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Simultaneous determination of the elimination profiles of the individual enantiomers of racemic isoproterenol using capillary electrophoresis and microdialysis sampling

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Abstract

Microdialysis sampling and capillary electrophoresis with electrochemical detection (CE-EC) were used in combination to simultaneously define the elimination profile of each enantiomer of isoproterenol (ISP) administered as a racemic mixture to Sprague-Dawley rats. Resolution of the enantiomers of ISP was accomplished using a running buffer containing methyl-*O*-β-cyclodextrin as a chiral recognition reagent. The CE-EC system provided a concentration limit of detection of 0.63 ng ml^{-1}, allowing monitoring of the elimination of ISP for up to six half-lives. Microdialysis sampling was capable of continuously monitoring the concentration of ISP with 60 s resolution. The concentration versus time data for the elimination of (+) and (−) ISP were fit to a biphasic first order elimination model yielding average apparent distribution half-lives of 0.52 ± 0.07 min and 0.55 ± 0.08 min and average apparent elimination half-lives of 9.8 ± 2.2 and 8.8 ± 2.0 min for (−) and (+) ISP, respectively ($n = 3$ rats). No statistically significant difference in the average half-lives was found. However, because each enantiomer was simultaneously determined in each animal a paired two-sample *t*-Test could also be done. This statistical analysis demonstrated that a difference in the elimination half-lives of the enantiomers of ISP does exist.

Keywords: Microdialysis sampling; Chiral analysis; Capillary electrophoresis; Isoproterenol

1. Introduction

Determination of the elimination kinetics of potential therapeutic entities is a primary concern in the development of new drugs. When the drug is a chiral compound and is not to be developed in an enantiomerically pure form, it is important to establish the elimination rates of both enantiomers. As most quantitative methods do not distinguish between enantiomers, this must be accomplished by administering enantiomerically pure forms of the drug to different groups of experimental animals. Small differences in the kinetics of the individual enantiomers can be hard to distinguish from the inter-animal variation by this approach. The situation is even more complex if the elimination kinetics are relatively rapid. In this case, sampling with sufficient frequency to establish the kinetics with high confidence is

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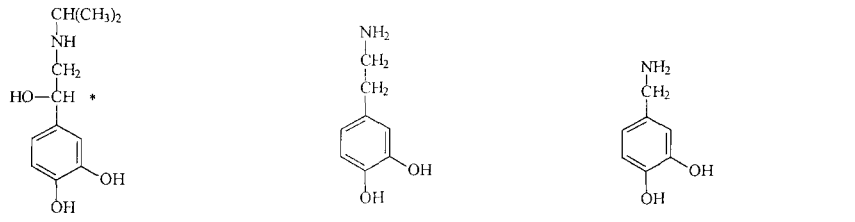

Isoproterenol (ISP) Dopamine (DA) 3,4-dihydroxybenzylamine (DHBA)

Fig. 1. Structure of isoproterenol, dopamine and 3,4-dihydroxybenzylamine.

difficult. This report describes the use of capillary electrophoresis in conjunction with microdialysis sampling to simultaneously determine the elimination kinetics of the individual enantiomers of a chiral compound with half-lives of less than 10 min.

Microdialysis sampling has been shown to be a powerful tool for the determination of drug pharmacokinetics [1–3]. Microdialysis sampling is performed by implanting a small dialysis fiber at the site of interest in an experimental animal. The fiber is perfused at a low flow rate, typically 1 μl min^{-1}. Low molecular weight compounds in the extracellular fluid can diffuse across the membrane and into the probe lumen. These compounds are flushed through the probe and collected for analysis. Because the microdialysis process is based on diffusion through the dialysis membrane no volume of fluid is removed from the experimental animal. Therefore, continuous sampling with high temporal resolution is possible without affecting the pharmacokinetics.

While a wide range of analytical methods can be coupled to microdialysis sampling, liquid chromatography (LC) has been the most common because microdialysis produces highly ionic aqueous samples often with multiple analytes in each sample. While chromatography is well suited for achiral separations, the efficiency of the achiral column packing materials is not sufficient to resolve enantiomers and available chiral packing materials typically do not provide sufficient capacity to resolve the compounds of interest from interferences in complex biological samples. Capillary electrophoresis (CE) has become an attractive separation technique due to its high separation efficiency and extremely small sample volume requirement. The separation principle of CE is based on differences in the electrophoretic mobilities of analytes in an electric field. However, since enantiomers have identical electrophoretic mobilities, a chiral complexing reagent must be added to the separation buffer to form labile diastereomeric complexes with the analytes. The enantiomer with the stronger affinity for the chiral selector will have a different average electrophoretic mobility than its partner and resolution can be achieved. In CE, metal complexes [4], proteins [5], cyclodextrins [6–11], crown ethers [12], and oligosaccharides [13] have been successfully used as chiral selectors. The use of these chiral recognition agents in the electrophoretic run buffer results in a mixed mode separation capable of resolving enantiomers from each other based on interactions with the chiral recognition agent and from sample interferences based on differences in free solution electrophoretic mobility.

Isoproterenol (ISP) was chosen as a model compound to explore the use of microdialysis sampling and capillary electrophoresis to determine the elimination kinetics of a rapidly eliminated chiral compound (Fig. 1). Because of the rapid elimination half-lives of both enantiomers of ISP, a sampling interval of one minute was required to accurately define the elimination profiles. While this would be extremely difficult to achieve when collecting blood samples, it was readily accomplished by microdialysis sampling. The small volume of dialysate collected could then be analyzed by CE to provide quantitation of each enantiomer of ISP. With the use of the

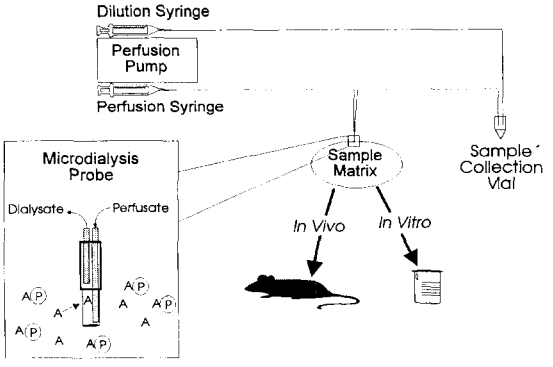

Fig. 2. Schematic diagram of the microdialysis system.

chiral analytical method, racemic ISP could be administered in order to simultaneously determine the elimination half-lives of the individual enantiomers in each experimental animal.

2. Experimental

2.1. Materials

Disodium ethylenediaminetetraacetic acid dihydrate, sodium chloride and glacial acetic acid were obtained from Fisher Scientific (Fairlawn, NJ, USA) and used as received. Racemic isoproterenol·HCl (ISP), (+) and (−) isoproterenol bitartrate, dopamine·HCl (DA), 3,4-dihydroxybenzylamine (DHBA) and methyl-*O*-β-cyclodextrin (MβCD) were obtained from Sigma (St. Louis, MO, USA). All other compounds were reagent grade or better and used as received. All solutions were prepared in deionized water (Nanopure water purification system, Barnstead, Dubuque, IA, USA $R \geqslant 17.5$ MΩ) and were filtered through an Acrodisc 0.22 μm nylon syringe filter (Fisher Scientific, Pittsburgh, PA, USA) before use. The Ringer's solution consisted of 155 mM NaCl, 2.3 mM $CaCl_2$, 5.5 mM KCl [14].

Table 2
Free fraction of ISP in rat plasma[a]

[ISP] (μM)	(+)	(−)
100	0.59 ± 0.02	0.54 ± 0.04
10	0.54 ± 0.03	0.56 ± 0.06
2.5	0.57 ± 0.02	0.52 ± 0.06
Average	0.57 ± 0.04	0.54 ± 0.09

[a] $n = 5$.

2.2. Microdialysis system

A schematic diagram of the microdialysis system is shown in Fig. 2. A CMA/102 microinjection pump (Carnegie Medicin/Bioanalytical Systems, West Lafayette, IN, USA) was used to perfuse the microdialysis probe and the dilution capillary. Microdialysis probes of the flexible cannulla design were constructed according to the method described previously [2] except that a 5 mm long regenerated cellulose membrane fiber with an inner diameter of 200 μm, an outer diameter of 222 μm, and a molecular weight cutoff of 12 000 Da was used. The fiber was a gift from Ivan Mefford of the National Institutes of Health. The perfusion syringe was connected to the microdialysis probe and was used to perfuse the microdialysis probe with Ringer's solution. The dilution syringe was required to maintain good resolution

Table 1
Reproducibility of current response ($n = 8$)

	Absolute current response for calibration standards (nA)				Normalized current ratios		
	0.60 μM DHBA	0.60 μM DA	0.57 μM (−) ISP	0.63 μM (+) ISP	(−) ISP/DHBA	(+) ISP/DHBA	DA/DHBA
Average	0.172	0.229	0.239	0.254	1.39	1.48	1.33
S.D.	0.027	0.034	0.034	0.035	0.07	0.09	0.04
rsd (%)	15.9	15.0	14.2	13.7	5.3	6.4	2.8

by reducing the ionic strength of the sample matrix relative to that of the background electrolyte, prevent concentration of small volume samples by evaporation, and to prevent the oxidative degradation of ISP in the microdialysates. To allow for good mixing of the dialysate with the dilution solution the fused silica outlet capillary of the microdialysis probe and the fused silica outlet capillary of the dilution solution were connected so that the ends of each were equally positioned when inserted into 250 μl polyethylene collection vials.

2.3. In vitro calibration

Calibration of microdialysis probes in vitro was performed by inserting a microdialysis probe into a stirred 60 ml glass jar filled with 2.5 μM of ± ISP·HCl, 8.0 mM disodium EDTA, 97 μM $NaHSO_3$ and Ringer's solution. The jar was placed in a thermostatted dry bath at 37°C (Thermolyne type 17600 Dri-bath, Fisher Scientific, Pittsburgh, PA, USA). Before sample collection, the microdialysis probe was perfused for 30 min to allow the temperature of the sample solution to stabilize. The probe perfusion solution consisted of 8.0 mM disodium EDTA, 97 μM $NaHSO_3$ and 5.0 μM DA. The perfusion flow rate was 0.5 μl min^{-1}. Samples were diluted with a solution consisting of 8.0 mM disodium EDTA, 97 μM $NaHSO_3$ and 0.8 μM DHBA as shown in Fig. 1. The dilution flow rate was 1.5 μl min^{-1}. Five samples were collected at 10 min intervals and analyzed by the chiral CE-EC method. Na_2EDTA and $NaHSO_3$ serve to stabilize ISP, DA, and DHBA by preventing auto-oxidation. DA served as a marker for microdialysis probe function and DHBA served as an internal standard for calibrating and monitoring the performance of the CE-EC system. The structures of the two internal standards are shown in Fig. 1. The in vitro recovery for (−) and (+) ISP was calculated as the ratio of the concentration in the dialysate sample relative to the concentration in the standard. Similarly, the delivery for DA was calculated as the ratio of the difference in the concentration in the standard and dialysate sample relative to the concentration in the standard.

Table 3
In vitro recoveries of isoproterenol

Probe	(−) ISP	(+) ISP
A	0.64 ± 0.02	0.69 ± 0.03
B	0.65 ± 0.01	0.69 ± 0.01
C	0.60 ± 0.02	0.64 ± 0.02

2.4. Capillary electrophoresis with electrochemical detection

The CE-EC system was built in-house as described previously [15]. The development of the chiral CE method used in this study has been described in detail previously [16]. To minimize the amount of MβCD used, only the anodic buffer reservoir contained the chiral running buffer. The cathodic buffer reservoir contained the same solution without MβCD. To maintain good reproducibility in elution time between analytical runs, the capillary was flushed by pressurizing the anodic buffer reservoir to 40 psi with argon. When the CE-EC system was idle, the capillary was filled with 125 mM lithium acetate pH 4.75.

On the very first analysis of the day the capillary was flushed for 300 s with 500 mM sodium EDTA pH 13 followed by 60 s of running buffer. On all subsequent runs the capillary was flushed for 60 s with 500 mM sodium EDTA pH 13 followed by 60 s of running buffer. After flushing the capillary, the carbon fiber electrode was activated by applying a 6.5 kHz square wave of −1.0–1.5 V to the working electrode for 30 s.

For pharmacokinetics experiments the sample injection procedure was varied as a function of time after dosing. Since the concentration range of samples obtained from a pharmacokinetics experiment can span up to four orders of magnitude, it was necessary to use two injection protocols, electrokinetic injection and pH-mediated stacking electrokinetic injection. pH-Mediated stacking allowed a longer injection time for samples with high ionic strength relative to the run buffer ionic strength as described previously [16]. The microdialysis sample collected immedi-

ately after dosing was electrokinetically injected by applying a potential of 18 kV for 3 s. The reason for treating this one sample differently from all others was that the concentration of (−) and (+) ISP in the microdialysate was so high that pH-mediated stacking was not practical. All other samples were injected electrokinetically for 15 s by applying a potential of 18 kV and then concentrated on-column by pH-mediated stacking by injecting 100 mM HCl electrokinetically at 18 kV for 20 s. The electrophoretic analysis of all microdialysis samples was conducted at 18 kV. The chiral background electrolyte (BGE) consisted of 0.1 g ml^{-1} MβCD dissolved in 100 mM lithium acetate pH 4.75.

The stability of the CE-EC system response was monitored using a standard solution containing 0.6 μM DHBA, 0.6 μM DA, 0.57 μM (−) ISP, and 0.63 μM (+) ISP in 25% (v:v) Ringer's, 8.0 mM Na_2EDTA, and 97 μM $NaHSO_3$. This standard was analyzed before the start of each pharmacokinetics experiment and then once every five microdialysis samples. The current ratio of DA and (−) or (+) ISP to DHBA was used for quantitation.

2.5. Surgery

The surgical procedure for microdialysis probe implantation into the vena cava was as described previously [2] with the following modifications. Male Sprague-Dawley rats (385–400 g) were anesthetized by an intramuscular injection of a solution containing 150 mg kg^{-1} ketamine and 10 mg kg^{-1} xylazine. The incision site was prepared by shaving away as much hair as possible, then washed with Prodine Scrub (0.75% aqueous iodine, Phoenix Pharmaceutical, St. Joseph, MO, USA) and with a 70% (v:v) solution of aqueous ethanol. Microdialysis probes were sterilized by soaking in 70% aqueous ethanol 1 h before use. All solutions injected into the animal (ISP bolus and Ringer's) were filter sterilized using a disposable 0.2 μm nylon syringe filter (Acrodisc filters, Fisher Scientific). Surgical tools, drapes, suture, cannula and rinsing water used for surgery were sterilized by autoclave. All animals were allowed to recover from surgery for at least 8 h prior to the start of a pharmcokinetics experiment. For pharmacokinetics experiments the rat was given a 10 mg kg^{-1} i.v. dose of (±) ISP in Ringer's, 8.0 mM disodium EDTA and 97 μM $NaHSO_3$ through the jugular vein cannula.

Rat plasma used for protein binding experiments was obtained by cannulating the femoral vein of an anesthetized rat with a 25 cm length of sterilized tygon tubing (0.03 in. o.d., 0.01 in. i.d.). The femoral vein was nicked and the cannula was pushed 6–10 cm toward the heart through the vena cava and ligated in place. Whole blood was withdrawn from the cannula using sterilized disposable 1.0 ml syringes fitted with a 20 gauge syringe needle and stored in 1.5 ml sterile capped conical polyethylene vials. The vials were centrifuged and the plasma was removed with a sterile disposable pasteur pipette.

2.6. Protein binding

The apparatus for protein binding experiments using microdialysis sampling was the same as that used for in vitro calibration experiments except a conical bottom 1.0 ml reaction vial was used instead of a 60 ml glass jar. Rat plasma (950 μl) was pipetted into a 1.0 ml reaction vial, then spiked to the desired concentration with 50 μl of either (+) or (−) ISP bitartrate in 8.0 mM disodium EDTA, 97 μM $NaHSO_3$ and incubated at 37°C. The microdialysis probe was inserted into the mixture and perfused for 30 min with Ringer's solution, 8.0 mM disodium EDTA and 97 μM $NaHSO_3$ before collecting samples to allow time for the system to equilibrate. After probe equilibration, six samples were collected and analyzed for the free fraction of ISP.

2.7. Pharmacokinetics experiments

For pharmacokinetics experiments samples were collected manually at 1 min intervals for the first 10 min after dosing, then at 2 min intervals for the next 50 min. Samples were immediately frozen on dry ice for later analysis. When ready for analysis, the samples were thawed and then sonicated for 5 s prior to injection. It is important to note that the sample volume was only 2 μl per

sample for the first 10 samples and 4 μl for subsequent samples. The composition of the perfusion and dilution/stabilization solutions and the flow rates the infusion pump was operated at were identical to those described above for in vitro probe calibrations. Pharmacokinetics parameters were determined using non-compartmental analysis ([17]). The AUC was extrapolated to infinite time using the terminal elimination rate constant and clearance was determined as $Cl = D/AUC$ where D is the dose administered.

3. Results and discussion

3.1. Capillary electrophoresis

The high separation efficiency and low concentration detection limits of CE-EC, when used with a chirally selective run buffer constituent such as MβCD, provide both the resolution and detection limits necessary to follow the elimination of the individual enantiomers of racemic isoproterenol. Concentration detection limits of 2.1 μg ml^{-1} (s/n 3:1) with normal electrokinetic injection and 0.63 ng ml^{-1} with pH-mediated stacking were achieved. The response was linear from the limit of detection to 1.06 μg ml^{-1} with pH-mediated stacking and 12.7 μg ml^{-1} without stacking. Furthermore, 2 μl samples could be analyzed as the injection volume of the CE-EC apparatus was only 10 nl.

The reproducibility of response of the CE-EC system was very good ($\leq 2\%$ rsd) when 10 μl standard solutions were used as samples. However, it was found that injection reproducibility was highly dependent on the ability of the operator to position the CE anode and separation capillary into the sample vial. While this was not difficult for 10 μl samples, it became a significant problem when injecting from 2 or 4 μl microdialysis samples. To minimize the variation in the analytical system DHBA was used as an internal standard for all samples. DHBA could be added directly to the dialysis sample during collection using a second syringe mounted on the microinfusion pump used for microdialysis sampling. As microdialysis requires precise control of flow, the microinfusion pumps used provide excellent flow control for addition of the internal standard. The use of response ratios of each analyte relative to DHBA resulted in a 3-fold improvement in the precision of the measurement as shown in Table 1.

Table 4
Comparison of in vitro and in vivo deliveries of DA

Rat	Average in vitro relative delivery ($n = 5$)	Average in vitro relative delivery ($n = 33$)
1	0.70 ± 0.01	0.71 ± 0.04
2	0.71 ± 0.03	0.52 ± 0.04
3	0.58 ± 0.02	0.15 ± 0.06

3.2. Plasma binding of ISP

It has been shown [17–19] that microdialysis sampling is suitable for the determination of the free fraction of drugs in plasma. Over the range of 2.5–100 μM the binding of ISP was concentration independent as shown in Table 2. The average free fraction was 0.57 ± 0.04 for (+) ISP and 0.54 ± 0.09 for (−) ISP. Statistical analysis comparing the average free fractions of the enantiomers using a Student's t-Test revealed no significant difference ($P = 0.224$) at the 95% confidence level. From the free fraction, the average protein bound fraction (f_B) in rat plasma was calculated to be 0.43 ± 0.04 and 0.46 ± 0.09 for (+) and (−) ISP, respectively. These values are between the $65.8 \pm 3.2\%$ reported by Kelly and McDevitt [20] and the 14–18% for racemic ISP in human plasma reported by Tariq and Al-Badr [21].

3.3. Microdialysis sampling of isoproterenol

The initial stage was to determine the relative recoveries of the individual enantiomers of ISP. The recovery of (+) ISP was consistently higher than that of (−) ISP (Table 3). This may be due to the chiral nature of the microdialysis fiber which is composed of regenerated cellulose. It has been shown that oligopolysaccharides can serve as chiral selectors [13]. The recovery determined in

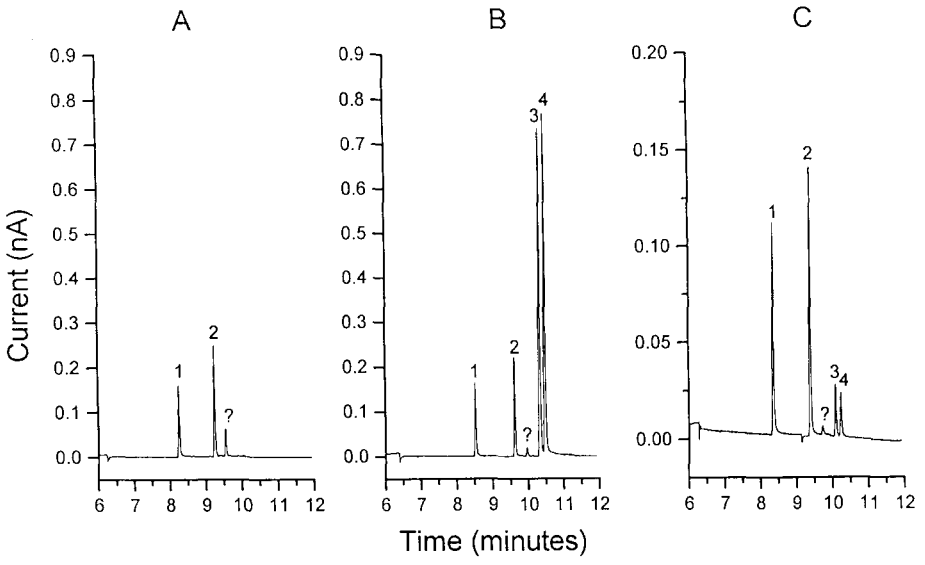

Fig. 3. Typical electropherograms of plasma microdialysate samples. A, prior to dosing; B, 10 min after dosing; C, 60 min after dosing.

vitro for each enantiomer was used to calculate the concentration of that enantiomer in the plasma microdialysis samples.

It has been shown that recoveries determined under hydrodynamic conditions in vitro are valid for calibration of microdialysis probes used for intravenous sampling [2,22]. However, this is only true if the effective surface area of the microdialysis membrane is not changed by absorption of components in the blood [23] or occlusion by resting against the wall of the blood vessel. To account for this possibility a calibrator was used in a manner similar to that suggested by Larsson [24] and Wang et al. [25]. In this case DA was added to the perfusion solution and the delivery of DA out of the microdialysis probe was used as a marker of probe performance. Any change in the membrane surface area following implantation will be reflected in a change in the delivery of DA. Because the effective surface area of the membrane equally effects the recovery and the delivery, the delivery of DA can be used as a calibrator for changes in the recovery of ISP in vivo.

A comparison of the delivery of DA in vitro to that determined in vivo is listed in Table 4. Three cases were examined to determine the effect of experimental protocols on the relationship between delivery in vitro and in vivo. In the first case, Rat 1, the probe was carefully implanted well into the vena cava. In this case, good agreement between the in vitro and in vivo deliveries was found as has been previously reported [2]. In the second case, Rat 2, the probe was purposely not implanted completely into the vena cava. In this case the in vivo delivery was somewhat lower than the in vitro delivery. This is likely to be due to part of the dialysis membrane being occluded by resting against the wall of the blood vessel. This problem is avoided by proper implantation. After each experiment, the probe was carefully removed from the animal and examined under a microscope. Proteinaceous material was observed to be adhered to the epoxy connecting the microdialysis membrane to the fused silica of the probe but not to the membrane itself. For the third case, the probe used in Rat 2 was carefully rinsed with water and implanted in Rat 3 after being recalibrated in vitro. In this case, a significant difference between the in vitro and in vivo deliveries was observed. However, the second in vitro delivery, determined after use in Rat 2, was not different than the initial in vitro delivery. Examination of this probe after the experiment revealed signifi-

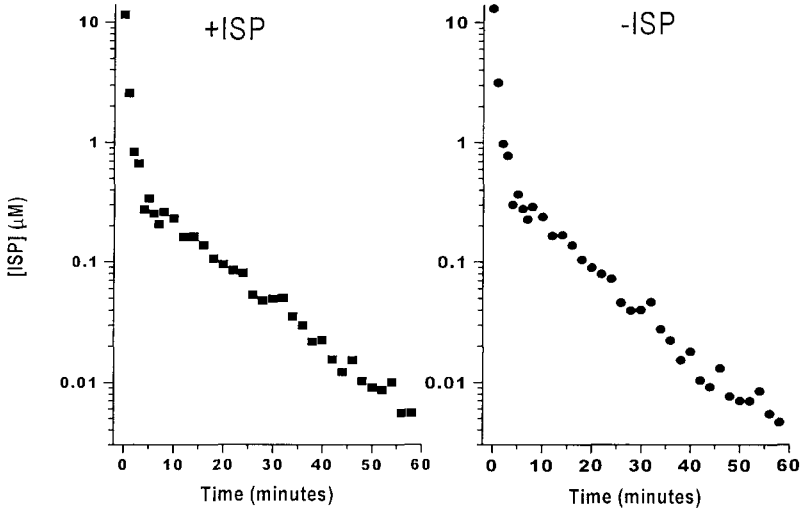

Fig. 4. Typical plasma free concentration versus time curves for isoproterenol. A, (+) isoproterenol; B, (−) isoporterenol.

cant adhesion of proteinaceous material to both the expoxy and microdialysis membrane. In all cases, correction of the in vitro recovery of ISP for changes in effective microdialysis membrane surface area were made using the ratio of the in vivo to in vitro delivery of DA.

3.4. Determination of the pharmacokinetics of racemic ISP

Fig. 3 shows electropherograms of typical microdialysates analyzed by CE-EC. The electropherogram in Fig. 3A is of a blank microdialysate taken prior to dosing with ISP. As can be seen, one endogenous compound is detected but this does not interfere with ISP or either of the internal standards. Fig. 3B and C show electropherograms of a microdialysates taken 10 and 60 min, respectively, after ISP dosing. The concentration of ISP in the dialysates was determined from a standard curve using DHBA as an internal standard for analytical system response. The free concentration of ISP in the plasma was then calculated by accounting for the recovery of ISP using the in vitro recovery corrected by the ratio of the in vivo to in vitro delivery of DA.

Fig. 4 shows representative plasma free concentration versus time curves for (+) and (−) ISP. The pharmacokinetics data for the individual enantiomers is summarized in Table 5. Using a t-Test assuming equal variance (an F-Test was used to test the variances), no significant difference was found between the enantiomers using the averaged half-lives. The inter-animal variation is larger than any difference between the individual enantiomers. However, because each enantiomer was determined simultaneously in each animal following administration of a racemic mixture, a paired two-sample t-Test was possible. Using this form of analysis revealed that the difference between the elimination half-lives was significant at the 95% confidence level ($P = 0.003$).

4. Conclusion

The use of microdialysis sampling coupled to chiral CE-EC allowed the simultaneous determination of the elimination profiles of the individual enantiomers of isoproterenol following administration of a racemic mixture. Microdialysis sampling was able to monitor the in vivo concentration of ISP with a temporal resolution of one minute. Capillary electrophoresis with a chiral recognition reagent provided resolution of the enantiomers of ISP. The use of electrochemi-

Table 5
Pharmacokinetic parameters for (+) and (−) isoproterenol

Rat	AUC (mg*min ml^{-1})		Cl(ml min^{-1})		V_D (ml)		Dist $t_{1/2}$ (min)		Elim $t_{1/2}$ (min)		Δ^a
	−	+	−	+	−	+	−	+	−	+	
1	0.36	0.39	4.2	4.1	9.1	8.7	0.58	0.60	8.3	7.5	0.8
2	1.61	1.06	1.1	1.8	2.7	3.4	0.53	0.58	12.3	11.1	1.2
3	0.79	0.84	2.2	2.3	3.0	3.1	0.44	0.46	8.8	7.8	1.0
Average	0.92	0.76	2.5	2.7	4.9	5.1	0.52	0.55	9.8	8.8	1.0
S.D.	0.64	0.34	1.6	1.2	3.6	3.1	0.07	0.08	2.2	2.0	0.3

[a] Δ The difference between the elimination half-lives of (+) and (−) ISP.

cal detection provided sufficiently low detection limits that the elimination of ISP could be followed for several half-lives. DHBA was used as an internal standard to calibrate the analytical system. DA was added to the microdialysis prefusion fluid to calibrate the microdialysis system. Finally, the ability to determine the pharmacokinetics of the individual enantiomers of ISP following administration of a racemic mixture allowed inter-animal variation to be eliminated in the comparison of the two enantiomers.

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