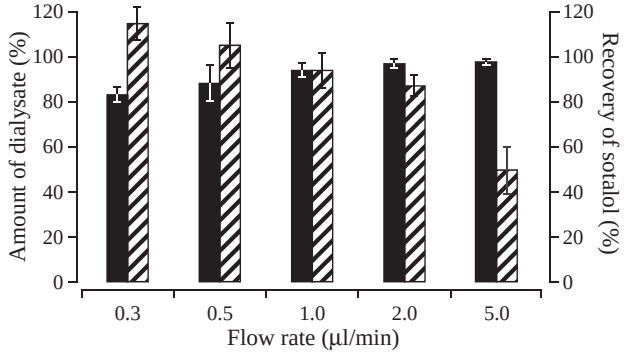

Fig. 4. Recovery of sotalol (hatched bars) and relative amount of dialysate (black bars) at flow rates of 0.3 up to 5.0 μl/min with a polyamide microdialysis membrane with 20 kD molecular weight cut-off and an outer diameter of 0.6 mm using Ringer's solution with 4% dextran (medium: whole blood, sample interval: 35 min). Note that the optimal flow rate was chosen at the intersection of both parameters at a flow rate of 1.0 μl/min. Data are mean±SD, n=18 each bar.

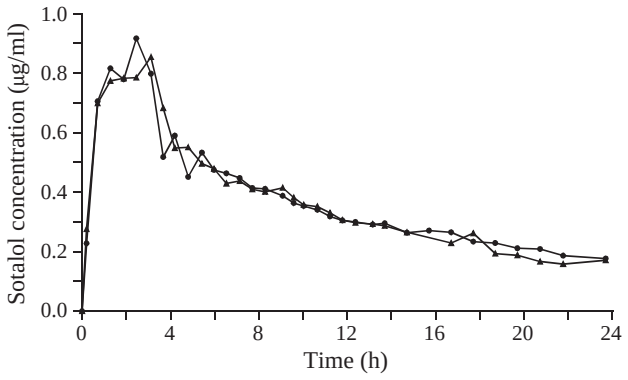

Fig. 5. Concentration/time profiles of sotalol after oral application of 160 mg sotalol in one healthy volunteer. Filled triangles represent the sotalol concentrations from in vivo microdialysis experiments inserted into the cubital vein, filled circles represent the sotalol concentrations in the corresponding plasma samples.

Table 2
Pharmacokinetic parameters (PK) of sotalol from microdialysis or conventional plasma sampling in one healthy volunteer after oral ingestion of 160 mg sotalol

PK parameter	Conventional plasma sampling	In vivo microdialysis	Ratio microdialysis/plasma [%]
$AUC_{0\text{-}24h}$	9.98 [μg · h/ml]	9.82 [μg · h/ml]	98.4
C_{max}	0.76 [μg/ml]	0.77 [μg/ml]	101.3
t_{max}	1.46 [h]	1.47 [h]	100.7
$t_{½}$	8.02 [h]	7.77 [h]	96.9
CL/*F*	0.18 [L/h · kg]	0.18 [L/h · kg]	100
*V*d/*F*	2.1 [L/kg]	2.0 [L/kg]	95.2

AUC=Area under the curve; C_{max}=maximal plasma concentration, t_{max}=time of maximal plasma concentration; $t_{½}$=terminal half-life, *F*=bioavailability, CL/*F*=oral clearance; *V*d/*F*=apparent volume of distribution.

method with 94% was therefore comparable with the relative recovery of 88% from the retrodialysis.

3.2. In vivo microdialysis

Comparison of the sotalol concentrations from intravenous microdialysates and from plasma samples after oral ingestion of 160 mg sotalol showed nearly overlapping sotalol concentration/time curves (Fig. 5). Sotalol microdialysate concentrations amounted to 100±11% (percentage of corresponding plasma concentration). The comparability of both plasma and microdialysate concentrations remained for at least 24 h and was stable over the whole range of concentrations (0.2-0.9 μg/ml). The respective pharmacokinetic parameters of the microdialysate sotalol concentrations versus the sotalol concentrations obtained with the conventional method are given in Table 2. The recovery of sotalol was estimated in the in vivo experiments from the recovery calculated from the internal standard atenolol (2.6) and was on average 55.5±3.0% in the microdialysates.

4. Discussion

The stepwise development of an intravenous microdialysis method using a microdialysis cell helped us to evaluate in vitro conditions before starting the in vivo experiment. For our method two crucial parameters were closely evaluated to achieve accurate and reliable drug concentrations in the dialysates. Firstly, a constant and high amount of dialysate was necessary to obtain short sampling intervals for a sufficient number of data points. Secondly, a continuous assessment of the relative recovery throughout the microdialysis experiment was taken into account to calculate drug concentrations from dialysates accurately. Atenolol was evaluated as the calibrator for the retrodialysis. Flow rate and composition of the perfusion fluid were optimized and validated, and two membrane types were tested before starting the in vivo application. In a pilot in vivo application in one healthy volunteer comparable sotalol concentrations and pharmacokinetic parameters throughout the full microdialysis procedure between microdialysate and plasma concentrations were achieved. This can be regarded as a starting point for further in vivo experiments. Although the recovery in the in vivo experiment of sotalol was lower than expected from the in vitro experiment, the incorporation of the continuously monitored retrodialysis by calibrator method enabled us to monitor and calculate the sotalol concentrations precisely. The pharmacokinetic results of sotalol in this single healthy volunteer fit to pharmacokinetic parameters obtained by others (Table 3, Läer, Neumann, Scholz, 1997, Läer et al., 2001a,b).

Constant and high amounts of dialysate were achieved by adding the macromolecule dextran to the perfusion fluid. The relative dialysate volume depends on the osmotic gradient between surrounding medium and perfusion fluid. Adding dextran to the perfusion fluid when the microdialysis membrane was located in Ringers solution resulted in a net flux into the perfusion medium with an amount of dialysate exceeding 100%. This was primarily evident at low flow rates because the time to reach steady state between both sides was longer at low flow rates. Exchanging the Ringer's solution with plasma or blood led to homeostasis resulting in a dialysate amount of about 100% (Edsman & Sundelöf, 1987). Similar experiences were reported when adding dextran to the perfusion fluid while performing microdialysis experiments in skeletal muscle and adipose tissue (Hamrin et al., 2002; Rosdahl et al., 1997), but not for microdialysis in blood (Castejon et al., 1999; Evrard & Verbeeck, 1994; Stjernström et al., 1993). Since diffusion conditions are optimal in case of homeostasis, oncotic pressure should be compensated. Addition of dextran is a simple and effective method to achieve this aim.

To avoid plugging of the HPLC-system, the macromolecule dextran was digested prior to injection into the HPLC-system. Other techniques to remove macromolecules from solutions are filtration, centrifugation, liquid–liquid extraction, and solid phase extraction (Gilar, Bouvier, & Compton, 2001). But apart from one recently published miniaturized method designed for sample volumes of 1–5 μl, the other methods have the disadvantage of the loss of substance and volume which does not allow quantification of the drug in the dialysate. Using the technique described in this paper, however, constant and reliable quantification of the drug is possible.

The digestion of dextran, however, was not complete to isomaltose. It is well known that every biochemical reaction

Table 3
Pharmacokinetic parameters from Läer et al. (2001a,b)

Pharmacokinetic parameter	Adult data from Läer et al. (2001a,b), *n*=5
CL/F	0.11–0.15 [L/h · kg]
Vd/F	1.2–2.4 [L/kg]
$t_{1/2}$	6.6–9.5 [h]

F=bioavailability, CL/F=oral clearance; *V*d/*F*=apparent volume of distribution, $t_{½}$=terminal half life.

leads to a balance of educt and product depending on the surrounding conditions such as pH, temperature or solubility. Degradation of dextran to smaller molecules should avoid sufficient plugging of tubes. Dextranase as a macromolecule itself might plug the HPLC system as well, but the concentration of 30 μg/ml (10 U/ml) is far lower than the concentration of dextran (40 mg/ml). Furthermore, about 1500 samples have been analyzed after digestion so far and no problem has ever occurred with the HPLC system that could be attributed to a plugged HPLC system. Dextran or dextranase did not affect the sotalol or atenolol concentration. The binding of substances to dextran or dextranase and their impact on the drug analysis, however, has to be investigated as part of the method development.

Calibration methods are often the difficult part of a quantitative microdialysis experiment. A sufficient calibration was reached by continuous retrodialysis using atenolol as a calibrator. It was shown that diffusion of sotalol and atenolol did not differ throughout the dialysis process (88.0±1.4% vs. 88.8±0.7%, PO probe). The method enabled us to adjust the measured sotalol dialysate concentrations for the recovery, as it was shown for brain microdialysis for different drugs (Bouw & Hammarlund-Udenaes, 1998). Compared to other calibration methods like the No Net Flux method or retrodialysis by drug, the special value of internal recovery control using retrodialysis by calibrator is its continuous recovery determination. Changes in recovery during the experiment will be taken into account, whereas the other methods can only determine recovery at the beginning and in the end of an investigation. No time-consuming calibration process is necessary before starting the experiment, as calibration is controlled throughout the whole experiment. The in vitro experiments showed that the relative recoveries of sotalol from the direct and the retrodialysis method were similar. This provides the scientific basis for using in vitro direct measurements to optimize the microdialysis conditions.

The recovery in the in vivo experiment of sotalol was lower than the in vitro experiments. This might confirm that in clinical microdialysis, in vitro relative recovery is not predictive for in vivo relative recovery (Glick et al., 1994). One explanation might be that the magnetically shirred in vitro experiment might create a sink condition in the cell medium. This may artificially change the driving force which may be different from the in vivo blood circulation. However, considering data from one single healthy volunteer, a speculation on reasons explaining such differences might be misleading. Furthermore, the main determinant for precise and accurate calculations of the in vivo drug concentrations is a reliable and robust internal standard resulting in a recovery on average with 55±3% which was demonstrated in the experiments. If the internal standard behaves similarly to the substance of interest, differences in the in vitro and in vivo relative recoveries are compensated. Therefore, this experiment is a starting point showing that in vitro evaluation steps are a safe procedure to achieve comparable plasma and microdialysate concentrations in vivo which in the present investigation was convincingly successful in the “first in man” experiment. Without these in vitro assessments and the testing of the suitability of an internal standard, in vivo intravenous investigations of drug concentrations cannot be performed on ethical grounds.

A limitation of the used calibration method is that a calibrator might exert a pharmacological effect in the organism. This may raise concerns regarding safety aspects of the in vivo microdialysis experiment. In fact, the maximum concentration of atenolol that may be reached in plasma in this experimental setting will not exceed 2 ng/ml, considering the special case of a neonate with a supposed blood volume of 400 ml and an atenolol concentration of 1.0 μg/ml in the perfusion fluid. Two nanogram per milliliter of atenolol is far below any pharmacological activity of this drug (Tabacova & Kimmel, 2002).

The in vivo microdialysis investigation worked successfully with a sterile microdialysis probe suitable for adults. For investigations in infants, the smaller CUP probe could be used but has to be sterilized first with ethylene oxide. The probe should be placed in one of the head veins as they are easier to puncture than the cubital veins in infants. For a further miniaturization, a probe with a shorter membrane, e.g. 10 mm (Baumeister, Rolinski, Busch, & Emmerich, 2001) could be used. Since recovery will drop, a more sensitive analytical system like HPLC coupled with mass spectroscopy might be applied.

A second limitation of the current investigation is that sotalol is predominantly not bound to plasma proteins. Since microdialysis samples the unbound drug concentration, one might ask whether the microdialysis cell is also applicable to moderately or highly protein bound drugs. The protein binding, however, does not affect the microdialysis diffusion process qualitatively, only quantitatively. This means a lower unbound drug fraction diffuses through the membrane and reduces the absolute amount of the drug which needs to be detected. In cases of high protein binding, the temporal resolution of intravenous microdialysis might be limited by the sensitivity of the analytical system. However, others have already shown in rat experiments that intravenous microdialysis is well applicable to drugs with moderate or high protein binding (Opezzo et al., 2001). Therefore our system should be transferable to drugs of different protein binding.

In summary, this stepwise approach using the microdialysis cell seems suitable in vitro method to evaluate parameters for intravenous microdialysis before starting the in vivo experiments. The pilot in vivo application suggests the suitability of the intravenous microdialysis for pharmacokinetic investigations in humans, but this has to be confirmed and proven in some further in vivo investigations. Moreover, extending this test system to different membrane types and sizes a large spectrum of drugs and indications may be covered. Since the CUP probe even fits

in the smallest i.v. catheter, this method should hopefully be applicable to newborns and infants. The very detailed pharmacokinetic information gathered from intravenous microdialysis would be of great advantage for this population.

Acknowledgment

The authors gratefully acknowledge the financial support of the Deutsche Stiftung für Herzforschung. SL is a Heisenberg fellow of the Deutsche Forschungsgemeinschaft (German Research Foundation, DFG).

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